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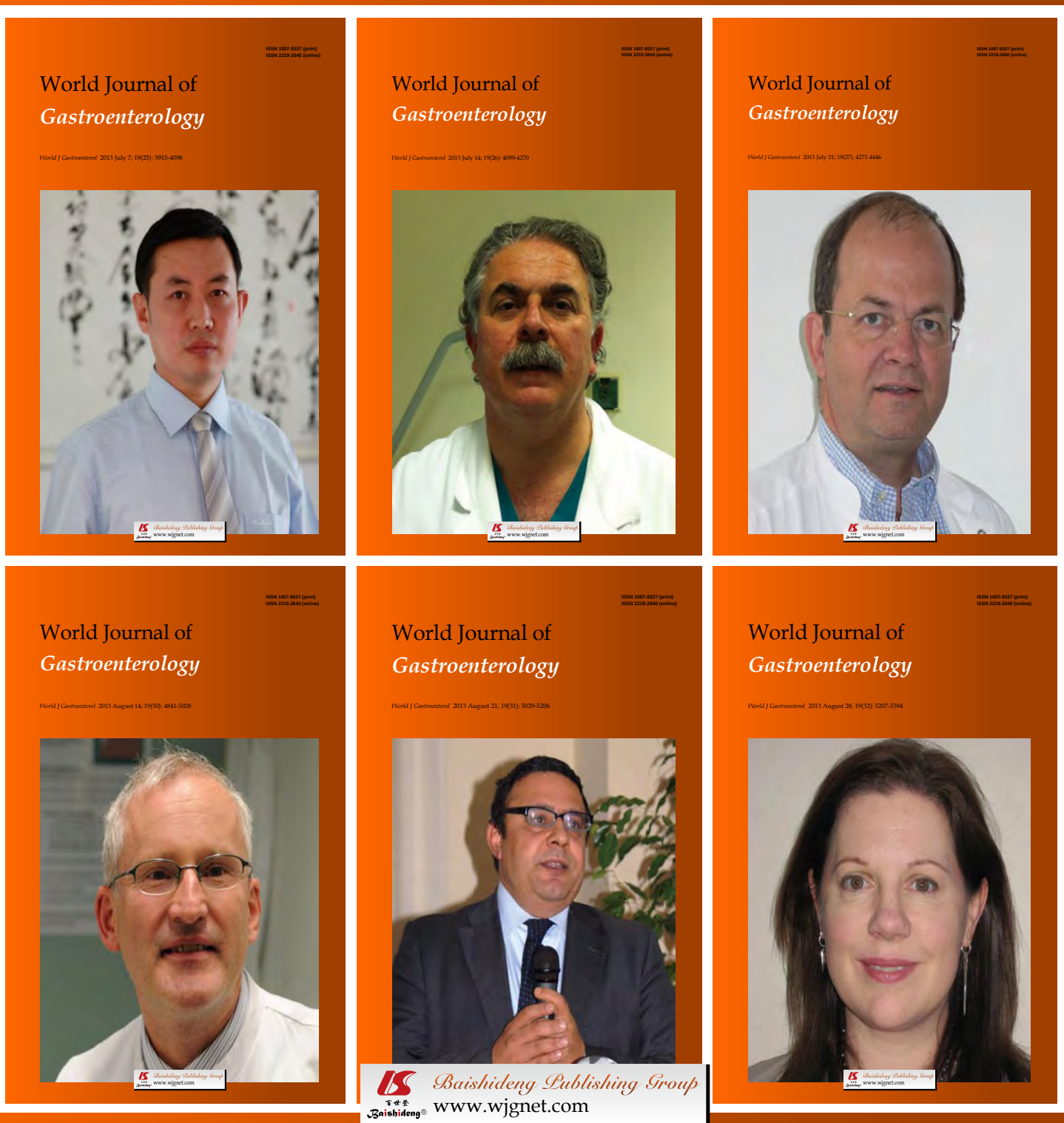

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FIELD OF VISION

Neonatal colon perforation due to anorectal malformations: Can it be avoided?

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Abstract

Anorectal malformations (ARM) are common anomalies in neonates. Diagnostic and therapeutic delays in the management of ARM may lead to colonic perforation, and even death. Physical examination of the perineum is often sufficient to diagnose ARM in neonates. Notwithstanding, delayed diagnosis of ARM has become increasingly familiar to surgeons, as evidenced by the number of recent publications on this topic in the literature. In this commentary, we discuss spontaneous colonic perforation due to delayed diagnosis of ARM in neonates, and highlight the importance of early diagnosis in assuring good outcomes with surgical management. At this point, a thorough examination of the perineum during the initial newborn assessment is mandatory, particularly in those patients presenting with abdominal signs or symptoms.

Key words: Anorectal malformation; Imperforate anus; Bowel perforation; Colon

Core tip: Anorectal malformations (ARM) are common anomalies observed in neonates. The delay in diagnosing a neonate with ARM results in significant complications, occasionally life-threatening morbidity, such as colon perforations. However, delayed diagnosis of ARM seems not the unique factor leading to colonic perforation, deficiency of musculature in the gut wall may also contribute. Colonic perforation due to ARM may not be avoided completely; however, early diagnosis is essential in assuring better outcomes with surgical management.

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COMMENTARY ON HOT TOPICS

We have read with great interest the recent article by Kapadnis *et al* describing a 2.5 kg neonate presenting after 72 h with sigmoid colon perforation due to anorectal malformation (ARM). Delayed diagnosis of ARM has become increasingly familiar to surgeons, as evidenced by the number of recent publications on this topic in the literature[1,2]. Despite the recommendations for peri-natal assessment[3], the overall incidence of a delayed diagnosis has recently been reported to be as high as 21.2%[2]. The delay in diagnosing a neonate with ARM results in significant complications, occasionally life-threatening morbidity, such as colon perforations. Spontaneous perforation of the colon is estimated to occur in 2% of neonates

Table 1 Classification of intestinal perforations complicated with anorectal malformations (n = 25)

Type of perforation	Frequency[1]	Description	Recommended management
Type 1		Perforation occurring before relief of obstruction	
Type 1a	16%	Involving cecum or proximal ascending colon	Cecostomy + distal colostomy
Type 1b	8%	Involving transverse colon including the 2 flexures	Exteriorization of perforation (as colostomy)
Type 1c	60%	Involving distal sigmoid or rectum	Closure of perforation + proximal colostomy
Type 1d	4%	Other sites such as vagina in cloaca	Closure of perforation + proximal colostomy
Type 2	12%	Perforation occurring in the postoperative period	Exteriorization of the perforation site

[1]Calculated by combining the 17 cases reported in the literature and the authors' series.

with ARM, and the incidence rises to 9.5% when the diagnosis is delayed[2]. Thus, it seems crucial to diagnose and treat ARM early to avoid colon perforation.

ARMs are common anomalies observed in neonates[4]. The reported incidence ranges between 1:3300 and 1:5000 live births. In Western countries , there is a male preponderance with 55%-70% of the patients in larger series being males[6]. They vary in severity from mild anal stenosis to complete caudal regression. These disorders usually require surgical intervention in the neonatal period and postoperative follow-up to obtain and maintain fecal and urinary continence. Diagnostic and therapeutic delays in the management of ARM may lead to complications such as sepsis, aspiration, abdominal distension, colonic perforation, respiratory embarrassment, electrolyte imbalance, and even death. The diagnosis of ARM is usually made at birth or shortly thereafter physical examination. Standardized national and international guidelines recommend a routine physical examination of all newborns within the first 48 h of life[3,5]. It has been reported that the median age at diagnosis of perforation in ARM cases was 48 h[6]. Generally, delayed diagnosis of ARM is defined as a diagnosis made after the first 48 h[2]. Undoubtedly, the necessity to diagnose ARM in a timely manner is reliant on a comprehensive neonatal examination performed by a pediatrician or pediatric trainee with sufficient experience. Furthermore, neonatal examination of all newborns should be made within the first 48 h of life. Increasing the awareness among pediatricians of the challenges and complications due to delayed ARM diagnosis may be the important first step. Additional training to adequately diagnose ARM, or change current guidelines to explicitly rule out ARM is also required. Some researchers believe that a higher incidence of associated anomalies may promote earlier diagnosis of the ARM[2], whereas others failed to confirm this hypothesis[7]. Wilson *et al*[7] believed that the only significant predictor of delayed diagnosis of ARM was a failure to receive a comprehensive neonatal examination within 48 h, reiterating that timely diagnosis of ARM is best achieved by adequate clinical examination.

However, colonic perforations cannot be simply attributed to the delayed diagnosis or treatment of ARM, because there are a few case reports of bowel rupture occurring during intrauterine life[8]. Based on their research and review of the literature, Raveenthiran[6] summarized two distinct patterns of perforations involving four different sites and recommended management (Table 1). Approximately 88% of perforations are of type 1, whereas only 12% are of type 2. Among the type 1 perforations, 60% occur in the rectum and sigmoid colon[6]. This difference suggests that the mechanism of perforation could be different for the two types. A higher ratio of rectosigmoid perforation in ARM implies an embryologic origin. As ARM is a developmental field defect, the tail end of the gut can be expected to have deficiency of musculature. The downstream obstruction leads to increased intraluminal pressure, and this, along with the muscular deficiency, is probably responsible for more frequent rupture of the rectum in ARM. Mathur *et al*[9] reported five perforations (6.5%) among 77 cases of ARM with congenital pouch colon (CPC). A high incidence of bowel perforation in CPC also favors the muscular deficiency theory. At this point, delayed diagnosis of ARM seems not the unique factor leading to colonic perforation.

Despite the fact that not all colonic perforations are the result of delayed diagnosis of ARM, the majority are, and early diagnosis is essential so that surgical management can commence to achieve better outcomes. At this point, a thorough examination of the perineum during the initial newborn assessment is mandatory, particularly in those patients presenting with abdominal signs or symptoms.

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REVIEW

Caustic injury of the upper gastrointestinal tract: A comprehensive review

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Abstract

Prevention has a paramount role in reducing the incidence of corrosive ingestion especially in children, yet this goal is far from being reached in developing countries, where such injuries are largely unreported and their true prevalence simply cannot be extrapolated from random articles or personal experience. The specific pathophysiologic mechanisms are becoming better understood and may have a role in the future management and prevention of long-term consequences, such as esophageal strictures. Whereas the mainstay of diagnosis is considered upper gastrointestinal endoscopy, computed tomography and ultrasound are gaining a more significant role, especially in addressing the need for emergency surgery, whose morbidity and mortality remains high even in the best hands. The need to perform emergency surgery has a persistent long-term negative impact both on survival and functional outcome. Medical or endoscopic prevention of stricture is debatable, yet esophageal stents, absorbable or not, show promising data. Dilatation is the first therapeutic option for strictures and bougies should be considered especially for long, multiple and tortuous narrowing. It is crucial to avoid malnutrition, especially in developing countries where management strategies are influenced by malnutrition and poor clinical conditions. Late reconstructive surgery, mainly using colon transposition, offers the best results in referral centers, either in children or adults, but such a difficult surgical procedure is often unavailable in developing countries. Possible late development of esophageal cancer, though probably overemphasized, entails careful and long-term endoscopic screening.

Key words: Caustic ingestion; Corrosive stricture; Developing countries; Surgical management; Endoscopic management

Core tip: The incidence of corrosive ingestion is high and largely unreported in developing countries, where prevention is lacking. Computed tomography and endoscopic ultrasound are gaining a more meaningful role in addressing the need for emergency surgery. The need to perform emergency surgery has a persistent long-term negative impact both on survival and functional outcome. Prevention of stricture is still a debatable issue, yet esophageal stents may offer promising outcomes. It is crucial to avoid malnutrition, especially in developing countries where management strategies are conditioned by poor clinical conditions. Late reconstructive surgery is often unavailable in developing countries.

Contini S, Scarpignato C. Caustic injury of the upper gastrointestinal tract: A comprehensive review. *World J Gastroenterol* 2013; 19(25): 3918-3930 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i25/3918.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i25.3918

INTRODUCTION

Ingestion of corrosive substances remain an important

public health issue in Western countries despite education and regulatory efforts to reduce its occurrence. These injuries are still increasing in developing countries[1,2], related to the social, economic, and educational variables and mainly to a lack of prevention[3,4]. The problem is largely unreported in these settings and its true prevalence simply cannot be extrapolated from the scarce papers or personal experience. Data available are heavily skewed towards well-resourced centers and do not mirror the full reality of the condition. Moreover, in industrialized and developing countries, the therapeutic approach and management strategies appear to be different, likely because of technology and endoscopic expertise.

Two independent MEDLINE and EMBASE searches from 1990-2012 were performed to identify relevant articles. The following medical subject headings terms were used in the searches: caustic ingestion, caustic lesions, corrosive injuries, esophagus, esophageal dilatation. Bibliographies of retrieved studies were reviewed and general medical and major gastroenterology journals manually searched over the previous 5 years.

Table 1 Most commonly ingested caustic substances

Caustic substance	Type	Commercially available form
Acids	Sulfuric	Batteries
		Industrial cleaning agents
		Metal plating
	Oxalic	Paint thinners, strippers
		Metal cleaners
	Hydrochloric	Solvents
		Metal cleaners
		Toilet and drain cleaners
		Antirust compounds
	Phosphoric	Toilet cleaners
Alkali	Sodium hydroxide	Drain cleaners
		Home soap manufacturing
	Potassium hydroxide	Oven cleaners
		Washing powders
	Sodium carbonate	Soap manufacturing
		Fruit drying on farms
Ammonia	Commercial ammonia	Household cleaners
	Ammonium hydroxide	Household cleaners
Detergents, bleach	Sodium hypochlorite	Household bleach, cleaners
	Sodium polyphosphate	Industrial detergents
Condy's crystals	Potassium permanganate	Disinfectants, hair dyes

EPIDEMIOLOGY AND PATHOPHYSIOLOGY

Worldwide, children represent 80% of the ingestion injury population globally[5], primarily due to accidental ingestion[6]. In contrast, ingestion in adults is more often suicidal in intent, and is frequently life-threatening.

Traditionally, ingested corrosive substances are either alkalis or acids (Table 1). Alkaline material accounts for most caustic ingestions in Western countries whereas injuries from acid are more common in some developing countries, like India, where hydrochloric acid and sulfuric acid are easily accessible[7]. Acids and alkalis produce different types of tissue damage. Acids cause coagulation necrosis, with eschar formation that may limit substance penetration and injury depth[8]. Conversely, alkalis combine with tissue proteins and cause liquefactive necrosis and saponification, and penetrate deeper into tissues, helped by a higher viscosity and a longer contact time through the esophagus. Additionally, alkali absorption leads to thrombosis in blood vessels, impeding blood flow to already damaged tissue[9]. Injury occurs quickly, depending on the agent's concentration and time of exposure (Figure 1)[10], with a 30% solution of sodium hydroxide being able to produce full thickness injury in 1 s[11]. Accordingly, alkali ingestion may lead to more serious injury and complications, but this distinction is probably not clinically relevant in the setting of strong acid or base ingestion, both being able to penetrate tissues rapidly, potentially leading to full-thickness damage of the esophageal/gastric wall. The conventional acceptance that acids preferentially damage the stomach, due to the protective esophageal eschar, has recently been questioned, with observation of extensive esophageal damage and perforations after acid ingestion[12]. Likewise, compared with alkali, ingestion of a strong acid may be associated with a higher incidence of systemic complications, such as renal failure, liver dysfunction, disseminated intravascular coagulation and hemolysis[13].

Esophageal injury begins within minutes and may persist for hours. Initially, tissue injury is marked by eosinophilic necrosis with swelling and hemorrhagic congestion[9]. Experimental findings suggest that arteriolar and venular thrombosis with consequent ischemia may be more important than inflammation in the pathogenesis of acute corrosive injury[10]. Four to 7 d after ingestion, mucosal sloughing and bacterial invasion are the main findings. At this time granulation tissue appears, and ulcers become covered by fibrin. Perforation may occur during this period if ulceration exceeds the muscle plane. Fibroblasts appear at the injury site around day 4, and around day 5, an "esophageal mold" is formed, consisting of dead cells and secretions. Esophageal repair usually begins on the 10th day after ingestion, whereas esophageal ulcerations begin to epithelialize approximately 1 mo after exposure. The tensile strength of the healing tissue is low during the first 3 wk since collagen deposition may not begin until the second week. Hence, endoscopy (and of course dilatation) is preferably avoided 5-15 d after ingestion[14]. Scar retraction begins by the third week and may continue for several months, resulting in stricture formation and shortening of the involved segment of the gastrointestinal tract. Additionally, lower esophageal sphincter pressure becomes impaired, leading to increased gastroesophageal reflux (GER), which in turn accelerates stricture formation[15]. GER is indeed a likely significant factor in persistent strictures not responding to sequential esophageal dilatations. Esophageal motility studies report low amplitude and nonperistaltic contractions, with a significantly higher exposure to pH below 4, compared with control groups[16]. Therefore, all caustic esophageal burn patients should be screened for GER

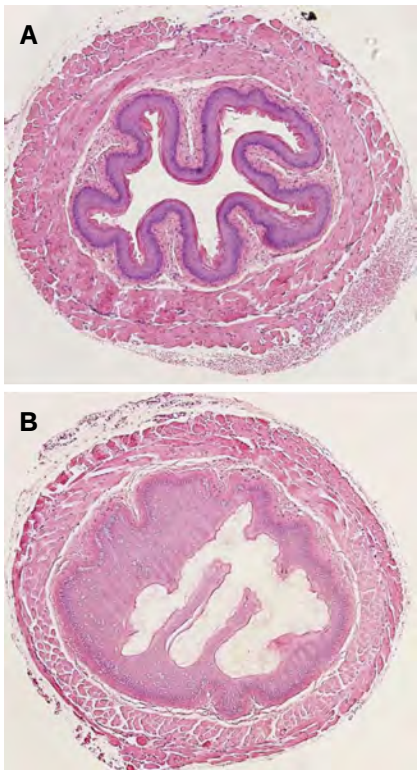

Figure 1 Murine esophagus exposed for 10 min to control (A) and 10% NaOH (B). Reproduced from Osman *et al*[10].

periodically, and GER should be controlled aggressively.

Reactive oxygen species generation with subsequent lipid peroxidation may contribute either to the initial esophageal injury, or to the subsequent stricture formation. Malondialdehyde, an end-product of lipid peroxidation, was found at significantly higher levels than normal in esophageal tissue exposed to sodium hydroxide, signifying the presence of reactive oxygen species at 24 h post exposure. These concentrations remained high for 72 h after exposure compared with no injured controls. Furthermore, significantly lower glutathione concentrations, a known endogenous free-radical scavenger, were found in the same tissues compared with controls, further supporting the presence of reactive oxygen species and free-radical damage[17].

CLINICAL PRESENTATION

Clinical features depend on the type of the substance, amount, physical form and time of presentation (early or delayed). Crystals or solid particles may adhere to the mucosa of the mouth, making them difficult to swallow and thereby diminishing the injury produced to the esophagus, but potentially increasing the damage to the upper airway and pharynx. Conversely, liquids are easily swallowed and are most likely to damage the esophagus and stomach, the extent of injury correlating directly with mortality and late sequelae[18,19]. Patients with oropharyngeal burns do not have significant damage to the esophagus in up to 70%, hence their presence is not a reliable index of esophageal damage[20]. Hoarseness and stridor suggest laryngeal or epiglottic involvement; dysphagia and odynophagia imply esophageal damage while epigastric pain and bleeding are more common in stomach involvement. The absence of pain does not preclude significant gastrointestinal damage. Later changes, such as appearance or worsening of abdominal or chest pain, should be carefully monitored and promptly investigated, since esophageal or gastric perforations can occur at any time during the first 2 wk after ingestion[5].

The relationship between symptoms and severity of injury is uncertain[21]. Stridor and drooling were considered 100% specific for significant esophageal injury[22,23], but no single symptom or symptom cluster can predict the degree of esophageal damage[20,24,25].

The incidence of coexistent gastric injury in the literature ranges from 20.0% to as high as 62.5%[26,27], extending from simple hyperemia/erosions to diffuse transmural necrosis. Delayed gastric emptying with consequent accumulation of food in the stomach (likely due to the contraction of the antropyloric region) may affect the severity of injuries. The most common presentation of an acute corrosive gastric burn is abdominal pain, vomiting, and hematemesis. Rarely, a full thickness burn can cause an immediate gastric perforation, which tends to present a few days after ingestion. Gastric perforation, early or delayed, carries a significant mortality[28], and is more rarely reported in children. Clinical examination and a careful follow-up with a computed tomography (CT) scan are likely more useful than endoscopy in assessing threatened or existing perforation[29]. Bleeding following corrosive ingestion is usually self-limiting: though massive hemorrhage from the stomach or duodenum has been reported a short time after corrosive ingestion[30], severe bleeding typically occurs at 2 wk after ingestion[29].

Respiratory complications from caustic ingestion may result in laryngeal injury and upper airway edema, which ultimately may require tracheotomy[31] and is usually coupled with extensive esophageal damage. Laryngeal injuries were diagnosed by flexible fiberoptic or rigid laryngoscopy in 38% of patients after caustic ingestion, but only few (8%) required immediate intubation and mechanical ventilation for respiratory distress on admission[11]. This low rate of lower airway and pulmonary complications suggests that the protective pharyngeal-glottic mechanism is highly efficient in preventing the caustic substance to reach the lower airway.

EVALUATION AND ASSESSMENT

Laboratory studies

Correlation between laboratory values and the severity/outcome of injury is poor. A high white blood cell count (> 20000 cells/mm^3), elevated serum C-reactive protein, age and the presence of an esophageal ulcer have been considered predictors of mortality in adults[32]; an arterial pH less than 7.22 or a base excess lower than -12 have been considered indication of severe esophageal injury

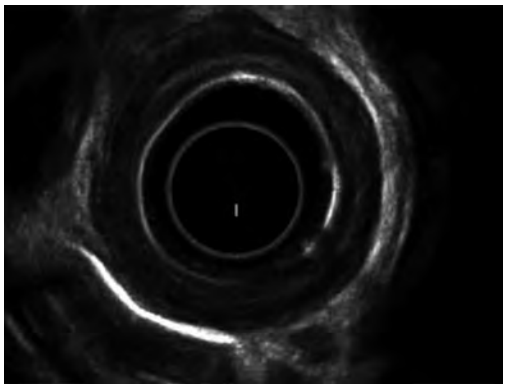

Figure 2 Endoscopic ultrasound showing involvement of the muscularis propria of esophageal wall. Reproduced from Kamijo *et al*[37].

and of emergency surgery[33]. Essentially, laboratory studies are more useful in monitoring and guiding patient management than in predicting morbidity or mortality[34].

Traditional radiology

Shortly after ingestion, a plain chest radiograph may reveal air in the mediastinum suggesting esophageal perforation, as well as free air under the diaphragm, indicating gastric perforation. If it is felt necessary to confirm a clinically suspected perforation, a water-soluble agent, such as Hypaque™ or Gastrografin™, and less irritant than barium sulphate, should probably be used, though both can be equally irritant[35]. Conversely, barium sulfate should be the preferred contrast agent in late barium swallowing, providing greater radiographic details than water-soluble contrast agents[22].

Ultrasounds

Evaluation of esophageal wall caustic damage by endoscopic ultrasound (EUS) using a miniprobe seems safe, though prolongs examination time without showing any difference with endoscopy in predicting early complications[36]. The destruction of the muscular layers of the esophagus observed at EUS seems a reliable sign of future stricture formation[37]; furthermore, ultrasound examination with a radial probe may predict the response to dilatation, which usually requires more sessions when the *muscolaris propria* is involved at EUS, as in Figure 2[38]. In spite of these encouraging reports, the role of US examination in caustic injuries is still under evaluation.

CT scan

A CT scan likely offers a more detailed evaluation than early endoscopy about the transmural damage of esophageal and gastric walls and the extent of necrosis[39]. It is more valuable than endoscopy in assessing threatened or established stomach perforation[29], and a CT grading system (Table 2 and Figure 3) has been proposed to predict esophageal stricture[40,41]. With the advantage of not being invasive, CT scan has a promising role in the early evaluation of caustic injury damage.

Table 2 Computed tomography grading system for caustic lesions

Grade	Features
Grade 1	No definite swelling of esophageal wall
Grade 2	Edematous wall thickening without periesophageal soft tissue involvement
Grade 3	Edematous wall thickening with periesophageal soft tissue infiltration plus well-demarcated tissue interface
Grade 4	Edematous wall thickening with periesophageal soft tissue infiltration plus blurring of tissue interface or localized fluid collection around the esophagus or descending aorta

Reproduced from Ryu *et al*[40].

Endoscopy

Esophagogastroduodenoscopy is considered crucial and usually recommended in the first 12-48 h after caustic ingestion, though it is safe and reliable up to 96 h after the injury[13,42]; gentle insufflation and great caution are mandatory during the procedure. Endoscopy and even dilatation have been performed without consequences from 5 to 15 d after corrosive ingestion[43], though potentially hazardous due to tissue softening and friability during the healing period. Adequate sedation (general anesthesia in children) is compulsory, yet endotracheal intubation is strictly required only for patients in respiratory distress. The constraint to stop the endoscope in the presence of a circumferential second or third degree esophageal burn is not mandatory[44,45].

When lip and oropharyngeal injuries are the main clinical findings, esophageal or gastric injuries are generally no greater than grade 1[46]. Although severe esophageal injuries have been reported in 12.0%[47] and 19.3%[48] of asymptomatic children, significant lesions at endoscopy are not usually observed when symptoms are absent after unintentional ingestion of less aggressive substances[24,49], thus making routine post-ingestion endoscopy questionable in this group of patients. All adult patients must undergo endoscopy after suicidal ingestion, because of the larger amount of more corrosive agents swallowed compared with unintentional injuries, where early esophagoscopy has been questioned[50]. Ultimately, though endoscopy is considered by most a cornerstone in the diagnosis of corrosive ingestions, which patients would clearly benefit from it is still debated. Considering that 10%-30% of caustic ingestions globally do not show any upper gastrointestinal injury[22,51], the indication for early endoscopy should be made on a case-by-case basis, with consideration of symptoms, otorhinolaryngeal injuries, and the amount and nature of the ingested substance.

Contraindications to endoscopy are a radiologic suspicion of perforation or supraglottic or epiglottic burns with edema, which may be a harbinger of airway obstruction, therefore indicating endotracheal intubation or tracheostomy. A third degree burn of the hypopharynx is a further contraindication for endoscopy[22].

Endoscopic classification[8] is important for prognosis and management (Table 3). Generally, grade 0 and 1 le-

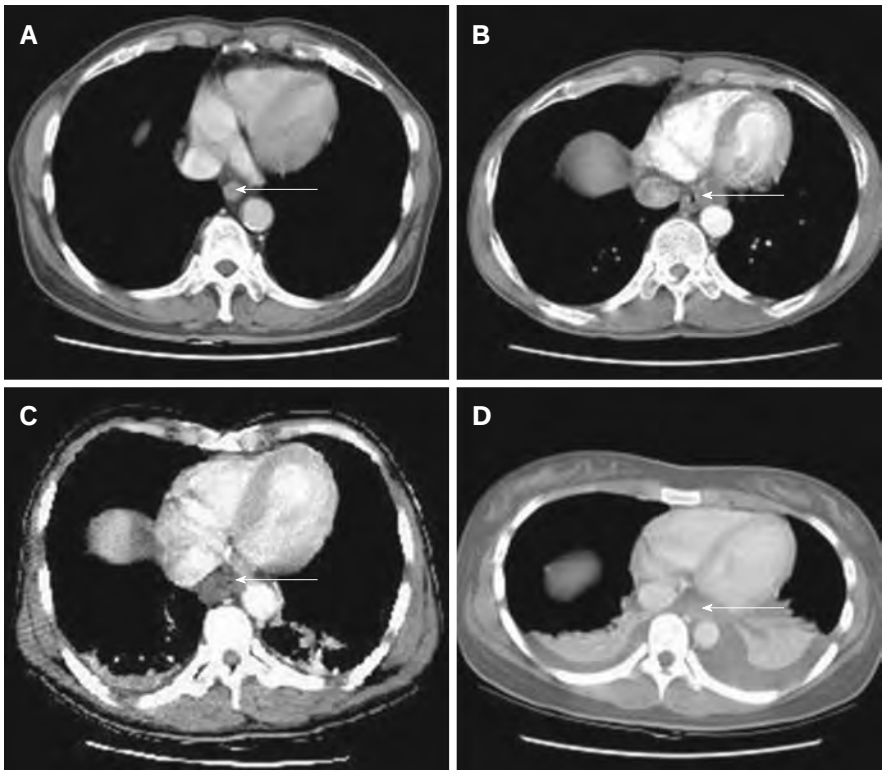

Figure 3 Computed tomography grading of esophageal caustic injuries. A: Grade 1; B: Grade 2; C: Grade 3; D: Grade 4. Reproduced from Ryu *et al*[40]. Arrows show the esophageal wall.

Table 3 Endoscopic classification of caustic injuries

Grade	Features
Grade 0	Normal
Grade 1	Superficial mucosal edema and erythema
Grade 2	Mucosal and submucosal ulcerations
Grade 2A	Superficial ulcerations, erosions, exudates
Grade 2B	Deep discrete or circumferential ulcerations
Grade 3	Transmural ulcerations with necrosis
Grade 3A	Focal necrosis
Grade 3B	Extensive necrosis
Grade 4	Perforations

Reproduced from Zargar *et al*[14].

sions do not develop delayed sequels, such as esophageal strictures or gastric outlet obstruction, whose incidence increases with the severity of the lesion. Additionally, the degree of esophageal injury at endoscopy is an accurate predictor of systemic complications and death, with each increased injury grade correlated with a 9-fold increase in morbidity and mortality[14]. Emergency surgery can be planned according to the endoscopic degree of burn, though an isolated black eschar does not always indicate full-thickness injury and the need for immediate surgical treatment: such patients may deserve further evaluation and careful observation. Recently, some concerns have been raised about the correlation between endoscopic findings and the extent of necrosis[39]: gastrectomy was considered unnecessary at laparotomy in 12% of patients with gastric injuries staged 3b at endoscopy, while the decision to perform esophagectomy based exclusively on endoscopic findings led to unnecessary esophagectomy in 15% of cases[52], suggesting the need for better criteria to improve patient selection for emergency surgery.

MANAGEMENT

Acute management

Immediate treatment is usually conservative, as the definitive extent of the injury is determined within minutes after ingestion. Hemodynamic stabilization and adequacy of the patient's airway are priorities. If the airway is unstable, fiberoptic laryngoscopy allows intubation under direct visualization, avoiding "blind" intubation with the risk of bleeding and additional injuries. In challenging patients, a surgical airway may be required. Gastric lavage and induced emesis are contraindicated for the risk of re-exposure to the corrosive agent and additional injury to the esophagus. The effectiveness of milk and water either as antidotes or to dilute the corrosive agents has never been proven. pH neutralization, with either a weak acid or base, is not recommended for fear of an exothermic reaction, which may increase the damage. Milk and activated charcoal are contraindicated because may obscure subsequent endoscopy. Nasogastric tubes may be applied to prevent vomiting and as stent in severe circumferential burns, but their validity has never be proven. In any case they should not be placed blindly because of the risk of esophageal perforation[53].

To date, the efficacy of proton-pump inhibitors and H_2 blockers in minimizing esophageal injury by suppressing acid reflux has not been proven, though an impressive endoscopic healing after *iv* omeprazole infusion has been observed in a small prospective study[54].

The utility of corticosteroid is controversial. A meta-analysis of studies between 1991 and 2004, and an ad-

ditional analysis of the literature over a longer period from 1956 to 2006 did not find any benefit of steroid administration in terms of stricture prevention. Steroids are usually reserved for patients with symptoms involving the airway[55,56].

The administration of broad-spectrum antibiotics is usually advised mainly if corticosteroids are initiated, as well as if lung involvement is identified[53,57].

Patients whose injuries are graded 1 and 2A are permitted oral intake and discharged within days with antacid therapy. In more severe cases (grade 2 or 3), observation in an intensive care unit and adequate nutritional support is required.

Early surgery

Patients with clinical or radiological evidence of perforation require immediate laparotomy, usually followed by esophagectomy, cervical esophagostomy, frequently concomitant gastrectomy and even more extensive resections, and jejunostomy feeding[58-60]. Some patients without features of perforation at admission may later develop necrosis, perforation and massive bleeding with disastrous results. Indications for emergency surgery rely more often on clinical grounds than on radiological findings; in the presence of doubtful clinical features a decision to perform laparotomy is likely more advantageous for patients than a conservative attitude especially in patients who ingested large amounts of corrosive substances[60].

Laboratory and endoscopic criteria for emergency surgery have been suggested, including disseminated intravascular coagulation, renal failure, acidosis and third degree esophageal burns[58,61]. Unfortunately, these are often late findings and surgery may improve mortality and morbidity in grade 3A injuries only[14].

Severe injuries of the stomach at endoscopy require careful monitoring with a low threshold for laparotomy. At surgery, a gastrotomy allows an accurate evaluation of the extent of damage, since mucosal (and transmural) necrosis may be more extensive than what is apparent from the serosal side. There is no role for procedures such as closure of a perforation. Conservative management of severe gastric injuries at laparotomy, with partial or total conservation of the stomach, has been recently advocated by some in the absence of clinical and biological signs of severity[62].

The need to perform surgery for caustic injuries has a persistent long-term negative impact both on survival and functional outcome. Moreover, esophageal resection *per se*, is an independent negative predictor of survival after emergency surgery[52].

Laparoscopy has been proposed when gastric perforation is highly suspected[63], but the mini-invasive approach has two caveats: unless in very expert hands, it is not a substitute for a comprehensive abdominal exploration, particularly in the posterior aspects of the stomach and duodenum, and it can extend the operative time excessively in a situation where time is a major determinant of outcome. However, it might be considered a useful tool when the stomach cannot be evaluated by endoscopy. Some authors have proposed routine laparoscopic examination in all injuries of second degree or greater[63,64] but the experience is still limited and laparoscopy may be neither feasible nor helpful in such dramatic circumstances.

All injured organs must be resected, if possible, during the first operation. Minimal resection followed by a planned second-look procedure is not recommended. However, secondary extension of caustic burns is unpredictable and re-exploration is indicated when in doubt. An extended resection to adjacent abdominal organs, even the pancreas, does not necessarily carry a prohibitive risk of death in referral centers[60], but an extensive colon resection may compromise future reconstruction and require vascular surgery for atypical transplants. A massive intestinal necrotic injury represents a reasonable limit for resection.

Emergency surgery may be required in the case of severe, uncontrolled late gastric bleeding, usually 1-2 wk after ingestion. Total gastrectomy may be necessary. In duodenal hemorrhages, under-running of the bleeding vessel through a duodenotomy is advised[29].

Acute surgery is quite exceptional in the pediatric population and most authors recommend exhausting all resources to try to preserve the child's native esophagus[25].

Late sequelae

Following a grade 2B and a grade 3 esophageal burn, stricture incidence may be 71%[14] and 100%, respectively[45,53]. Strictures usually develop within 8 wk after the ingestion in 80% of patients, but it can happen as early as after 3 wk or as late as after 1 year. Obviously, ingestion of powerful caustic substances (*e.g.*, sodium hydroxide) is followed by severe, long-standing strictures and dramatically altered esophageal motility[65].

Late sequelae of corrosive gastric injury include intractable pain, gastric outlet obstruction, late achlorhydria, protein-losing gastroenteropathy, mucosal metaplasia and development of carcinoma[66]. Gastric outlet obstruction has an incidence of 5%[67], mainly in the prepyloric area, where prolonged contact with the antral mucosa due to pyloric spasms and to resulting pooling of the caustic agent in this region[55] usually results in stricture in more than 60% of patients[68]. When the volume of the corrosive substance ingested is large, the entire stomach is scarred leading to a diffusely contracted stomach.

Stricture prevention

Steroids: Systemic administration of steroids seems ineffective in preventing strictures[55,56], especially in patients with 3rd degree esophageal burns. Intralesional triamcinolone injections have been proposed to prevent strictures[69], but optimal dose, frequency, and best application techniques are still to be defined[70].

Antibiotics: Though an old study reports a marked decrease in stricture formation with the use of antibiotics[71],

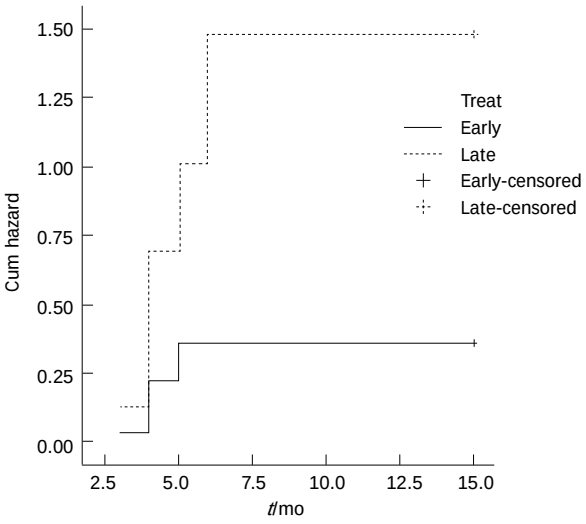

Figure 4 Significantly higher hazard of re-dilatation in patients submitted to late dilatation. $P = 0.0008$. Reproduced from Contini *et al*[97].

no prospective trial evaluated their utility, and their value in the setting of caustic ingestion, in the absence of concomitant infection, is unknown[18]. There is a consensus that patients treated with steroids should also be treated with antibiotics, but prophylactic antibiotics to prevent strictures, in the absence of steroid therapy, has not been advocated[72].

Nasogastric tube: Though a nasogastric tube may be helpful to ensure patency of the esophageal lumen, the tube itself can contribute to the development of long strictures and routine use is not uniformly recommended[22]. Any esophageal catheterization may be a nidus for infection and nasogastric placement may worsen gastroesophageal reflux in this patient population, with a consequent delay in mucosal healing. However, enteral nutrition through a nasogastric tube has been demonstrated to be as effective as jejunostomy feeding in maintaining nutrition in such patients, with a similar rate of stricture development[73]. Moreover, positioning a nasogastric tube has the advantage of providing a lumen for dilatation should a tight stricture develops. Therefore, after caustic injuries the placement of a nasogastric tube may be considered, but the decision should be made with caution and done on a case-by-case basis.

Mitomycin C: Mitomycin C, a chemotherapeutic agent with DNA crosslinking activity, when injected or applied topically to the esophageal mucosa, may be valuable in preventing strictures, but this drug has deleterious adverse effects, especially if systemic absorption occurs across the intact mucosa[74]. A recent systematic review indicated encouraging results in the long term[75], but prospective studies are clearly mandatory to determine the most effective concentration, duration and frequency of application[76]. The theoretical risk of secondary long-term malignancy should also be taken into account[77].

Intraluminal stent: Specially designed silicone rubber[78] or, more recently, polyflex stents[79] have been found helpful in preventing stricture formation but the efficacy is less than 50%, with a high migration rate (25%). Patient selection remains a challenge and the development of hyperplastic tissue is a concern. Home-made polytetrafluoroethylene stents have shown promising results with a 72% efficacy[80] at 9-14 mo, similar to home-made silicone stents positioned by endoscopy[81] or through laparotomy[82] for 4-6 mo. Biodegradable stents (poly-*L*-lactide or polydioxanone) are under evaluation for benign strictures[83,84], with a 45% success rate at 53 mo in a patient population with only two caustic strictures, a migration rate of around 10%, and a significant hyperplastic tissue response. Experimentally, biodegradable stents were not able to prevent strictures in pigs after circumferential submucosal resection[85]. Moreover, cost and minimal experience in caustic strictures make the use of biodegradable devices questionable, especially in developing countries.

Other modalities for stricture prevention under evaluation: Intraperitoneal injection of 5-fluorouracil has been effective in preventing strictures experimentally[86]. Anti-oxidant treatment (vitamin E, H_1 blocker, mast cell stabilizer, methylprednisolone) and phosphatidylcoline[87,88] inhibit collagen production and stricture formation by decreasing tissue hydroxyproline, the ultimate product of collagen degradation, but no human study is available. Octreotide and interferon-alfa-2b have been shown in animals to depress the fibrotic activity in the second phase of wound healing of the esophageal wall after a corrosive burn[89]. Cytokines have also been used experimentally with success to prevent stricture formation[90]. Until now, none of the above approaches, albeit appealing, has been tested in humans.

Stricture management

Endoscopic dilatation: Timely evaluation and dilatation of the stricture play a central role in achieving a good outcome[91]. Late management is usually associated with marked esophageal wall fibrosis and collagen deposition[5], which makes dilatation more complex. Maximal esophageal wall thickness, observed at CT scan, was associated with a higher number of sessions required for adequate dilatation[92], and recurrent strictures were significantly more frequent after delayed dilatation (Figure 4)[93-95]. Moreover, delayed presentation and treatment have been found to be strong predictors of future esophageal replacement[96]. This issue, which may entail different management strategies[3] for early or late patients, may be crucial in developing countries, where late presentations are more than 50%[2,97,98].

Dilatation can be carried out with balloon or bougies (usually Savary) without a clear advantage for each method[70]. However, the failure rate after pneumatic dilatation is higher in caustic ingestion-related strictures than in other benign strictures[99]; Savary bougies are considered more reliable than balloon dilators in consoli-

dated and fibrotic strictures such as old caustic stenosis or in long, tortuous strictures[100,101], and may offer the operator the advantage of feeling the dilatation occurring under his hands[102]. Dilatation should be avoided from 7 to 21 d after ingestion for the risk of perforation, though early, prophylactic dilatation with bougienage has been reported to be safe and effective even in this period[43]. The perforation rate after dilatation of benign esophageal strictures varies between 0.1% and 0.4%[70], but for caustic strictures it fluctuates from 0.4% to 32.0%, dropping from 17.6% to 4.5% with increased experience[103]. The 5%-8% perforation rate after balloon dilatation[104] may be as high as 32% in caustic strictures[105]. Indeed, radiological intramural and well-contained transmural esophageal ruptures were observed in 30% of balloon dilatation procedures[106]. In addition, balloon inflation may cause either extrinsic mechanical compression of the trachea or obstruction at the endotracheal tube tip[107]. Therefore, the use of the balloon catheter in children entails careful intraoperative monitoring and likely requires greater endoscopic skill and experience than for Savary bougies. If these requirements are not met, as is often the case in developing countries, pneumatic dilatations will carry a considerable risk and then require extra caution, so that bougie dilatation is preferred.

The interval between dilatations varies from less than 1 to 2-3 wk and usually 3-4 sessions are considered sufficient for durable results, although the number of dilatations required may be unpredictable and quite high[103]. In challenging strictures, a nylon thread left between the nose and the gastrostomy maintains luminal access and facilitates further dilatations when an expert endoscopist is not available[108,109]. A cut-off value for unsuccessful dilatation treatment may be difficult to define, especially in developing countries, where alternative surgical options are not widely available.

A good nutritional state is crucial for a successful outcome, especially in children, and both an improvement in nutritional status and sustained esophageal patency should be considered reference points for a successful dilatation[3]. Changes in feeding practices may be required in order to maintain an adequate nutritional status[110]. In developing countries, delayed presentation and severity of strictures due to the more corrosive substances usually ingested, together with poor nursing and surgical care make this target quite challenging. In such a scenario, feeding by nasogastric tube for long periods may be tolerated with difficulty and a gastrostomy is more effective and often necessary to attain an acceptable nutritional state. Moreover, gastrostomy allows a retrograde approach for dilatation, which is usually easier and safer[111,112].

RISK OF CANCER

Esophageal neoplasms (both adenocarcinoma and squamous cell carcinoma) may develop as a late complication of caustic injury at a rate 1000-3000 times higher than expected in patients of a similar age[113] and have actually been reported only 1 year after ingestion[114]. The reported incidence ranges from 2% to 30%, with an interval from 1 to 3 decades after ingestion[53]. Cancer is most commonly observed at the areas of anatomic narrowing, and may be related to increased exposure to the caustic substance. Esophageal bypass surgery does not prevent the development of esophageal cancer following caustic ingestion[53]. The problem may be overestimated, in accordance with the low number of esophageal cancer reported in a large series with long-term follow-up[9,115,116], yet endoscopic screening is still recommended for patients following caustic ingestion. Moreover, the role of other confounding factors, such as alcohol abuse or smoking habit, should be considered[39].

DISMOTILITY

Orocecal transit time is prolonged mainly in patients with lower third esophageal involvement of the burn[65], probably related to autovagotomy due to vagal entrapment in the cicatrization process involving the lower third of the esophagus. Moreover, impaired vagal cholinergic transmission, possibly due to the same mechanism[117] can explain the increased fasting gallbladder volume and decreased gallbladder emptying found in patients after lower esophageal damage.

Gastric emptying time of liquids after caustic ingestion, was found to be significantly prolonged in patients with lower esophageal strictures, but not in upper-middle esophageal strictures, even in the absence of symptoms suggestive of gastric outlet obstruction or gastroparesis[118].

Late surgery

Surgery for non-responding esophageal strictures: When esophageal dilatation is not possible or fails to provide an adequate esophageal caliber in the long-term, esophageal replacement by retrosternal stomach or, preferably, right colonic interposition should be considered. Mortality and morbidity are low in expert hands[119,120]. The more demanding pharyngoesophageal strictures may be treated with acceptable results, provided considerable expertise is available[121]. The native esophagus can be left or removed. Though resection of the scarred esophagus may be performed without a substantial increase in morbidity and mortality compared to by-pass[120], a 13% incidence of esophageal cancer after by-pass[93], the risk of infected esophageal mucocele in 50% of the patients after 5 years[94], and the impossibility of endoscopic follow-up for cancer are all arguments favoring esophageal resection. Removal of the native esophagus seems advisable in children because of the risk of cancer in a long life period. Conversely, the doubled mortality rate (11.0% *vs* 5.9%) of resection *vs* by-pass[122], the possible damage to the trachea and laryngeal nerve, and the low reported incidence (3.2%) of esophageal malignancy, could support a conservative strategy. In children, reconstruction with gastroplasty seems easier, and more functional failures can be expected with coloplasty[123-125]. In developing

countries, experienced pediatric surgical centers are not widely available and this should be considered before abandoning the conservative approach of dilatation.

Surgery for stomach injuries: The timing and type of elective surgery for gastric outlet obstruction is still controversial. Early surgery has been advised to decrease mortality and morbidity[67,126]. Conversely, elective surgery earlier than 3 mo has been considered risky because of poor nutritional state and the presence of adhesions and the edematous gastric wall[27]. Moreover, assessment of the limits of the gastric resection may be difficult, due to ongoing fibrosis. Endoscopic balloon dilatation and/or intralesional steroid injection have been proposed as alternatives[127,128]. However, endoscopic gastric dilatation should be considered a temporary substitute for surgical resection because gastric wall fibrosis usually diminishes the long-term functional result[129,130]. Moreover, although dilatation averts surgery in less than 50% of patients[127], perforation can occur in strictures longer than 15 mm[131]. Pyloroplasty has been recommended for moderate strictures[67], but progressive fibrosis causing recurrent stricture occurs frequently. Gastrojejunostomy is a safer alternative to gastric resection in the presence of extensive perigastric adhesion, an unhealthy duodenum, and poor general condition; marginal ulceration is rarely reported[27,132] possibly due to physiologic antrectomy resulting from mucosal damage[66]. Partial gastric resection is preferred by many[133,134] for the long-term risk of malignant transformation, though the need for gastric resection as prophylaxis against future malignancy has been overstated in the literature[29]. Previous reports of gastric carcinomas after acid ingestion are usually old and limited[135,136]. Regular follow-up and surveillance endoscopy is a more reliable approach.

Late reconstructive surgery after emergency esophagectomy: When the stomach has been removed or shows chronic injuries, the use of a gastric tube for esophageal reconstruction is obviously precluded. Reconstruction is probably advisable at the end of the evolving scarring process, usually after 6 mo, although the optimal timing of reconstruction has been reported from 2 mo to years[94,137,138]. The functional success rate after colon reconstruction at 5 years is 77% and the severity of the initial insult or a delay more than 6 mo, may strongly influence the outcome[119]. Coloplasty dysfunction is responsible for half of the failures, with an overall 70% success rate after revision surgery in expert hands. An emergency tracheostomy may have an adverse impact on the outcome of a colopharyngoplasty[139]. Secondary esophagocoloplasty should be considered with good results if intraoperative colon necrosis occurs at the time of primary reconstruction[140].

CONCLUSION

Ingestion of corrosive substances is increasingly reported in developing countries, due to lack of education and prevention. The relationship between symptoms and severity of injury may be vague, and patients should be carefully monitored, since esophageal or gastric perforations can occur at any time during the first 2 wk after ingestion. Endoscopy is considered a cornerstone in the diagnosis of corrosive ingestions, yet the indication for early endoscopy should likely be made on a case-by-case basis. Reported discrepancies between endoscopic findings and the extent of necrosis found at surgery suggest the need for better criteria to improve patient selection for emergency surgery. A CT scan may offer a promising role in assessing the evolution of the injury and impending perforations. In suicide attempts, mortality is still high and the need to perform emergency surgery for caustic injuries has a persistent long-term negative impact both on survival and functional outcome. However, timely and early surgery may be the only hope for patients with severe injuries, and a rather aggressive attitude should be considered in such patients.

Main late sequelae include esophageal strictures, often accompanied by undernourishment, especially in developing countries. The likelihood of a gastric outlet obstruction should always be kept in mind. The presence of severe GER and of esophageal dysmotility may worsen the prognosis. Stricture prevention by stents seems promising but the experience is still limited. Systemic corticosteroids offer no role. Endoscopic dilatation is usually successful in achieving a patent esophageal lumen, but in complex strictures several attempts must be carried out, and in such patients bougies may be preferred to balloon dilatation. A cut-off value for unsuccessful dilatation treatment may be difficult to define, especially in developing countries, where alternative surgical options are not widely available. Both an improvement in nutritional status and a sustained esophageal patency should be considered reference points for a successful dilatation. Gastrostomy may be lifesaving in this perspective. Mortality and morbidity of esophageal replacement in patients not responding to dilatation are low in expert hands. The preservation of the native esophagus is still debated. When late reconstructive surgery is carried out after early emergency surgical treatment, the outcome is strongly influenced by coloplasty dysfunction, responsible for half of the failures. Risk of esophageal cancer after caustic ingestion might be overestimated, yet endoscopic screening is still recommended.

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REVIEW

Why interleukin-10 supplementation does not work in Crohn's disease patients

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Abstract

Inflammatory bowel diseases (IBD) such as Crohn's disease (CD) or ulcerative colitis are chronic intestinal disorders, which are on the increase in "Westernised" countries. IBD can be caused by both genetic and environmental factors. Interleukin-10 (IL-10) is an immunoregulatory cytokine that has been identified as being involved in several diseases including IBD. Studies have shown that polymorphisms in the promoter region reduce serum levels of IL-10 and this reduction has been associated with some forms of IBD. Mouse models have shown promising results with IL-10 supplementation, as such IL-10 supplementation has been touted as being a possible alternative treatment for CD in humans. Clinical trials have shown that recombinant human IL-10 is safe and well tolerated up to a dose of 8 μg/kg. However, to date, the results of the clinical trials have been disappointing. Although CD activity was reduced as measured by the CD activity index, IL-10 supplementation did not result in significantly reduced remission rates or clinical improvements when compared to placebo. This review discusses why IL-10 supplementation is not effective in CD patients currently and what can be addressed to potentially make IL-10 supplementation a more viable treatment option in the future. Based on the current research we conclude that IL-10 supplementation is not a one size fits all treatment and if the correct population of patients is chosen then IL-10 supplementation could be of benefit.

Key words: Inflammatory bowel disease; Crohn's disease; Interleukin-10; Recombinant human interleukin-10

Core tip: Inflammatory bowel disease (IBD) is a chronic condition with no known cure. This review addresses the current available treatments for IBD before discussing a potential new treatment strategy using the immunoregulatory cytokine interleukin-10 (IL-10). To date clinical trial results have been disappointing. We highlight the limitations of current IL-10 supplementation treatment and suggest how, with changes to IL-10 delivery and the correct choice of patient, IL-10 supplementation could become a viable treatment option.

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INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic intestinal disorders that are typified by ulcerative colitis (UC) and Crohn's disease (CD). They are considered to be caused by an aberrant intestinal immune response to commensal microbiota in genetically susceptible individuals[1-3]. IBD

affects over 1.4 million people in the United States and over 2.2 million in Europe and is on the increase[4-7]. In New Zealand CD affects 16 per 100000 and UC 7 per 100000. Clinical symptoms include pain, diarrhoea, rectal bleeding and weight loss, which can have a debilitating effect on sufferers[8]. There are both environmental and genetic factors that have a role in the development and progression of IBD. IBD is more prevalent in "Westernised" countries, believed to be a result of diet and lifestyle and also an effect of improved sanitation[9-11].

Genome-wide association studies (GWAS) have highlighted the complexity of IBD. To date, 163 IBD susceptibility loci have been identified[12], 30 associated with CD, 23 with UC and 110 with both[10,12-14]. Some susceptibility genes have been identified, covering genes involved in autophagy (*ATG16L1* and *IRGM*), pattern recognition receptors, intestinal epithelium maintenance and immune response[4].

The anti-inflammatory cytokine interleukin-10 (IL-10) has been identified as being involved in IBD[15]. Studies[16-18] have shown that polymorphisms in the *IL-10* promoter alter IL-10 serum levels and have been linked to IBD. IL-10 supplementation has been tested as a potential therapy for CD[19-28]. This review will focus on the use of IL-10 supplementation explaining why it is currently ineffective at treating patients with CD and showing how that effectiveness could be improved.

IL-10

Functions of IL-10

IL-10 was first identified as a cytokine secreted by $CD4^+$ Th2-cells that inhibits cytokine production in antigen presenting cells[29], and was described as a cytokine synthesis inhibitory factor. The gene for human *IL-10* is located in the 1q32 band on chromosome 1 and encodes for 5 exons. The encoded protein is a homodimer with a mass of 37 kDa consisting of 160 amino acid monomers[16,19,30]. The structure of IL-10 resembles interferon gamma (IFN-γ) and both IL-10 receptor (IL-10R) subunits are members of the interferon receptor family[31,32].

IL-10 is a pluripotent cytokine and could be considered the most important anti-inflammatory cytokine found in the human immune response[4,19]. IL-10 is produced by different cell types including B- and T-lymphocytes, macrophages, monocytes, dendritic cells and mast cells[16,33]. IL-10 has the ability to differentially affect the function of different subsets of immune cells, affecting both the innate immune system and the adaptive immune system, and is therefore considered to have a broad effect in immunoregulation and host defense[34]. Broadly speaking, IL-10 inhibits pro-inflammatory mediator production while increasing the production of anti-inflammatory mediators[19,34].

Many of the pro-inflammatory cytokines suppressed by IL-10 are known to be regulated by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Dysregulation of NF-κB has been implicated in the pathogenesis of chronic inflammatory disease including IBD[35,36]. It has been shown that IL-10 can block IKK activation and directly inhibit the nuclear localisation ofthe NF-κB p65/p50 heterodimer[37,38]. It has also been shown that IL-10 can selectively induce nuclear translocation and DNA-binding of p50 homodimer, which has been shown to inhibit transcription[39].

IL-10 down-regulates major histocompatibility complex Ⅱ (MHC class Ⅱ) expression[40] and the expression of the co-stimulatory ligands CD80/CD86 (B7-1, B7-2) in monocytes[41], macrophages[42,43] and dendritic cells[44,45]. While both MHC class Ⅱ and co-stimulatory ligands are needed to effectively activate $CD4^+$ Th2 cells by antigen presentation, this results in decreased macrophage and T cell derived cytokine synthesis, *e.g.*, IL-1, IL-6, IL-8, IFN-α, tumor necrosis factor-α[25,46-48]. However IL-10 also has immunostimulatory effects, by up-regulating MHC class Ⅱ expression on B lymphocytes[49] and increasing the synthesis of several antibody isotypes *e.g.,* immunoglobulins (IgM, IgA and IgG)[50].

Despite unanswered questions, our current knowledge credits IL-10 with having a significant critical role in regulating intestinal immune homeostasis, this is highlighted by the fact that impaired IL-10 signalling contributes to IBD[4,51,52]. Rare homozygous mutations in *IL10RA* and *IL10RB*, resulting in defective IL-10 signalling were identified in children with early-onset IBD[52] thereby confirming IL-10's critical role in maintaining intestinal homeostasis.

IL-10 signalling

During IL-10 signalling the IL-10 homodimer binds to the tetrameric receptor IL-10R complex, consisting of 2 molecules of IL-10R α-chain (IL-10R1) and two molecules of the IL-10R β-chain (IL-10R2)[53-55]. This binding activates Janus Kinase 1 (JAK1) and tyrosine kinase 2 (Tyk2), which self-phosphorylate and subsequently phosphorylate IL-10R1 at tyrosine residues, 446 and 496, which recruits signal transducer and activator of transcription 3 (STAT3) *via* its SH2-domain. STAT3 is phosphorylated by JAK1 and Tyk2, causing dimerisation and translocation to the nucleus, where target genes are induced[2,4,20,53] (Figure 1).

There is contradictory evidence regarding the role of STAT3 in IBD[56], with studies showing that it can play both a pathogenic[57-60] or a regulatory[61-64] role in IBD depending on the specific activator and cell type[65]. STAT3 mediates mucosa-protective functions in epithelial and myeloid cells but can also contribute to inflammation if active in other cell types[66]. It has been shown that STAT3 is essential for all known functions of IL-10 and that STAT3 acts as a transcription factor for other genes within the anti-inflammatory response[67,68]. STAT3 is primarily recruited and activated in macrophages, and this activation is transient[69], which avoids the inflammation associated with an increase of activated STAT3 in IBD[70-72].

Genetic variants in *IL-10*, the IL-10 receptor and *STAT3* genes are associated with IBD, highlighting the

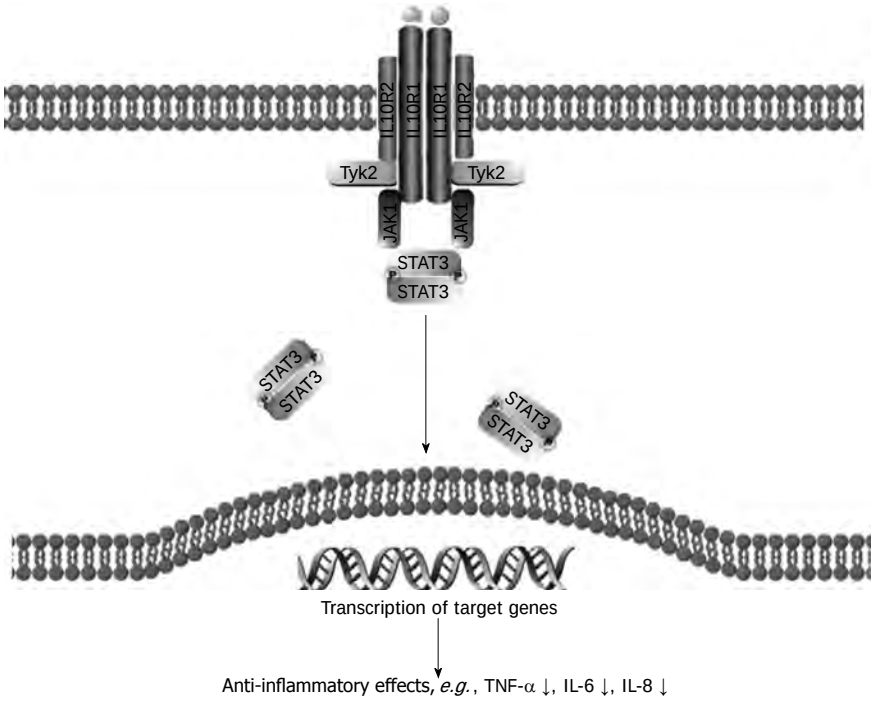

Figure 1 Interleukin-10 signalling pathway. Interleukin-10 (IL-10) binds to the tetrameric receptor IL-10 receptor (IL-10R) complex, this activates Janus kinase 1 (JAK1) and tyrosine kinase 2 (Tyk2), which self-phosphorylate resulting in the binding and phosphorylation of signal transducer and activator of transcription 3 (STAT3). STAT3 dimerises and translocates to the nucleus, inducing target genes. TNF-α: Tumor necrosis factor-α.

involvement of the IL-10 signalling cascade in the pathogenesis of CD and UC, further supporting the hypothesis that defective anti-inflammatory mechanisms may be key to IBD development[2,11,15,52,73-75].

IL-10 AND IBD

How IL-10 relates to IBD

The first evidence of a role of IL-10 in IBD, came from a GWAS study by Franke *et al*[15] that showed a significant ($P = 1.35 \times 10^{-12}$) association between a single nucleotide polymorphism (SNP) rs3024505 near the three-prime untranslated regions of the *IL-10* gene and UC, there was modest association with CD.

IL-10 knockout mice develop chronic enterocolitis, which is similar to human CD, if they are not kept in germ-free conditions. Administration of IL-10 ameliorates inflammation in both animal and *in vitro* models[76], indicating a potential role for IL-10 in the down-regulation of Th1-mediated mucosal inflammation[16,77].

Because IL-10 mediated immune responses are so important in maintaining intestinal homeostasis and commensal flora tolerance, it has been hypothesized that a defect in IL-10 production may be involved in the pathogenesis of CD[78]. In fact impaired IL-10 production has been found in severe cases of both UC[79] and CD[78]. Studies show that CD patients have normal[80,81] or high IL-10 levels[18,82]. However, low IL-10 production in intestinal mucosa has been shown to be associated with increased postoperative recurrence[22,83] and it has been shown that administration of recombinant human IL-10 in low IL-10 producers significantly reduced recurrence after surgery[22].

IL-10 mucosal levels

It has been shown that intestinal epithelial cells from healthy and inflamed colonic tissue express IL-10 mRNA and protein to the same extent. However during inflammation and also in patients with CD, there are significantly increased numbers of mononuclear cells producing IL-10[20,25]. Circulating levels of IL-10, as determined by serum levels of IL-10[82] and mRNA levels[84], have been shown to correlate with disease activity.

IL-10 serum levels

It is believed that circulating levels of IL-10 are critical in immune regulation. Basal levels of IL-10 modulate production of other cytokines and thus minor changes can affect the cytokine network, which in turn affects inflammation.

Studies have been inconsistent regarding serum levels of IL-10 in IBD, as stated earlier some studies show higher IL-10 levels in CD, Wang *et al*[18] found that CD patients had significantly higher levels of IL-10 compared to controls. Kucharzik *et al*[82] reported increased serum IL-10 concentrations in patients with active CD or UC compared to controls. Mitsuyama *et al*[85] showed an increase in serum IL-10 in active UC patients but not CD. In contrast, Nielsen *et al*[81] reported that serum IL-10 concentrations did not differ among UC, CD and healthy control subjects. These inconsistencies could be the result of variations, *e.g.,* age, severity of disease and ethnicity in the studied populations or in different methodological designs.

As IL-10 is an anti-inflammatory cytokine, we expect that high serum levels of IL-10 are likely to be good for patients with chronic inflammatory disease. In fact low IL-10 levels are known to increase disease severity in CD patients compared to high IL-10 levels[85,86]. From steroid treatment it has been shown that steroid non-responders have low IL-10 levels while steroid responders have sus-

tainable high IL-10 levels during and after treatment[87]. Sufficient IL-10 levels seem to be required for recovery but do not offer a cure.

We can hypothesize that IL-10 has an optimal level to be beneficial to reduce chronic inflammatory diseases, and may prove detrimental at too high or too low levels. Diseases associated with IL-10 SNPs such as psoriasis and rheumatoid arthritis are known to have high IL-10 serum levels[18,88,89], while in other diseases like UC, IL-10 levels vary between individuals and studies, with a trend toward increased IL-10 production, though the big studies are lacking[90-92].

IL-10 serum level and disease severity is not restricted to IBD, other diseases including autoimmune diseases, such as systemic lupus erythematosis, Behçets, type 1 diabetes mellitus[14], psoriasis[93], atherosclerosis[94] and rheumatoid arthritis[89] have all been shown to be associated with *IL-10* SNPs. Susceptibility to several cancers including prostate[95], breast[96], cervical[97] and more recently gastric[98,99] have been associated with *IL-10* promoter polymorphisms.

IL-10 promoter polymorphisms

SNPs are the most common form of genetic variation in humans. A SNP occurs at a location where more than one possible nucleotide occurs naturally within a population at a frequency >1%[100]. SNPs can be in both coding and non-coding regions of DNA. Due to the degeneracy of the genetic code. Even if the SNP is in a gene it may not change the amino acid and so has no effect on the protein (synonymous SNP), however non-synonymous SNPs do change the protein and are more commonly associated with disease. It is these variations that are most interesting to researchers as these can account for whether/how a person develops a disease, the severity of disease and how they respond to treatment.

Important variability in IL-10 secretion has been reported and is associated with SNPs in the *IL-10* promoter at 3 locations -592, -819, -1082[78,101,102]. The *IL-10* promoter polymorphisms C-592A (rs1800872), C-819T (rs1800871) and G-1082A (rs1800896) have been extensively studied. The most recent studies of Franke *et al*[15], Amre *et al*[17], Wang *et al*[18], Andersen *et al*[73], Fowler *et al*[103], Fernandez *et al*[104] and Tedde *et al*[105] reported a significant association between IL-10 rs1800896 and IBD.

The "A" allele of rs1800896 was found to be more common in IBD patients, especially in UC patients, individuals with the A/A genotype have lower IL-10 production than the G/G genotype[106], Koss *et al*[107] found that the -1082 AA is associated with decreased IL-10 production in both CD patients and controls. Wildtype -1082 (rs1800896) "G" and -592 (rs1800872) "C" are known to be associated with increased IL-10 levels; therefore we expect the GCC haplotype to show the highest IL-10 expression and ATA the lowest. This hypothesis was studied by Reuss *et al*[108] who showed in THP-1 monocyte cells that IL-10 expression was highest in the GCC haplotype compared to ACC and ATA (P = 0.042 and P = 0.0026). In the twin-study which followed, the haplotype showed no correlation with IL-10 serum levels. Wang *et al*[18] showed a significant (P = 0.001) increase in IL-10 production for TAT haplotype in healthy controls. The -592A allele was also shown to be associated with reduced transcription and decreased IL-10 secretion[16,102,109].

CURRENT IBD TREATMENTS

The current treatment options available for IBD, include: surgery, aminosalicylates, *e.g.,* 5-aminosalicylic acid, corticosteroids, *e.g.,* prednisone, immunosuppressants, *e.g.,* azathioprine, cyclosporine or biologicals, *e.g.,* infliximab[110,111]. The choice of treatment is dependent on phenotype, disease activity, characteristics of the drug and the patient. The choice should look to balance effectiveness with side effects and long term complications. As with any drug treatment there are side effects, these range from the usually well-tolerated upset stomach, nausea and headache to the more severe bone marrow and liver problems. As well as the associated side effects these treatments only work for some cases and can also result in a loss of response. Thus stronger treatments are required which have more severe side effects and long term consequences, and so alternative therapies are being investigated.

ALTERNATIVE TREATMENTS

Environmental and dietary factors are thought to play a role in the development of CD[112,113] and so changes to diet and lifestyle can have beneficial effects. Studies[114-118] have shown that specific foods are associated with IBD and that avoiding certain foods can reduce both the severity and frequency of symptoms. There are several classes of new drugs being developed: monoclonal antibodies, small molecules, fusion proteins and recombinant growth factors, as well as stem cell based therapies; one of these new therapies is IL-10 supplementation.

IL-10 supplementation for CD

Studies have suggested that IL-10 has huge therapeutic potential in intestinal inflammation, and that it should inhibit the up-regulated pro-inflammatory cytokines in CD and UC[25]. In most studies to date, Tenovil (Schering-Plough, Kenilworth, NJ, United States), has been used, which is the brand name of rhuIL-10. It is produced by a genetically engineered *Escherichia coli* strain, that expresses a 161 amino acid protein identical to human IL-10 with an additional amino-terminal methionine residue[119,120].

Why doesn't it work?

Based on the success of animal models[121-126] of intestinal inflammation, IL-10 therapy was heralded as a potential anti-inflammatory treatment in CD and several human trials have been undertaken. The first trial conducted by van Deventer *et al*[28] showed that IL-10 supplementation

Table 1 Summary of key findings from interleukin-10 trials in human and animal studies

Ref.	Model	Intervention	Outcome
	Human		
Colombel *et al*[22]	65 patients having recently undergone intestinal resection surgery	4 μg/kg daily or 8 μg/kg twice weekly for 12 wk	No clear evidence of effect
Fedorak *et al*[23]	95 mild to moderately active CD (CDAI 200-350)	1, 5, 10 or 20 μg/kg of daily for 4, 20 wk follow up	Improved clinical response (based on CDAI score) and improved endoscopic appearance
Schreiber *et al*[26]	329 therapy-refractory chronic active CD (CDAI 200-400)	1, 4, 8 or 20 μg/kg of Tenovil subcutaneously for 28 d	Non-significant clinical improvements
van Deventer *et al*[28]	46 patients with active steroid-resistant CD (CDAI 200-350)	0.5, 1, 5, 10 or 25 μg/kg daily for 1, 3 wk follow up	Reduction in the average score of CDAI
Braat *et al*[128]	10 patients with moderate to severe CD	10 enteric-coated capsules containing 10^{10} cfu of LL-Thy12 twice daily for 7 d	Clinical benefit observed in 8 of 10 patients, including 5 showing complete remission
	Animal		
Barbara *et al*[121]	DNB induced colitis Spf Sprague-Dawley rats	Ad5IL-10 (5×10^8-1×10^{10} pfu)	Improved colitis macroscopically and histologically and decreased MPO activity and LTB4 levels
Grool *et al*[123]	40 male NZ white rabbits formalin-immune complex induced colitis	100 or 500 μg/kg single IV infusion of rIL-10	Anti-inflammatory response as measured by decreased mucosal damage, leukocyte recruitment, MPO and LTB4
Ribbons *et al*[124]	TNBS induced colitis in 74 Sprague-Dawley rats	0.5, 5, 50, 500 μg/kg rIL-10 subcutaneous injection twice daily for 5 d	Mild anti-inflammatory effects Significant reduction in MPO
Sasaki *et al*[125]	3% DSS induced C57B6 mice	Intra-peritoneal administration of adIL-10	Significantly reduced disease activity and weight loss and completely prevented histopathologic injury to the colon
Tomoyose *et al*[126]	4% DSS induced colitis BALB/c mice	Recombinant mouse rIL-10 (1, 100, 1000 unit/mL)	Marked improvement in intestinal inflammation Inhibition of tissue damage and production of pro-inflammatory cytokines
Steidler *et al*[127]	DSS induced and spontaneous IL10$^{-/-}$ mouse models of colitis	Daily intragastric inocula of 2×10^7 or 10^9 LL-mIL10	Reduced histological score by 50% in DSS and prevented onset of colitis in IL-10$^{-/-}$ mice

CD: Crohn's disease; CDAI: Crohn's disease activity index; cfu: Colony forming units; MPO: Myeloperoxidase; LTB4: Leukotriene B4; TNBS: 2, 4, 6 trinitrobenzenesulfonic acid; DSS: Dextran sodium sulphate; adIL-10: Adenoviral IL-10; DNB: Dinitrobenzene sulphonic acid; Spf: Specific pathogen free; Ad5IL-10: Human type 5 adenovirus + murine IL-10; pfu: Plaque forming units; LL-mIL10: *Lactococcus lactis* secreting murine IL-10.

was safe and well tolerated. This was confirmed by subsequent studies[22,23,26]. van Deventer *et al*[28] showed a reduction in the average score of CD activity index (CDAI), but this was not significant. Fedorak *et al*[23] showed that 5 μg/kg of Tenovil given subcutaneously for 28 d to patients with mild to moderate CD activity resulted in improved clinical response (based on CDAI score) and improved endoscopic appearance of the disease. Schreiber *et al*[26] showed that 8 μg/kg of Tenovil given subcutaneously for 28 d to patients with mild to moderate CD activity resulted in a non-significant clinical improvement. However, Colombel *et al*[22] found no evidence that treatment with Tenovil for 12 wk in CD patients after intestinal resection prevented recurrence of CD. The key findings of these studies are summarised in Table 1.

These data show that IL-10 treatment did not result in significantly reduced remission rates or clinical improvements when compared to placebo[21,24]. In fact a Cochrane review in 2010[21] concluded that "Interleukin 10 does not appear to provide any treatment of active Crohn's disease. ...interleukin 10 does not increase the number of remissions (complete or clinical), but increases the rate of withdrawal due to adverse events relative to placebo." This review only included three of the studies mentioned above[23,26,28] and although more patients receiving IL-10 withdrew from studies there was no significant difference in the number of patients reporting adverse reactions between treatment and control.

However this is not the whole story, as other studies not included in the Cochrane analysis have shown that patients respond differently to IL-10 supplementation. Colombel *et al*[22] reported that endoscopic recurrence in patients with low IL-10 levels at time of surgery reduced to 47% with Tenovil treatment compared to 80% in the placebo group. Schreiber *et al*[26] found that patients responded differently to IL-10 treatment, with patients suffering from high disease activity having a greater rate of clinical improvement. These data suggest that IL-10 levels and disease activity are factors in how a patient responds to IL-10 supplementation. Also, as previously stated, some CD patients already have raised levels of IL-10[18,82]. These patients will not benefit from IL-10 supplementation and may suffer detrimental effects as high doses of systemically administered IL-10 induce the pro-inflammatory cytokine IFN-γ[27].

The different response to IL-10 supplementation is not surprising given the heterogenous nature of CD. A therapy that targets one step within a complex immunological pathway may only benefit a small proportion of patients but if you select the correct sub-population of patients who under-produce IL-10, for example those that have a penetrating phenotype who have a greater deficiency in IL-10[78], you may see a significant beneficial response to IL-10 therapy[25].

There are five potential explanations as to why IL-10 treatment has not been effective as a therapeutic strategy: (1) the administered dose of IL-10 results in an intestinal concentration of IL-10 that is too low to elicit a response; (2) there are differences among individuals depending upon disease phenotype/severity; (3) IL-10 is only successful at preventing and not treating an established disease; (4) IL-10 alone fails to suppress all the pro-inflammatory mediators involved in chronic inflammation; or (5) IL-10's immunostimulatory effects counterbalance its immunosuppressive properties.

Can IL-10 supplementation work?

Most of the potential explanations as to why IL-10 supplementation currently doesn't work can be overcome.

The modest therapeutic benefits[23,26] and adverse effects can potentially be attributed to limited mucosal bioavailability of IL-10 and the fact that the trials so far have not separated patients by genotype or disease phenotype/severity. To address the low bioavailability of mucosal IL-10 without resorting to the detrimental high levels of IL-10 systemic administration, *Lactococcus lactis* (*L. lactis*) was engineered to secrete IL-10, and this was used to successfully prevent the onset of colitis in the IL-10 KO model and caused a 50% reduction in inflammation in the DSS mouse model[127]. This study was followed up with a small phase 1 human trial using *L. lactis* modified to contain the human IL-10 sequence (LL-Thy12)[128]. 10 capsules containing 1×10^{10} cfu of LL-Thy12 were given to 10 patients with moderate to severe CD twice daily for 7 d. The results showed this approach is both safe and biologically contained, avoiding the side effects associated with high systemic doses while still retaining the ability to reduce disease activity. This was a small trial in a controlled environment without a control comparison and so further studies are needed to confirm the effectiveness of this treatment. However based on these initial results this form of IL-10 supplementation is showing promise as a treatment for patients with chronic intestinal inflammation.

Alternative ways to improve local delivery of IL-10 include gene therapy using replication-deficient adenoviral vectors delivered directly to the gastrointestinal epithelial cells. This approach has proven successful in two mouse studies[121,129] showing an effect on colitis without the associated side-effects of systemic administration. Gelatine microspheres containing IL-10 (GM-IL-10) were developed by Nakase *et al*[130] to deliver sustained IL-10 release locally without losing bioactivity. Colonic inflammation in mice treated with GM-IL-10 was reduced compared to mice treated with IL-10 alone.

The second point of selecting patients based on disease phenotype and/or severity can be easily addressed based on clinical diagnosis. Selecting patients based on genotype is slightly more complicated and would require that potential candidates for treatment be screened. Genotyping has become relatively quick and easy to perform and the cost is reducing as the technology advances. However who actually performs the genotyping service, who pays for the service and gaining patient consent may be problematic. This could potentially be overcome by having the patients enrol onto a research study. However IL-10 serum levels are determined 50% by genetics and 50% by environment[108] and so just because a person has the low IL-10 producing SNP doesn't necessarily mean they will have low IL-10 levels. Therefore a better measure to determine potential benefit of IL-10 supplementation would be to measure the serum level of IL-10, which can be done using commercially available ELISA kits. This should prove to be easier to conduct and in gaining patient consent.

As mouse models proved[121,131] IL-10 administration was only successful when administered prior to initiation of colitis and was unable to treat any established inflammation. Therefore IL-10 supplementation could be used to prevent relapses rather than to treat active inflammation.

If locally delivered IL-10 fails to have an effect, then it may be due to the fact IL-10 alone is unable to suppress all the pro-inflammatory mediators involved in chronic inflammation.Therefore it would be necessary to develop a combination treatment containing IL-10. However the evidence suggests IL-10 alone should have an effect and so it may be that IL-10 supplementation is not a suitable treatment for that disease phenotype.

CONCLUSION

Based on this knowledge, it is our opinion that a subpopulation of CD patients, who have lower expression of IL-10, and who have active disease could benefit from targeted IL-10 supplementation therapy. However further studies are needed to determine the exact population of patients who would benefit the most from this treatment and to determine if there are any long term detrimental effects of this treatment. Given that current treatments of IBD may not be beneficial to a patient or have severe side effects, we believe it is worth exploring this potential treatment avenue.

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REVIEW

Treatment options of inflammatory appendiceal masses in adults

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Abstract

At present, the treatment of choice for uncomplicated acute appendicitis in adults continues to be surgical. The inflammation in acute appendicitis may sometimes be enclosed by the patient's own defense mechanisms, by the formation of an inflammatory phlegmon or a circumscribed abscess. The management of these patients is controversial. Immediate appendectomy may be technically demanding. The exploration often ends up in an ileocecal resection or a right-sided hemicolectomy. Recently, the conditions for conservative management of these patients have changed due to the development of computed tomography and ultrasound, which has improved the diagnosis of enclosed inflammation and made drainage of intra-abdominal abscesses easier. New efficient antibiotics have also given new opportunities for nonsurgical treatment of complicated appendicitis. The traditional management of these patients is nonsurgical treatment followed by interval appendectomy to prevent recurrence. The need for interval appendectomy after successful nonsurgical treatment has recently been questioned because the risk of recurrence is relatively small. After successful nonsurgical treatment of an appendiceal mass, the true diagnosis is uncertain in some cases and an underlying diagnosis of cancer or Crohn's disease may be delayed. This report aims at reviewing the treatment options of patients with enclosed appendiceal inflammation, with emphasis on the success rate of nonsurgical treatment, the need for drainage of abscesses, the risk of undetected serious disease, and the need for interval appendectomy to prevent recurrence.

Key words: Appendicitis; Phlegmon; Abscess; Computed tomography; Antibiotics; Percutaneous drainage; Surgery

Core tip: The management of adult patients with inflammatory appendiceal masses is controversial. This report aims at reviewing the treatment options of these patients, with emphasis on the success rate of nonsurgical treatment, the need for drainage of abscesses, the risk of undetected serious disease, and the need for interval appendectomy to prevent recurrence. The debate arises over the importance of the complication rate of interval appendicectomy. Moreover, if appendicectomy is not performed, consideration needs to be given to what investigations should be undertaken and in which patients. It is also worth recalling that the appendix is used in reconstructive surgery.

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INTRODUCTION

Acute appendicitis is one of the most common causes of acute abdomen and can be classified into uncomplicated

and complicated. The life-time risk of appendicitis is 7%-8%, with the highest incidence in the second decade. The inflammation in acute appendicitis may sometimes be enclosed by the patients own defense mechanisms, by the formation of an inflammatory phlegmon or a circumscribed abscess. The management of these patients is controversial. Immediate appendectomy may be technically demanding because of the distorted anatomy and the difficulties to close the appendiceal stump because of the inflamed tissues. The exploration often ends in ileocecal resection or a right-sided hemicolectomy due to the technical problems or a suspicion of malignancy because of the distorted tissues[1-9]. Recently, the conditions for conservative management of these patients has changed due to the development of computed tomography (CT) and ultrasound (US), which has improved the diagnosis of enclosed inflammation and made drainage of intra-abdominal abscesses easier[10-15]. New efficient antibiotics have also given new opportunities for nonsurgical treatment of appendicitis[16-21]. The traditional management of these patients is nonsurgical treatment followed by interval appendectomy to prevent recurrence. The need for interval appendectomy after successful nonsurgical treatment has recently been questioned because the risk of recurrence is relatively small[22-27]. After successful nonsurgical treatment of an appendiceal mass, the true diagnosis is uncertain in some cases and an underlying diagnosis of cancer or Crohn's disease (CD) may be delayed[27].

This report reviews the treatment options of patients with enclosed appendiceal inflammation, with emphasis on the success rate of nonsurgical treatment, the need for drainage of abscesses, the risk of undetected serious disease, and the need for interval appendectomy to prevent recurrence. The debate arises over the importance and level of the complication rate of interval appendicectomy. Moreover, if appendicectomy is not performed, consideration needs to be given to what investigations should be undertaken and in which patients. It is also worth recalling that the appendix is occasionally used in reconstructive surgery[26,28].

DEFINITIONS

Acute appendicitis is inflammation of the vermiform appendix and remains the most common cause of the acute abdomen in young adults. The term complicated appendicitis is often used to describe a palpable appendiceal mass, an appendiceal phlegmon, or a localized abscess without distinction. A phlegmon is an inflammatory tumor consisting of the inflamed appendix, its adjacent viscera and the greater omentum, whereas an abscess is a pus-containing appendiceal mass[27-31]. The diagnosis of enclosed inflammation is made by finding a palpable mass at clinical examination before or after anesthesia, or by finding an inflammatory mass or a circumscribed abscess by CT, US or at surgical exploration of the abdomen. We consider that nonsurgical treatment has failed when the patient undergoes appendectomy during the same hospital stay after attempted nonsurgical treatment. The patients treated with drainage are those who had drainage (without appendectomy) of an abscess either percutaneously or by surgical exploration. Morbidity includes postoperative infectious complications, intestinal fistula, small bowel obstruction, and recurrence after initially successful nonsurgical management[27].

TREATMENT OPTIONS OF NONCOMPLICATED ACUTE APPENDICITIS

Although the etiology of acute appendicitis is poorly understood, it is probably caused by luminal obstruction in the majority of cases. Luminal obstruction can be caused by fecaliths, lymphoid hyperplasia, foreign bodies, parasites and both primary (carcinoid, adenocarcinoma, Kaposi sarcoma and lymphoma) and metastatic (breast and colon) tumors. Once appendiceal obstruction occurs, the continued secretion of mucus results in elevated intraluminal pressure and luminal distention. This eventually exceeds capillary perfusion pressure, which leads to venous engorgement, arterial compression, and tissue ischemia. As the epithelial mucosal barrier becomes compromised, luminal bacteria multiply and invade the appendiceal wall, which causes transluminal inflammation. The most common bacteria that can cause acute appendicitis are intestinal bacteria including *Escherichia coli* and bacteria belonging to the *Bacteroides fragilis* group. Continued ischemia results in appendiceal infarction and perforation[29-31]. However, the observation of spontaneous resolution of acute appendicitis cases and some reports of a good outcome in patients treated with antibiotics suggest that not all cases of acute appendicitis are caused by mechanical obstruction and progression to complicated disease. Some researchers have suggested that uncomplicated and complicated forms of appendicitis are two distinct diseases, with different etiologies. As in other intra-abdominal infections, such as salpingitis, diverticulitis and enterocolitis, which are often treated only with antibiotics, the infectious etiology of acute appendicitis is advocated by some scholars. Conservative treatment is most effective when administered within 12 h of symptom onset, ideally within the first 6 h[16-21,29-33]. Antibiotic therapy is associated with a 68%-84% success rate and a trend toward decreased risk of complications without prolonging hospital stay. The authors have described a low morbidity and mortality rate, and a recurrence rate between 5% and 15%[25-33].

At present, the treatment of choice for uncomplicated acute appendicitis in adults continues to be surgical (open or laparoscopy) and it is the gold standard. The most common operative complications are wound infection, intra-abdominal abscess, and ileus caused by intra-abdominal adhesions (Dindo *et al*[34] classification), which vary in frequency between open and laparoscopic appendectomy. The overall complication rates for open and laparoscopic appendectomy are respectively 11.1% and 8.7%, with a mortality rate < 0.5%[35-41]. The exclusive treatment with antibiotics cannot be routinely recommended in current

medical practice and should only be considered in selected patients or conditions in which surgery is contraindicated or in the context of clinical studies[18,19,31,32].

PROPORTION OF PATIENTS WITH APPENDICITIS WHO DEVELOP ENCLOSED APPENDICEAL INFLAMMATION AND CLINICAL PRESENTATION

Circumscribed appendiceal inflammation is common and often undiagnosed preoperatively. The proportion of all patients with appendicitis treated for enclosed inflammation is 3.8%-5.0%. The risk of perforation is negligible within the first 12 h of untreated symptoms, but then increases to 8.0% within the first 24 h. It then decreases to 1.3%-2.0% during 36-48 h, and subsequently increases again to 5.8%-7.6% for each ensuing 24-h period[42-47].

The diagnosis is suspected in patients with a palpable mass or with symptom duration > 3 d and is more common in children, especially in those aged < 5 years. Delay in presentation, age > 55 years, and elevated temperature (> 38.8 ℃) on admission are predictors of perforated appendicitis. Additionally, patients older than 55 years of age have a 29% prevalence of perforated appendicitis in the first 36 h from symptom onset. Patients with hyperbilirubinemia and clinical symptoms of appendicitis should be identified as having a higher probability of appendiceal perforation than those with normal bilirubin levels[48,49].

Enclosed inflammation is found more often in studies in which the diagnosis is based on CT or US than in those based on clinical diagnosis (14.2% *vs* 5.1%). It is also more common in children than in adults as shown by the trend of 8.8% in children, 6.5% in patients of all ages, and 4.8% in adults. There is an early risk of perforation even within the first 36 h of symptom onset, which may be higher in men than women. This suggests that diagnostic imaging should be used more frequently in children, in patients with a long duration of symptoms, and in patients with a palpable mass. Appendectomy should be performed without delay in adults, especially men and those aged > 55 years once diagnosis is confirmed[42-47].

RADIOLOGICAL DIAGNOSIS

There is continued debate about the relative merits of US and CT[10-15,50-59]; the latest meta-analysis has concluded that CT[60-69] is significantly more sensitive than US for the diagnosis of appendicitis, but that US should be considered in children. Sonography has high sensitivity (86%-100%), specificity (88%-95%), and accuracy (91%-92%) in diagnosing acute appendicitis. CT is comparable to sonography with respect to sensitivity, specificity, and accuracy for adults (90%-97%, 93%-100%, and 94%-99%, respectively) and children (95%-97%, 91%-99%, and 96%, respectively) with appendiceal diameter > 6 mm, although some studies have revealed lower diagnostic rates in children than in adults. The major area of debate is regarding which patients suspected of having acute appendicitis should have a CT scan before appendectomy. There are several articles in the literature that argue against routine preoperative imaging of patients with suspected acute appendicitis. In these articles, the routine use of imaging has not been shown to decrease the rate of negative appendectomy, and may actually delay the diagnosis and appropriate intervention in cases of acute appendicitis. Other studies have shown a benefit from preoperative imaging in suspected acute appendicitis, and the development of guidelines for CT in patients with an equivocal presentation has decreased the rate of negative appendectomy from 25% to 6%. A review of a large, prospectively gathered database of general surgical procedures in Washington state has found the negative appendectomy rate to be 9.8% in patients with no preoperative imaging and only 4.5% in those who had a preoperative CT scan. This difference was statistically significant. Based on these findings, CT scans seem to have significant benefit in the evaluation of patients with suspected acute appendicitis, to exclude other pathology, in selected patients such as elderly people[52,70].

Various CT techniques have been described for diagnosing acute appendicitis, including enhanced CT with rectally administered colon contrast medium, enhanced focused CT with thin collimation (3-5 mm), nonfocused technique with oral and intravenous contrast material, focused technique with oral contrast medium, and focused helical CT with colonic contrast medium, and have a high diagnostic accuracy. CT provides a rapid complete diagnostic evaluation of the right lower quadrant, with reported accuracy rates in the diagnosis of appendicitis of up to 95%-100%[11,52,66]. The obvious disadvantages of CT include exposure to ionizing radiation and the potential for contrast medium reactions. Those who benefit most from preoperative imaging are those with an atypical presentation and women of childbearing age. However, it is recognized that this is not without increased cost, radiation exposure and a potential delay in diagnosis. The use of US is particularly important in children and can be of use in premenopausal women[50-52,58]. Institution of a clinical pathway using CT can lead to a substantial decrease in the number of negative appendectomies from 16% to 4%. CT has greater potential than US to reveal alternative diagnoses and complications, such as perforation and abscess formation. US has lower sensitivity than CT in the setting of appendiceal perforation. The appendix is significantly larger in diameter in perforated appendicitis than in appendicitis with no perforation (15 mm *vs* 11 mm). Direct CT signs (*i.e.*, phlegmon, abscess, and extraluminal air) are more specific for perforated appendicitis. Indirect signs (bowel wall thickening, ascites, ileal wall enhancement, intraluminal air, and combined intraluminal air and appendicolith) are also found with higher incidence in appendiceal perforation[13,53,54,61,63]. Intraluminal appendiceal air in the setting of acute appendicitis is a marker of perforated or necrotic appendicitis.

Recognition of this finding in otherwise uncomplicated appendicitis at imaging should raise suspicion for image-occult perforation or necrosis[56]. Defect in the enhancing appendiceal wall allows excellent sensitivity (94.9%) and specificity (94.5%) for the diagnosis of perforated appendicitis when evaluated in a group of patients with known appendicitis. A defect in the enhancing appendiceal wall has the highest sensitivity (64.3%) of any individual finding[53]. Detecting a defect in the enhancing appendiceal wall by using cine mode display of transverse thin-section CT images allows 96.1% accuracy for diagnosing appendiceal perforation[55]. In one series, appendicolith, free fluid, a focal defect in the enhancing appendiceal wall, and enlarged abdominal lymph nodes were not sensitive or specific for the presence of perforation. That study has concluded that unless abscess or extraluminal gas is present multidetector CT cannot establish the diagnosis of perforation[63].

The range of diagnoses that can mimic appendicitis is wide and includes right ureteric calculus, epiploic appendagitis, torsion of Meckel's diverticulum, mesenteric adenitis, inflammatory bowel disease, colitis, gynecological disorders, and right-sided diverticulitis. CT is useful in differentiating between these disorders[63].

Magnetic resonance imaging (MRI) has had little role in the evaluation of acute abdominal pain. However, increasing concerns over the potentially hazardous effects of ionizing radiation associated with CT have made MRI the study of choice to evaluate pregnant women and children with symptoms of appendicitis and equivocal US findings. MRI is highly accurate with a sensitivity of 100%, specificity of 98%, positive predictive value of 98%, and negative predictive value of 100%. Although MRI may be used in any patient with suspected acute appendicitis, there is a special role for MRI in pregnant women with new-onset abdominal pain. MRI has many advantages. It is valuable in the imaging of pregnant women and children because there is no exposure to ionizing radiation. Although MRI is safe during pregnancy, no intravenous contrast should be used during pregnancy because gadolinium is a category C drug and potentially teratogenic. However, noncontrast MRI provides detailed images, which usually provide the correct diagnosis. MRI is operator independent and the results are highly reproducible. MRI is more useful than US in obese patients and in patients with a retrocecal appendix, which is difficult to visualize on US. Drawbacks of MRI are that it is more expensive than other imaging modalities and not as widely available. The examination itself takes longer to perform and may be degraded by motion artifact. There are concerns that, with the exception of trained radiologists, other health care providers are not comfortable interpreting MRI findings[52,70-73].

IMMEDIATE SURGICAL TREATMENT *VS* NONSURGICAL TREATMENT

Emergency appendectomies are still considered the primary means of treating acute appendicitis, with mortality rates of 0.5%-2.4% and 0.07%-0.7% for patients with and without perforation, respectively. Overall, post-appendectomy complication rates are typically 10%-19% for acute appendicitis without perforation and reach 12%-30% for perforated acute appendicitis[19]. Perforation increases the mortality rate of acute appendicitis from 0.0002% to 3% and increases the morbidity from 3% to 47%[52]. Perforated appendicitis may be treated first by conservative treatment or percutaneous abscess drainage with great improvement of the clinical symptoms[74-80]. This is in contrast to nonperforated appendicitis, which requires operation as early as possible in order to reduce morbidity. Immediate surgical treatment of enclosed appendiceal inflammation is associated with a > 3-fold increase in morbidity compared with conservative management, and may result in an unnecessary ileocecal resection or right-sided hemicolectomy for technical reasons or suspicion of malignancy in about 3% of patients[9,27]. Nonsurgical treatment is successful in about 93% of patients, but may need percutaneous drainage of abscesses in about 20%. Most perforated appendicitis give way to generalized peritonitis and cannot be drained. Indications of drainage are absence of generalized peritonitis and presence of percutaneously or surgically drainable abscess[75-78]. Nonsurgical treatment is associated with lower morbidity and shorter hospital stay compared with immediate appendectomy. The results of immediate surgery compared with those of nonsurgical treatment, eventually followed by interval appendectomy, have been reported in 19 retrospective studies[27]. Right-sided hemicolectomy for suspicion of a malignant disease or for technical reasons, but where only inflammatory changes could be found at histopathological examination, has been reported in 17 of 493 adult patients. In all but three of the studies, the authors have concluded that nonsurgical treatment is to be recommended. Conservative treatment is associated with significantly fewer overall complications, wound infection, abdominal/pelvic abscess, ileus/bowel obstruction, and reoperation. No significant difference has been found in the duration of first hospitalization, overall duration of hospital stay, and duration of intravenous antibiotics[79]. Immediate surgery is associated with morbidity in 35.6% of patients compared with 13.5% in nonsurgical treatment and an additional 11.0% after interval appendectomy. The majority of the studies have practiced elective interval appendectomy after successful nonsurgical treatment.

PRIMARY NONSURGICAL TREATMENT FOLLOWED BY DELAYED OR INTERVAL APPENDECTOMY OR WITHOUT APPENDECTOMY

The results of primary nonsurgical treatment followed by delayed appendectomy during the same hospital stay have been compared with those of interval appendec-

tomy and with or without surgical intervention 6-12 wk later (interval appendectomy)[80-88]. Delayed appendectomy[89-93] is associated with morbidity in 18.2% compared with 12.4% after interval appendectomy. The return to work takes longer for patients treated with interval appendectomy, mainly because the patients want to have the planned interval appendectomy done before they are willing to return to work. One prospective study[7] has randomized patients to primary nonsurgical treatment followed by delayed or interval or no appendectomy. The group with nonsurgical treatment without appendectomy had the lowest morbidity and the shortest length of stay. In patients with an appendiceal mass, the authors have concluded that conservative treatment without interval appendicectomy is the best treatment.

FAILURE RATE OF NONSURGICAL TREATMENT AND NEED FOR ABSCESS DRAINAGE

All studies have reported a low failure rate for nonsurgical treatment without appendectomy; some of them even without giving antibiotics[75-80]. The failure rate for all the studies was 7.2%. Failure was associated generally with abscess diameter > 4-5 cm[77-79]. The proportion of patients in need of abscess drainage is strongly related to how the diagnosis is made, with 100% in studies of patients selected because of a drained abscess, 47.5% in patients with a palpable mass or preoperatively found abscess, 27.6% in patients with an abscess or phlegmon diagnosed by CT or US, 9.5% in patients with a palpable mass, and no need for drainage in studies of patients with a phlegmon diagnosed by CT or US. There is no association between the need for drainage and patient age.

COMPLICATIONS FOLLOWING INTERVAL APPENDICECTOMY

The morbidity of interval appendectomy has been reported in a few studies with a pooled value of 11.0%[94-97]. The age of the included patients had no influence on the results. The complication rate following interval appendicectomy is a consideration to be balanced against the recurrence rate. The complication rate varies from 8% to 23%. True surgical complications include wound infection (15.0%), pelvic abscess (5.0%), and aspiration pneumonia (1.5%). Another retrospective study reported a complication rate of 13%, but a prolonged fever, which others may not have cited as a true complication, accounted for almost half of these complications and only one wound infection occurred in 38 interval appendicectomies. An 8% complication rate was reported in a review of 50 interval appendicectomies, but about 25% of these were prolonged fever, about 50% cecal damage, and the remainder subcutaneous abscesses. Laparoscopic interval appendicectomy may decrease the complication rate and length of hospital stay[36,92]. A small retrospective study of 10 patients undergoing laparoscopic interval appendicectomy reported no complications and all patients were discharged on the day after surgery. A prospective study of open and laparoscopic appendicectomy for acute appendicitis in 65 patients showed a significantly lower wound infection rate in the laparoscopic group; however, it is not possible to extrapolate directly this finding to interval appendicectomy, even though one would expect a lower wound infection rate. In one study, the morbidity rates, particularly for intra-abdominal abscesses and wound infection, were lower for laparoscopic appendectomy in complicated appendicitis than those reported in the literature for open appendectomy, whereas operating times and hospital stays were similar[88].

RISK OF RECURRENCE

The recurrence rate of appendiceal pathology if appendicectomy is not performed is central to the debate over the use of routine interval appendicectomy. For some authors, the risk of recurrence after successful nonsurgical treatment was about 10% (3%-25% in the literature) and was often associated with an appendicolith. The majority of recurrences occur within 6 mo after initial hospital stay. Recurrence is characterized by a milder course than the primary attack in most cases. Elective interval appendectomy is associated with morbidity in about 11% (0%-23%) of patients. These results do not motivate routine elective interval appendectomy after successful nonsurgical treatment[16,20,27,98]. The literature review shows that at least 75%-90% of routine interval appendicectomies in adults are unnecessary. It would be reasonable and perhaps safer, as malignancy can be missed at appendicectomy, to replace routine interval appendicectomy with adequate follow-up of symptoms, performing appendicectomy only if symptoms recur or persist. Appropriate investigation should be done if the appendix is not removed, provided the patient has access to surgical care should symptoms recur[27].

HISTOLOGY

Several studies have examined the microscopic changes in the interval appendicectomy specimen. Many specimens show chronic inflammatory changes (52%)[5] and acute inflammation (50%)[3,8]. However, this may be of little clinical importance in the asymptomatic patient. The real concern is whether leaving the appendix *in situ* will prevent the detection of a cecal carcinoma or an ileal or appendicular malignancy[27].

RISK OF MISSING OTHER DIAGNOSES

Nonsurgical treatment is associated with a risk of missing or delaying an underlying cancer diagnosis or CD in about 2% of patients. The concern of failing to diagnose a rare case of appendiceal malignancy without interval appendicectomy may persist even with colonic investi-

gation, although it is likely that these patients will have recurrent symptoms[99-101]. Most of the cancer cases occur in patients aged > 40 years. The risk of missing an important alternative diagnosis is probably lower if imaging is used for the diagnosis of enclosed appendiceal inflammation. This underlines the need of follow-up after nonsurgical treatment, especially in patients aged > 40 years. By tradition, this follow-up consists of colonoscopy or a barium study of the colon, but a virtual colonoscopy, CT scan, or US is probably more accurate to detect malignant conditions outside the colon or CD. Malignant disease was detected during follow-up in 1.2% of patients. This risk was related to age at diagnosis with 0.2% in children, 1.8% in studies of all ages, and 1.4% in adults. There was no difference in relation to how the diagnosis was done. CD was detected in 0.7% during follow-up after nonsurgical treatment. This risk was related to age with 0.1% in children, 0.8% in all ages, and 1.5% in adults. There was no difference in relation to how the diagnosis was done. Appendicular malignancy is rare and may be missed if appendicectomy is not performed; however, it is likely that such patients will have either a nonresolving mass or early recurrence. Colonic malignancy is a more common concern, but interval appendicectomy is not a reliable method of detecting a cecal tumor. Imaging is needed when cecal malignancy is possible. Colonic investigation should be a consideration regardless of whether interval appendicectomy is performed[27].

CONCLUSION

In patients with suspicion of contained appendiceal inflammation, based on a palpable mass or long duration of symptoms, the diagnosis should be confirmed by imaging techniques, especially CT scan. The patient should receive primary nonsurgical treatment with antibiotics and abscess drainage as needed. After successful nonsurgical treatment, no interval appendectomy is indicated in some cases, but the patient should be informed about the risk of recurrence especially in the presence of appendicolith. The risk of missing another underlying condition (cancer or CD) is low, but motivates a follow-up with a colon examination and/or a CT scan or US, especially in patients above the age of 40 years.

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MINIREVIEWS

Exposure to ambient air particulate matter and non-alcoholic fatty liver disease

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Abstract

The present study was designed to alert the public opinion and policy makers on the supposed enhancing effects of exposure to ambient air particulate matter with aerodynamic diameters < 2.5 mm ($PM_{2.5}$) on non-alcoholic fatty liver disease (NAFLD), the most common chronic liver disease in Western countries. For far too long literature data have been fixated on pulmonary diseases and/or cardiovascular disease, as consequence of particulate exposure, ignoring the link between the explosion of obesity with related syndromes such as NAFLD and air pollution, the worst characteristics of nowadays civilization. In order to delineate a clear picture of this major health problem, further studies should investigate whether and at what extent cigarette smoking and exposure to ambient air $PM_{2.5}$ impact the natural history of patients with obesity-related NAFLD, *i.e.*, development of non alcoholic steatohepatitis, disease characterized by a worse prognosis due its progression towards fibrosis and hepatocarcinoma.

Key words: Non-alcoholic fatty liver disease; Particulate matter with aerodynamic diameters < 2.5 μm; Cytochrome P-450; Reactive oxygen species

Core tip: Important arguments Diesel exhaust particles are known to be major constituents of atmospheric particulate matter (PM) in metropolitan areas. Exposure to PM is positively associated with increases in the morbidity and daily mortality. Obesity-related health complications include cardiovascular disease, type 2 diabetes, hyperlipidemia, hypertension and non-alcoholic fatty liver disease (NAFLD). Exposure to ambient air PM may induce/worsen NAFLD.

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INTRODUCTION

The rising incidence of obesity in today's environment is associated with many obesity-related health complications, including cardiovascular disease, type 2 diabetes, hyperlipidemia, hypertension, and non-alcoholic fatty liver disease (NAFLD)[1-4]. This constellation is also recognized as the metabolic syndrome and is characterized by underlying insulin resistance. NAFLD or generally speaking hepatic steatosis is defined as the accumulation of lipid, primarily in the form of triacylglycerols in individuals who do not consume significant amounts of alcohol

(< 20 g ethanol/d) and in whom other known causes of steatosis, such as certain drugs and toxins, have been excluded[5]. The spectrum of NAFLD includes simple fatty liver, non alcoholic steatohepatitis (NASH) characterized by inflammation, apoptosis, ballooning degeneration, Mallory hyaline, fibrosis, cirrhosis post NASH, hepatocellular carcinoma and advanced liver disease, which leads to liver-related death[5-10].

Some epidemiological studies, deeply informed and full of insights, have demonstrated that exposure to ambient particulate matter (PM) is positively associated with increases in the morbidity and daily mortality caused by diseases, including ischemic heart disease[11,12] and chronic obstructive pulmonary disease[13,14], which are closely related to life habits. Diabetes mellitus and its complications are the other typical diseases related to life habits. Over the past several decades, prevalence of type 2 diabetes mellitus has reached epidemic levels in Western countries[15], which is a significant public health interest. The prognosis of patients with diabetes mellitus is worsened generally by a variety of complications including macro- or micro-angiopathy[16], fatty liver[17-20], nephropathy and infection in the presence or absence of overweight/obesity. Some epidemiological studies have reported a positive association between mortality in patients with diabetes mellitus and ambient levels of PM[21,22].

Air pollutants expelled from diesel engine-powered automobiles include diesel exhaust particles (DEP), which are known to be major constituents of atmospheric PM in metropolitan areas. DEP generate reactive oxygen species (ROS)[23], through a non enzymatic process[24], or enzymatic reactions catalyzed by cytochrome P-450 (Cyp)[25]. Furthermore, DEP enhance the gene expression for Cyp enzymes[25]. DEP induce a variety of biological damage at least partly through oxidative stress[25].

The present study was designed to alert the public opinion, international media and policy makers on the negative effects of exposure to ambient air particulate matter with aerodynamic diameters < 2.5 mm on NAFLD, a most common chronic liver disease in Western countries, which represents the first indication of liver transplantation.

SMOKING AND NAFLD

A growing body of evidence supports the potential effects of exposure to some environmental factors on liver diseases. Environmental exposure related to toxic waste sites was associated with an increased prevalence of autoimmune liver disease[26,27]. Therefore, increasing attention is being given to the effects of environmental factors on liver diseases, including NAFLD. Several recent studies have too reported the association of smoking with the incidence of and acceleration of disease progression in NAFLD, as well as with advanced fibrosis in this process[28-32].

Cigarette smoke exposure, whether passive or active, carries a high disease burden worldwide[33] and is considered a worldwide major cause of preventable morbidity and mortality[34].

Yuan *et al*[35] provide novel evidence demonstrating that tobacco smoke exposure may accelerate the development of experimental NAFLD. The study extends an earlier report from the group showing that in apo B transgenic mice, chronic environmental (second-hand) smoke exposure is associated to features of atherosclerotic plaque initiation[36]. Using the same model, the former authors now show that exposure to second-hand smoke potentiates steatogenesis elicited by a high-fat diet, as assessed by red oil staining and hepatic triglyceride quantification[35]. Since increased hepatic lipogenesis has been shown to account for about 30% of triglyceride accumulation in steatotic livers[37], the investigators subsequently review the impact of second-hand smoke on liver lipogenic pathways. Interestingly, cultured hepatocyte cell lines exposed to second-hand smoke display enhanced accumulation of triglycerides and increased expression of acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS), two key enzymes governing hepatic synthesis of fatty acids. These data therefore indicate that the steatogenic properties of tobacco smoke are at least partly explained by a direct effect on hepatocytes.

In deciphering molecular determinants underlying tobacco-dependent activation of lipogenesis, the research focus on two key regulators of lipid metabolism, Sterol regulatory element binding protein-1c (SREBP-1c) and AMP-activated protein kinase (AMP kinase). SREBPs are a family of basic-helix-loop-helix-leucine zipper transcription factors synthesized as inactive precursors embedded in the endoplasmic reticulum[38]. Activation of SREBPs requires proteolytic cleavage, thereby allowing nuclear translocation and transcriptional activation of target lipogenic genes[39]. Whereas SREBP-2 governs synthesis of cholesterol, SREBP-1c promotes biosynthesis of fatty acids by upregulating enzymes such as ACC and FAS. The serine/threonine protein kinase AMP kinase is an energy sensor that acts as a metabolic master switch[40]. The phosphorylated active form of the enzyme simultaneously inhibits energy-consuming biosynthetic pathways such as lipogenesis and activates ATP-producing catabolic pathways such as fatty acid oxidation[40]. It has been shown that AMP kinase inhibits fatty acid synthesis both by phosphorylating target lipogenic enzymes and downregulating expression of transcription factors such as SREBP-1c[41-43]. In accordance with these data, Yuan *et al*[35] demonstrated that second-hand smoke exposure inhibits phosphorylation and activation of AMP kinase, thereby resulting in increased SREBP-1 activity and enhancement of fatty acid synthesis. Zein *et al*[44] showed that cigarette smoking were associated with increased fibrosis severity in human NALFD, suggesting it may accelerate disease progression.

Moreover, Yuan *et al*[35] extends this assumption to NAFLD and provides compelling evidence indicating that tobacco smoke might alter the regulatory effect of AMP kinase on lipid metabolism. Future studies should

closely investigate the clinical relevance of these findings. Nevertheless, in the meantime, tobacco cessation might be considered in the management of patients with NAFLD.

NAFLD AND AIR POLLUTION

The harmful effects of air pollutants on atherosclerotic cardiovascular diseases are well-documented[31]. These effects might be mediated through oxidative stress and insulin resistance[45], which are also known to have pivotal roles in the pathogenesis of fatty liver[46]. Therefore, it can be assumed that such environmental factors might be too associated with NAFLD. It is well-documented that DEP, which are major constituents of atmospheric PM in urban areas, generate ROS[47]. The ROS are generated *via* enzymatic reactions catalyzed by Cyp[48], or by a non-enzymatic route[49].

Folkmann *et al*[50] assessed the effects of oxidative stress elicited by DEP in the aorta, liver, and lungs of dyslipidemic ApoE(-/-) mice, at the age when visual plaques appeared in the aorta. Vascular effects secondary to pulmonary inflammation were omitted by injecting DEP into the peritoneum. Six hours later, the expression of inducible nitric oxide synthase mRNA increased in the liver. Injection of DEP did not induce inflammation or oxidative damage to DNA in the lungs and aorta. Therefore, the study proposed a direct effect of DEP on inflammation and oxidative damage to DNA in the liver of dyslipidemic mice[50].

Another study[51] evaluated the effects of following exposure of male C57BL/6 mice fed high fat chow to concentrated air particulate matter or filtered air for 6 wk, progression of NAFLD was evaluated by standardized histological assessment of hepatic inflammation and fibrosis. Progression of NAFLD was evaluated by histology cal examination of hepatic inflammation and fibrosis. Tan *et al*[51] indicated that ambient PM that reaches the liver has the potential to induce Kupffer cell cytokine secretion. Circulating fine PM may then accumulate in both atherosclerotic plaques and hepatic Kupffer cells[51]. The activation of cytokine release by Kupffer cells may then trigger inflammation and hepatic stellate cell collagen synthesis[51]. It is extraordinary that interleukin-6, the concentration of which increased up to 7-fold in the above-mentioned study, is too significantly abundant in cases of human NAFLD[52]. Some human studies confirmed the harmful effects of environmental toxins on liver diseases.

Cave *et al*[53] has showed that non-obese chemical workers highly exposed to vinyl chloride may develop insulin resistance and toxicant-associated steatohepatitis. Limited data exists on the potential role of environmental pollution on liver disease in the general population. Another study was conducted, by Cave *et al*[54] always, on 4582 adult participants without viral hepatitis, hemochromatosis, or alcoholic liver disease, from the National Health and Nutrition Examination Survey in 2003-2004, to investigate whether environmental pollutants are associated with an elevation in serum alanine aminotransferase (ALT) and suspected NAFLD. The ORs for ALT elevation were estabilished across exposure quartiles for 17 pollutants, after adjustments for age, race/ethnicity, sex, body mass index, poverty income ratio, and insulin resistance[54]. It showed that exposure to polychlorinated biphenyls in addition heavy metals, evident lead and mercury, was correlated with unexplained ALT elevation, and increased adjusted ORs for ALT elevation in a dose-dependent form[54].

Therefore, a growing number of studies suggest that air pollution can aggravate the adverse effects of obesity and insulin resistance[29,55,56]. Similarly, some other studies have documented the association of exposure to air pollutants with metabolic syndrome, as well as predisposition to diabetes mellitus and aggravation of its complications[57-59]. Given the inflammatory and oxidative properties of air pollutants, in addition their association with insulin resistance and metabolic syndrome, and considering the interaction of the latter conditions with fatty changes in liver, more studies about the effects of environmental factors, notably air pollution, on NAFLD are warranted. The high susceptibility of the young age group to the harmful effects of air pollutants, especially pertaining to early stages of chronic diseases[13,60-64], further stresses that more attention should be given to preventing late-onset effects of air pollutants.

FUTURE DIRECTIONS

It has been reported that ambient PM containing elementary carbon, sulfate, heavy metals, and organic compounds can cause and enhance cardiopulmonary diseases[65,66]. DEP form a large constituent of ambient urban PM. Inhalation or intratracheal instillation of DEP or the components of DEP has been shown to enhance lung inflammation and asthma[67,68], and to deteriorate biological cardiovascular functions[69]. On the other hand, cardiovascular disorders are critical participants in life habit diseases. Diabetes mellitus is another typical life habit disease and is characterized by complicated cardiovascular risk factors[70-72]. In epidemiological studies, individuals with diabetes mellitus have higher risk for death from exposure to polluted ambient air[73,74]. However, few experimental studies have elucidated the association between ambient air pollution and NAFLD.

In fact, Zein *et al*[44] showed that smoking may accelerate the progression of human NALFD and these observations may support a recommendation of smoking cessation in patients with NAFLD. This recommendation is added to the general recommendations of dietetic or lifestyle approach right for NAFLD patients[75].

As reported by Tan *et al*[51], exposure to ambient air particulate matter with aerodynamic diameters < 2.5 μm ($PM_{2.5}$) may be a significant risk factor for NAFLD progression. In other words, could air pollution be the so-called "second hit", according to the well-known theory?

A better understanding of the impact of ambient PM

exposure on NAFLD progression may require studies utilizing a variety of ambient PM sources[51].

CONCLUSION

So far it has been known that $PM_{2.5}$ result from fuel combustion (motor vehicles, power generation, industrial facilities), residential fireplaces and wood stoves. $PM_{2.5}$ are usually selected as indicators of air pollution since those particles cause morbidity[76]. In fact, $PM_{2.5}$ alone exposure could cause inflammation *via* tumor necrosis factor alpha[77], endothelial function and autonomic nervous system injuries, ozone potentiating these effects[78].

Further studies should investigate the effects of long-lasting exposures to cigarette smoking and to ambient air $PM_{2.5}$ on specific pathways of the hepatic metabolism, better delineating the cellular and molecular mechanisms involved. Importantly, very informative reports should clarify whether cigarette smoking, habit started at very young age, and early exposure to ambient air $PM_{2.5}$ impact the obesity, also the adolescents' one, and the obesity-related NAFLD, favouring development of NASH, disease characterized by a worse prognosis due its progression towards fibrosis, liver cirrhosis and hepatocarcinoma.

By modifying the natural history of patients with NAFLD, air pollution adds a new argument in the debate of regulating the toxic emissions.

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ORIGINAL ARTICLE

Differential mucin phenotypes and their significance in a variation of colorectal carcinoma

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Abstract

AIM: To investigate mucin expression profiles in colorectal carcinoma (CRC) histological subtypes with regard to clinicopathologic variables and prognosis.

METHODS: Mucin (MUC)2 and MUC5AC expressions were assessed by immunohistochemistry for a total of 250 CRC cases that underwent surgical resection. CRCs included 63 well-to-moderately differentiated adenocarcinomas (WMDAs), 91 poorly differentiated adenocarcinomas (PDAs), 81 mucinous adenocarcinoma (MUAs), and 15 signet-ring cell carcinomas (SRCCs). MUC2 and MUC5AC were scored as positive when $\geqslant$ 25% and $\geqslant$ 1% of cancer cells were stained positive, respectively. The human mutL homolog 1 and human mutS homolog 2 expressions were assessed by immunohistochemistry in PDAs to investigate mismatch-repair (MMR) status. Tumors that did not express either of these two were considered MMR-deficient. Results were analyzed for associations with clinicopathologic variables and the prognosis in individual histological CRC subtypes.

RESULTS: MUC2-positive and MUC5AC-positive WMDA percentages were 49.2% and 30.2%, respectively. In contrast, MUC2-positive and MUC5AC-positive PDA percentages were 9.5% and 51.6%, respectively. MUC2 levels tended to decrease and MUC5AC levels tended to increase from WMDA to PDA. In 21 tumors comprising both adenoma and adenocarcinoma components in a single tumor (4 WMDAs, 7 PDAs, and 10 MUAs), MUC2 was significantly downregulated in PDA and MUC5AC was downregulated in PDA and MUA in the adenoma-carcinoma sequence. These results suggested that MUC2 levels might be associated with malignant potential and that MUC5AC expression was an early event in tumorigenesis. Despite worse prognoses than WMDA, high MUC2 expression levels were maintained in MUA (95.1%) and SRCC (71.5%), which suggested a pathogenesis for these subtypes distinct from that of WMDA. No significant associations were found between MUC2 expression and any clinicopathologic variables in any histological subtype. MUC5AC expression in PDA was closely associated with right-sided location (P = 0.017), absence of nodal metastasis (P = 0.010), low tumor node metastasis stage (P = 0.010), and MMR deficiency (P = 0.003). MUC2 expression in WMDA was a marginal prognostic factor for recurrence/metastasis-free survival (RFS) by univariate Cox analysis (P = 0.077) but not by multivariate Cox analysis (P = 0.161). MUC5AC expression in PDA was a significant prognostic factor for RFS by univariate Cox analysis (P = 0.007) but not by multivariate Cox analysis (P = 0.104). Kaplan-Meier curves and log-rank tests revealed that MUC2 expression was marginally associated with a better WMDA prognosis [P = 0.064 for RFS and P = 0.172 for overall survival (OS)] but not for PDA. In contrast, MUC5AC expression was significantly and marginally associated with a better PDA prognosis in terms of RFS and OS, respectively

(P = 0.004 for RFS and P = 0.100 for OS), but not for WMDA and MUA.

CONCLUSION: Mucin core protein expression profiles and clinical significance differ according to histological CRC subtypes. This may reflect different pathogeneses for these tumors.

Key words: Mucin 2; Mucin 5AC; Microsatellite instability; Mismatch repair; Colorectal carcinoma; Poorly differentiated adenocarcinoma; Pathogenesis; Adenoma-carcinoma sequence; Prognosis

Core tip: Altered mucin expression may be correlated with biological behavior and possibly with the prognosis of colorectal carcinoma (CRC). However, many contradictory results make it difficult to interpret its clinical significance, possibly because of CRC variations. Therefore, we examined mucin (MUC)2 and MUC5AC expressions in different pathological CRC subtypes by immunohistochemistry to determine their true clinical significance. Our results suggest that the expression profiles and the clinical significance of these mucin core proteins are different according to histological subtypes. This may reflect different pathogeneses for these tumors.

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INTRODUCTION

Mucins are a diverse family of high-molecular-weight glycoproteins that are widely expressed in epithelial tissues and are characterized by the presence of tandem repeat sequences that are rich in highly *O*-glycosylated serine and threonine residues[1]. Mucins can be classified as either membrane-associated or secretory glycoproteins. To date, a total of 20 human mucins have been identified. Secreted mucins can be gel-forming or non-gel-forming and include mucin (MUC)2, MUC5AC, MUC5B, MUC6, MUC7, MUC8, MUC9, and MUC19. Transmembrane mucins include MUC1, MUC3A, MUC3B, MUC4, MUC11, MUC12, MUC13, MUC15, MUC16, MUC17, MUC20, and MUC21; these are anchored to the plasma membranes of various cells through a transmembrane domain. These mucin proteins are encoded by various *MUC* genes[2]. The genes for gel-forming mucins MUC2 and MUC5AC are found in a cluster on chromosome 11p15.5[3]. The *MUC2* gene codes for a typical secretory mucin, which is predominantly found in colorectal goblet cells, and the *MUC5AC* gene is mainly expressed in gastric and tracheal-bronchial mucosa.

Altered expressions of MUC2 and MUC5AC may be significantly correlated with the biological behavior of and, possibly, the prognosis for colorectal carcinoma (CRC). However, many contradictory results make it difficult to interpret their clinical significance. For example, MUC2 expression is significantly decreased according to CRC disease progression[4-6]. MUC2-positive CRC shows a relatively good prognosis or a low incidence of liver and nodal metastasis[7,8]. Suppressing the *MUC2* gene expression in colon carcinoma cell lines in vitro was associated with methylation of its promoter region[7]. In contrast, other studies reported that MUC2 expression was not a significant marker of tumor invasion depth, liver metastasis, or overall survival[9,10]. However, the absence of MUC5AC expression can be a prognostic indicator of a more aggressive colorectal tumor. Highly villous adenoma with severe dysplasia expressed a less MUC5AC than larger adenomas of moderate villous histology and dysplasia[11]. Carcinomas with low grade atypia exhibited a higher incidence of MUC5AC expression as compared with carcinomas showing high grade atypia[6]. Consistently, MUC5AC expression analysis combined with survival analysis has demonstrated that those patients with MUC5AC-negative CRC had lower rates for disease-free status and of overall survival[12].

Most studies analyzed CRC without detailed classifications. However, in the World Health Organization (WHO) classification, CRC consists of various histological subtypes, such as conventional adenocarcinoma, mucinous adenocarcinoma (MUA), signet-ring cell carcinoma (SRCC), squamous cell carcinoma, adenosquamous carcinoma, medullary carcinoma, undifferentiated carcinoma, and other very rare variants[13]. Conventional adenocarcinoma is further sub-classified into well-to-moderately differentiated adenocarcinoma (WMDA) and poorly differentiated adenocarcinoma (PDA) based on the percentage of the area showing a gland-like structure[13]. Most CRCs encountered in the clinic are WMDA and poorly differentiated/undifferentiated carcinomas are rare, accounting for up to 16% of all CRCs in the United States[14,15]. The purpose of this study was to assess MUC2 and MUC5AC expressions in different pathological CRC subtypes by immunohistochemistry and to determine their true clinical significance.

MATERIALS AND METHODS

Patients and tumor samples

For this study, WMDA included all consecutive cases that were surgically resected in Tokyo Kosei Nenkin Hospital from April 1998 to March 2000, but excluded 10 other histological CRC subtypes. These cases included 63 tumors from 63 patients. In addition, a total of 187 histological CRC subtypes other than WMDA were collected from all CRC cases resected in Dokkyo Medical University Koshigaya Hospital between 1990 and 2011 and Tokyo Kosei Nenkin Hospital between 1991 and 2010.

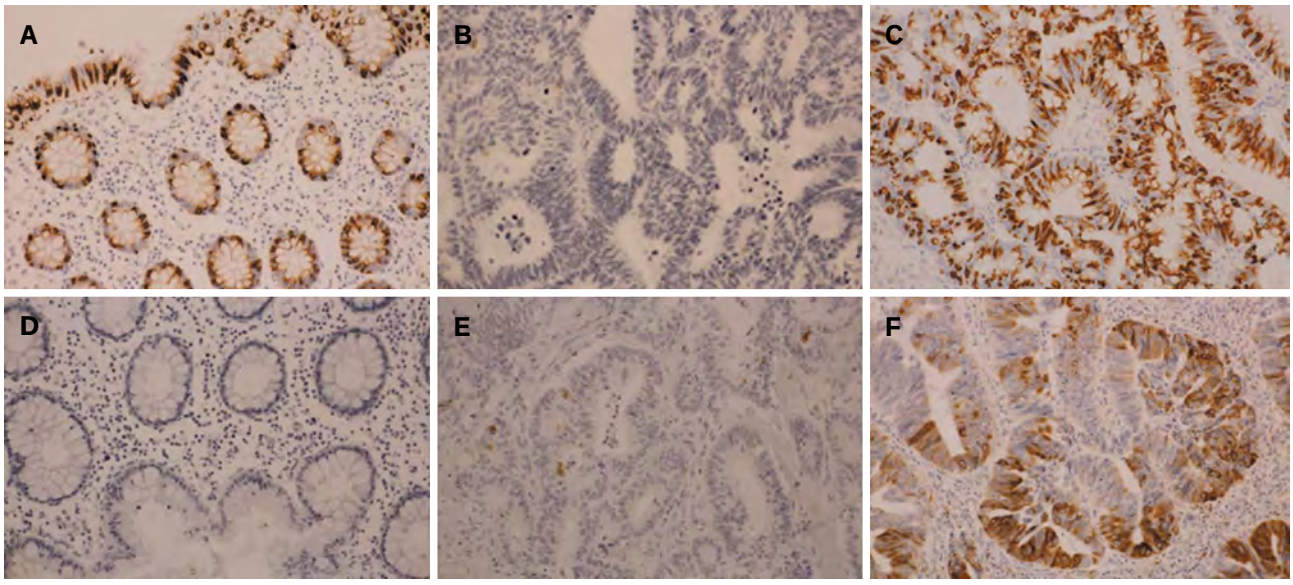


Figure 1 Expression of mucin 2 and mucin 5AC in colorectal carcinomas. A: Mucin (MUC)2 expression in normal colonic mucosa; B: MUC2 expression in cancer: level 0; C: MUC2 expression in cancer: level 4; D: MUC5AC expression in normal colonic mucosa; E: MUC5AC expression in cancer: level 1; F: MUC5AC expression in cancer: level 4 (immunohistochemical staining, × 10).

Formalin-fixed, paraffin-embedded tissue blocks were obtained from the archival material stored in the pathology departments of the both hospitals. A sufficient number of samples to provide for complete investigations were available for all these cases. Patients whose medical records were sufficiently complete were included in survival analysis. Patients with invasive cancers originating from other sites were excluded from the analysis. Clinicopathologic classifications and stage groupings were based on the WHO classification of colorectal tumors and the tumor node metastasis (TNM) staging by the American Joint Committee on Cancer[13,16]. Our study protocol was approved by the ethical review boards of the participating hospitals.

Immunohistochemistry

Tumor specimens were fixed in 10% neutral-buffered formalin for 48 h, embedded in paraffin, and cut into 4-μm-thick sections, and then mounted on silane-coated glass slides. Antigen-retrieval was done by autoclaving (121 ℃) for 5 min in pH 9 Antigen Retrieval Liquid (Nichirei, Tokyo, Japan) for MUC2, MUC5AC, and hMSH2, and by microwave irradiation for 10 min in pH 9 Antigen Retrieval Liquid for human mutL homolog 1 (hMLH1). Primary antibodies used were the mouse monoclonal antibody for MUC2 (1:100 dilution; clone Ccp58, Novocastra, Newcastle Upon Tyne, United Kingdom), the mouse monoclonal antibody for MUC5AC (1:100 dilution; CLH2, Novocastra), the rabbit anti-MLH1 monoclonal antibody (1:400 dilution; EPR3894, GeneTex, San Antonio, TX, United States), and the rabbit anti-MSH2 polyclonal antibody (1:200 dilution; 15520-1-AP, Proteintec, Chicago, IL, United States). Samples were treated overnight with each primary antibody at 4 ℃. Immunostaining was performed blindly by an investigator (Fukuda K) who was unaware of the clinical information using an N-Histofine Simple Stain MAX-PO kit (Nichirei).

The immunostaining results for mucin core proteins were assessed semi-quantitatively: 0, no staining; 1, < 5% of cells; 2, 5% to < 25% of cells; 3, 25% to < 50% of cells; 4, ≥ 50% of cells (Figure 1). The immunostaining results for mismatch repair (MMR) proteins were either completely negative (negative) or nearly 100% positive (positive). Immunoreactivity was independently evaluated by two investigators (Fukuda K and Imai Y), and discrepancies were resolved by discussion.

In light of their expression levels in normal colonic mucosa, levels 3-4 for MUC2 and levels 1-4 for MUC5AC were evaluated as positive.

Statistical analysis

Comparisons of two cohorts with or without a specific clinicopathologic variable were made by a χ^2 test with/without a Yates' correction or Fisher's exact probability test based on the expected values in a contingency table. Age was compared with Mann-Whitney *U* test. Comparisons of the mucin expression levels between adenoma and adenocarcinoma components in a single tumor were made by Wilcoxon signed-rank test for sample numbers of ≥ 6. Univariate analysis by Cox regression analysis was used to identify possible prognostic predictors. Variables for which *P* values were < 0.10 were entered into multivariate regression analysis (forced entry method). Survival curves were generated using the Kaplan-Meier method, and curves were compared by log-rank test. *P* value < 0.05 was considered significant. Statistical analysis was performed using IBM SPSS Statistics 20 (IBM, Armonk, NY, United States).

Table 1 Clinicopathologic characteristics in colorectal carcinoma *n* (%)

Variables		WMDA (*n* = 63)	PDA (*n* = 91)	MUA (*n* = 81)	SRCC (*n* = 15)
Gender	Male	39 (61.9)	45 (49.5)	46 (56.8)	6 (40.0)
	Female	24 (38.1)	46 (50.5)	35 (43.2)	9 (60.0)
Age (yr)	Range	32-87	35-92	26-90	30-82
	Median	65	64	71	70
Family history of CRC	Yes	5 (7.9)	5 (6.1)	5 (6.3)	2 (13.3)
	No	58 (92.1)	77 (93.9)	75 (93.7)	13 (86.7)
	Unknown		9	1	
Location	Left-sided	43 (68.3)	38 (41.8)	40 (49.4)	6 (40.0)
	Right-sided	20 (31.7)	53 (58.2)	41 (50.6)	9 (60.0)
Depth	Up tp MP	10 (15.9)	4 (4.4)	5 (6.2)	1 (6.7)
	Beyond MP	53 (84.1)	87 (95.6)	76 (93.8)	14 (93.3)
Venous invasion	Yes	48 (76.2)	77 (85.6)	41 (50.6)	14 (93.3)
	No	15 (23.8)	13 (14.4)	40 (49.4)	1 (6.7)
	Unknown		1		
Lymphatic invasion	Yes	35 (55.6)	82 (91.1)	54 (66.7)	12 (80.0)
	No	28 (44.4)	8 (8.9)	27 (33.3)	3 (20.0)
	Unknown		1		
Nodal metastasis	Yes	34 (54.0)	69 (78.4)	37 (46.8)	9 (64.3)
	No	29 (46.0)	19 (21.6)	42 (53.2)	5 (35.7)
	Unknown		3	2	1
Chemotherapy	Yes	17 (27.0)	42 (53.8)	31 (41.9)	7 (46.7)
	No	46 (73.0)	36 (46.2)	43 (58.1)	8 (53.3)
	Unknown		13	7	
Irradiation	Yes	4 (6.3)	2 (2.6)	3 (4.1)	1 (7.1)
	No	59 (93.7)	76 (97.4)	71 (95.9)	14 (92.9)
	Unknown		13	7	
TNM stage	Ⅰ/Ⅱ	29 (46.0)	17 (18.9)	40 (50.0)	4 (26.7)
	Ⅲ/Ⅳ	34 (54.0)	73 (81.1)	40 (50.0)	11 (73.3)
	Unknown		1	1	0

CRC: Colorectal carcinoma; WMDA: Well-to-moderately differentiated adenocarcinoma; PDA: Poorly differentiated adenocarcinoma; MUA: Mucinous adenocarcinoma; SRCC: Signet-ring cell carcinoma; MP: Muscularis propria; TNM: Tumor node metastasis.

RESULTS

Clinicopathologic characteristics

WMDA cases included 63 tumors from 63 patients. Five of these patients (5 tumors) had a family history of CRC. Additional chemotherapy and irradiation were administered for 17 tumors in 17 patients and 4 tumors in 4 patients, respectively. CRCs other than WMDA included a total of 187 tumors: 91 PDAs (90 patients); 81 MUAs (81 patients); and 15 SRCCs (15 patients). One patient had triple cancers (No. 148: two PDAs and one MUA) and one patient had double cancers (No. 170: one PDA and one MUA). Ten of these patients (12 tumors) had a family history of CRC, one of which was proven to be a hereditary non-polyposis colorectal cancer pedigree (No. 148). Predominant occurrence in females, right-sided location, depth of tumors beyond muscularis propria, lymphatic invasion, nodal involvement (except for MUA), TNM stage Ⅲ/Ⅳ (except for MUA) were more frequent in CRCs other than WMDA as compared with WMDA (Table 1). In addition to surgery, chemotherapy and irradiation were administered for 80 tumors in 80 patients and 6 tumors in 6 patients, respectively.

The clinicopathologic characteristics of each CRC subtype are summarized in Table 1. CRC patient prognosis was significantly associated with these histological subtypes (Figure 2).

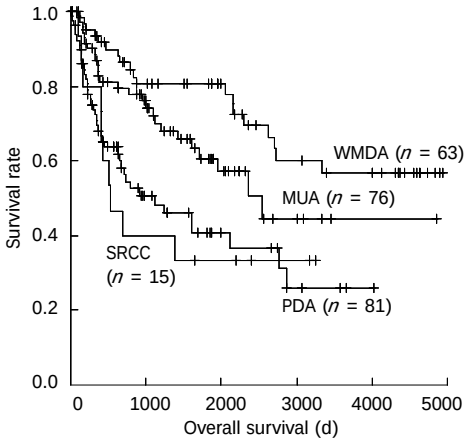


Figure 2 Colorectal carcinoma overall survival curves generated by the Kaplan-Meier method. WMDA: Well-to-moderately differentiated adeno-carcinoma; PDA: Poorly differentiated adenocarcinoma; MUA: Mucinous adenocarcinoma; SRCC: Signet-ring cell carcinoma.

Expression of MUC2 and MUC5AC in CRCs

Expression of the mucin core proteins was assessed individually in different pathological subtypes. MUC2 was expressed in the perinuclear cytoplasm of goblet cells in normal colonic mucosa and diffusely in the cytoplasm of cancer cells. MUC5AC was not expressed in normal colonic mucosa but was occasionally expressed in pericancerous normally appearing colonic mucosa. About half of WMDA cases (49.2%) were positive for MUC2 (levels 3-4), and 30.2% of these cases were positive for MUC5AC (levels 1-4). In contrast, only one tenth of PDA cases (9.5%) were positive for MUC2 and 51.6% of these cases were positive for MUC5AC. PDA was more frequently negative for MUC2 and positive for MUC 5AC than was WMDA. Nearly all MUA cases (95.1%) were positive for MUC2, and over half of these cases (54.3%) aberrantly expressed MUC5AC. Although small in number, 71.5% of SRCC cases were positive for MUC2 and nearly half (46.7%) expressed MUC5AC. These results are summarized in Figure 3.

Expression of the mucin core proteins in the adenoma-carcinoma sequence

Among our study subjects, 21 tumors in 20 patients had an adenoma component indicative of originating from the adenoma-carcinoma sequence. We investigated the sequential expression status of the mucin core proteins. MUC2 expression was found in the adenoma component in all cases, except for one PDA case. MUC2 expression was relatively well maintained in the carcinoma component of WMDA and mildly decreased in some MUA cases. MUC2 expression significantly decreased in the carcinoma component of PDA. MUC5AC was aberrantly expressed in the tubular/tubulovillous adenoma components in all but three cases, and there was a signifi-

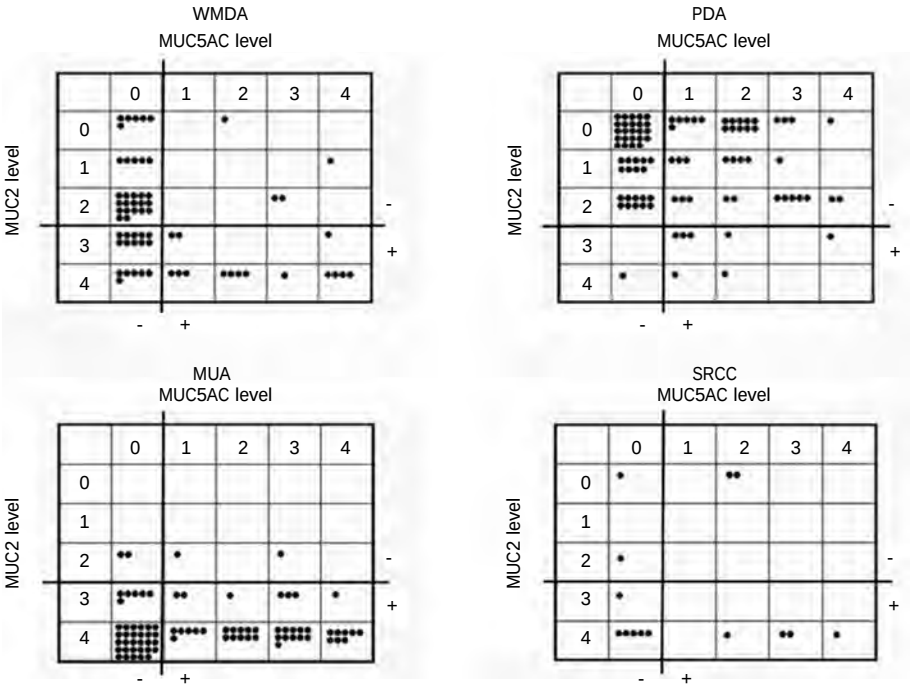


Figure 3 Expression profiles of mucin 2 and mucin 5AC in each colorectal carcinoma histological subtype. Each closed circle indicates one tumor. MUC: Mucin; WMDA: Well-to-moderately differentiated adenocarcinoma; PDA: Poorly differentiated adenocarcinoma; MUA: Mucinous adenocarcinoma; SRCC: Signet-ring cell carcinoma.

Table 2 Expression levels of the mucin core proteins in the adenoma carcinoma sequence

	Histology		MUC2			MUC5AC		
No. of patient	Adenoma	Carcinoma	Adenoma	Carcinoma	*P* value[1]	Adenoma	Carcinoma	*P* value[1]
208	TV	WMDA	4	3	ND	4	1	ND
225	T	WMDA	4	4		3	2	
237	TV	WMDA	4	4		2	4	
243	TV	WMDA	4	4		4	4	
84	TV	PDA	4	2	0.016	4	0	0.042
86	TV	PDA	4	0		1	0	
87	TV	PDA	4	0		0	0	
102	TV	PDA	4	0		4	0	
116	T	PDA	2	0		2	0	
138	T	PDA	4	0		3	0	
148	T	PDA	3	0		0	0	
15	TV	MUA	4	4	0.063	3	0	0.016
36	TV	MUA	4	4		3	3	
37	TV	MUA	4	4		0	0	
51	TV	MUA	4	4		3	3	
131	T	MUA	4	2		3	1	
148	TV	MUA	4	2		4	0	
151	TV	MUA	4	3		3	1	
155	TV	MUA	4	4		4	2	
175	TV	MUA	4	4		4	2	
191	TV	MUA	4	3		3	0	

[1]Wilcoxon signed-rank test. MUC: Mucin; T: Tubular adenoma; TV: Tubulovillous adenoma; WMDA: Well-to-moderately differentiated adenocarcinoma; PDA: Poorly differentiated adenocarcinoma; MUA: Mucinous adenocarcinoma; ND: Not determined.

cant decrease in MUC5AC expression in the carcinoma components as compared with the adenoma components in PDA and MUA (Table 2).

Expression of the mucin core proteins and clinicopathologic variables

Expression status of the mucin core proteins was analyzed in association with clinicopathologic variables in each histological CRC subtype that had sufficient numbers for statistical analysis. In a contingency table analysis, no statistically significant associations were found between MUC2 expression and any clinicopathologic variables in any of the histological subtypes. In contrast, MUC5AC expression was significantly associated with right-

Table 3 Expression of the mucin core proteins and clinicopathologic variables *n* (%)

		WMDA			PDA			MUA		
		MUC2			MUC2			MUC2		
		-	+	*P* value	-	+	*P* value	-	+	*P* value
Median age (range), yr		64 (32-82)	64 (34-87)	0.783	64 (35-92)	66.5 (55-86)	0.633	65 (52-88)	71 (26-90)	0.842
Gender	Male	21 (33.3)	18 (28.6)	0.537	41 (45.1)	4 (4.4)	1.000	1 (1.2)	45 (55.6)	0.311
	Female	11 (17.5)	13 (20.6)		42 (46.1)	4 (4.4)		3 (3.7)	32 (39.5)	
Locus	Right-sided	10 (15.9)	10 (15.9)	0.932	47 (51.6)	6 (6.6)	0.461	1 (1.2)	40 (49.4)	0.359
	Left-sided	22 (34.9)	21 (33.3)		36 (39.6)	2 (2.2)		3 (3.7)	37 (45.7)	
Venous invasion	Yes	24 (38.1)	24 (38.1)	1.000	71 (79.0)	6 (6.6)	0.325	3 (3.7)	38 (46.9)	0.616
	No	8 (12.7)	7 (11.1)		11 (12.2)	2 (2.2)		1 (1.2)	39 (48.1)	
Lymphatic invasion	Yes	21 (33.3)	14 (22.2)	0.102	76 (84.4)	6 (6.7)	0.148	1 (1.2)	51 (63.0)	1.000
	No	11 (17.5)	17 (27.0)		6 (6.7)	2 (2.2)		3 (3.7)	26 (32.1)	
Nodal metastasis	Yes	20 (31.7)	14 (22.2)	0.167	64 (72.7)	5 (5.7)	0.362	2 (2.5)	35 (44.3)	1.000
	No	12 (19.0)	17 (27.0)		16 (18.2)	3 (3.4)		2 (2.5)	40 (50.6)	
TNM stage	Ⅰ/Ⅱ	12 (19.0)	17 (27.0)	0.167	14 (15.6)	3 (3.3)	0.171	2 (2.5)	38 (48.1)	1.000
	Ⅲ/Ⅳ	20 (31.7)	14 (22.2)		68 (75.5)	5 (5.6)		2 (2.5)	37 (46.8)	
dMMR	Yes	ND			16 (17.6)	3 (3.3)	0.356	ND		
	No				67 (73.6)	5 (5.5)				
		MUC5AC			MUC5AC			MUC5AC		
		-	+	*P* value	-	+	*P* value	-	+	*P* value
Median age (range), yr		64 (32-83)	69 (47-87)	0.099	63 (36-87)	70 (35-92)	0.096	67 (34-87)	72 (26-90)	0.061
Gender	Male	26 (41.3)	13 (20.6)	0.677	22 (24.2)	23 (25.3)	0.919	23 (28.4)	23 (28.4)	0.371
	Female	18 (28.6)	6 (9.5)		22 (24.2)	24 (26.3)		14 (17.3)	21 (25.9)	
Locus	Right-sided	10 (15.9)	10 (15.9)	0.410	20 (22.0)	33 (36.3)	0.017	12 (14.8)	29 (35.8)	0.003
	Left-sided	34 (54.0)	9 (14.3)		24 (26.4)	14 (15.4)		25 (30.9)	15 (18.5)	
Venous invasion	Yes	34 (54.0)	14 (22.2)	0.757	38 (42.2)	39 (43.3)	1.000	16 (19.8)	25 (30.9)	0.224
	No	10 (15.9)	5 (7.9)		6 (6.7)	7 (7.8)		21 (25.9)	19 (23.5)	
Lymphatic invasion	Yes	26 (41.3)	9 (14.3)	0.560	41 (45.6)	41 (45.6)	0.714	25 (30.9)	29 (35.8)	0.875
	No	18 (28.6)	10 (15.9)		3 (3.3)	5 (5.6)		12 (14.8)	15 (18.5)	
Nodal metastasis	Yes	23 (36.5)	11 (17.5)	0.892	40 (45.5)	29 (33.0)	0.010	17 (21.5)	20 (25.3)	0.950
	No	21 (33.3)	8 (12.7)		4 (4.5)	15 (17.0)		19 (24.1)	23 (29.1)	
TNM stage	Ⅰ/Ⅱ	21 (33.3)	8 (12.7)	0.892	3 (3.3)	14 (15.6)	0.010	18 (22.8)	22 (27.8)	0.918
	Ⅲ/Ⅳ	23 (36.5)	11 (17.5)		41 (45.6)	32 (35.6)		18 (22.8)	21 (26.6)	
dMMR	Yes	ND			3 (3.3)	16 (17.6)	0.003	ND		
	No				41 (45.1)	31 (34.1)				

MUC: Mucin; WMDA: Well-to-moderately differentiated adenocarcinoma; PDA: Poorly differentiated adenocarcinoma; MUA: Mucinous adenocarcinoma; dMMR: Mismatch-repair deficiency; ND: Not determined; TNM: Tumor node metastasis.

sided location, absence of nodal metastasis, and lower TNM stage in PDA, and right-sided location in MUA. Furthermore, MUC5AC expression tended to be associated with older age in WMDA, PDA, and MUA, although the difference was not statistically significant. MUC5AC expression was not associated with any clinicopathologic variables in SRCC. These results are summarized in Table 3 (partly not shown).

Expression of the MMR proteins and the mucin core proteins in PDA

In PDA cases, MUC5AC positivity was significantly associated with right-sided location and lower TNM stage, and marginally associated with older age. Survival curve analysis also suggested a better prognosis for MUC5AC-positive PDA cases than for negative ones as described below. These are clinical features associated with high levels of microsatellite instability (MSI; MSI-H)[17-19]. A subset of sporadic CRC cases (approximately 10%-15%) is MSI-H; this is caused by inactivation of the DNA MMR system. Identifying MSI previously required molecular testing, although immunostaining for hMLH1 and hMSH2 has come to be accepted as a practical test to detect MSI[20,21]. Therefore, we investigated MMR status in PDA cases using immunohistochemistry. Tumors that did not express either of these two were considered MMR deficiency (dMMR).

dMMR was found in 19 PDA cases, 16 of 47 MUC5AC-positive cases and 3 of 44 MUC5AC-negative cases ($P = 0.003$). In contrast, there was no significant association between MUC2 expression and dMMR. Thus, dMMR showed statistically significant association with MUC5AC positivity, although it should be noted that dMMR was found in only one third of MUC5AC-positive tumors and dMMR was also found in one tenth of MUC5AC-negative tumors (Table 3).

Expression of the mucin core proteins and prognosis

The effects of clinicopathologic variables on recurrence/metastasis-free survival (RFS) and overall survival (OS) were investigated using Cox regression analysis for each CRC subtype. For WMDA, TNM stage was the only significant prognostic factor for RFS by univariate and multivariate analysis, but no significant predictor for OS was identified. Next, we included dMMR in the survival analysis for PDA. Univariate Cox regression analysis showed that TNM stage, MUC5AC expression, and dMMR were significant prognostic factors for RFS; however none of

Table 4 Prognostic significance of clinicopathologic variables in well-to-moderately differentiated adenocarcinoma and poorly differentiated adenocarcinoma

	Recurrence/metastasis				Death			
	Parameter		HR (95%CI)	*P* value	Parameter		HR (95%CI)	*P* value
WMDA	Univariate analysis				Univariate analysis			
		Age (over 65 yr)	2.215 (0.974-5.037)	0.058		Age (over 65 yr)	1.984 (0.794-4.955)	0.142
		Gender (male)	0.887 (0.398-1.975)	0.769		Gender (male)	2.194 (0.727-6.617)	0.163
		Location (right-sided)	1.268 (0.559-2.875)	0.570		Location (right-sided)	1.335 (0.524-3.402)	0.545
		TNM stage (Ⅲ/Ⅳ)	3.642 (1.446-9.170)	0.006		TNM stage (Ⅲ/Ⅳ)	2.632 (0.990-6.996)	0.052
		MUC2 positive	0.477 (0.210-1.082)	0.077		MUC2 positive	0.527 (0.207-1.341)	0.179
		MUC5AC positive	1.389 (0.613-3.145)	0.431		MUC5AC positive	1.803 (0.723-4.497)	0.206
	Multivariate analysis				Multivariate analysis			
		Age (over 65 yr)	2.220 (0.965-5.017)	0.061				
		TNM stage (Ⅲ/Ⅳ)	3.473 (1.370-8.805)	0.009				
		MUC2 positive	0.554 (0.243-1.265)	0.161				
PDA	Univariate analysis				Univariate analysis			
		Age (over 65 yr)	0.891 (0.495-1.602)	0.700		Age (over 65 yr)	0.985 (0.955-1.016)	0.331
		Gender (male)	0.624 (0.343-1.138)	0.124		Gender (male)	0.899 (0.484-1.667)	0.734
		Location (right-sided)	0.857 (0.474-1.549)	0.609		Location (right-sided)	0.686 (0.371-1.270)	0.231
		TNM stage (Ⅲ/Ⅳ)	2.647 (1.109-6.320)	0.028		TNM stage (Ⅲ/Ⅳ)	3.208 (1.236-8.330)	0.017
		MUC2 positive	0.936 (0.368-2.379)	0.890		MUC2 positive	1.009 (0.357-2.850)	0.987
		MUC5AC positive	0.433 (0.237-0.793)	0.007		MUC5AC positive	0.599 (0.323-1.112)	0.105
		dMMR	0.373 (0.164-0.847)	0.018		dMMR	0.352 (0.153-0.810)	0.014
	Multivariate analysis				Multivariate analysis			
		TNM stage (Ⅲ/Ⅳ)	1.698 (0.672-4.289)	0.263		TNM stage (Ⅲ/Ⅳ)	2.385 (0.886-6.421)	0.085
		MUC5AC positive	0.586 (0.307-1.117)	0.104		dMMR	0.466 (0.195-1.111)	0.085
		dMMR	0.228 (0.260-1.308)	0.175				

WMDA: Well-to-moderately differentiated adenocarcinoma; PDA: Poorly differentiated adenocarcinoma; dMMR: Mismatch-repair deficiency; TNM: Tumor node metastasis; MUC: Mucin.

these was significant by multivariate analysis. TNM stage and dMMR were also significant predictors for OS by univariate analysis but were not significant by multivariate analysis (Table 4).

For MUA, MUC2 expression was excluded from the analysis because there were very few MUC2-negative MUA cases (n = 4 out of 81). The TNM stage was the only significant predictor for RFS, and no variable was a significant predictor for OS (data not shown).

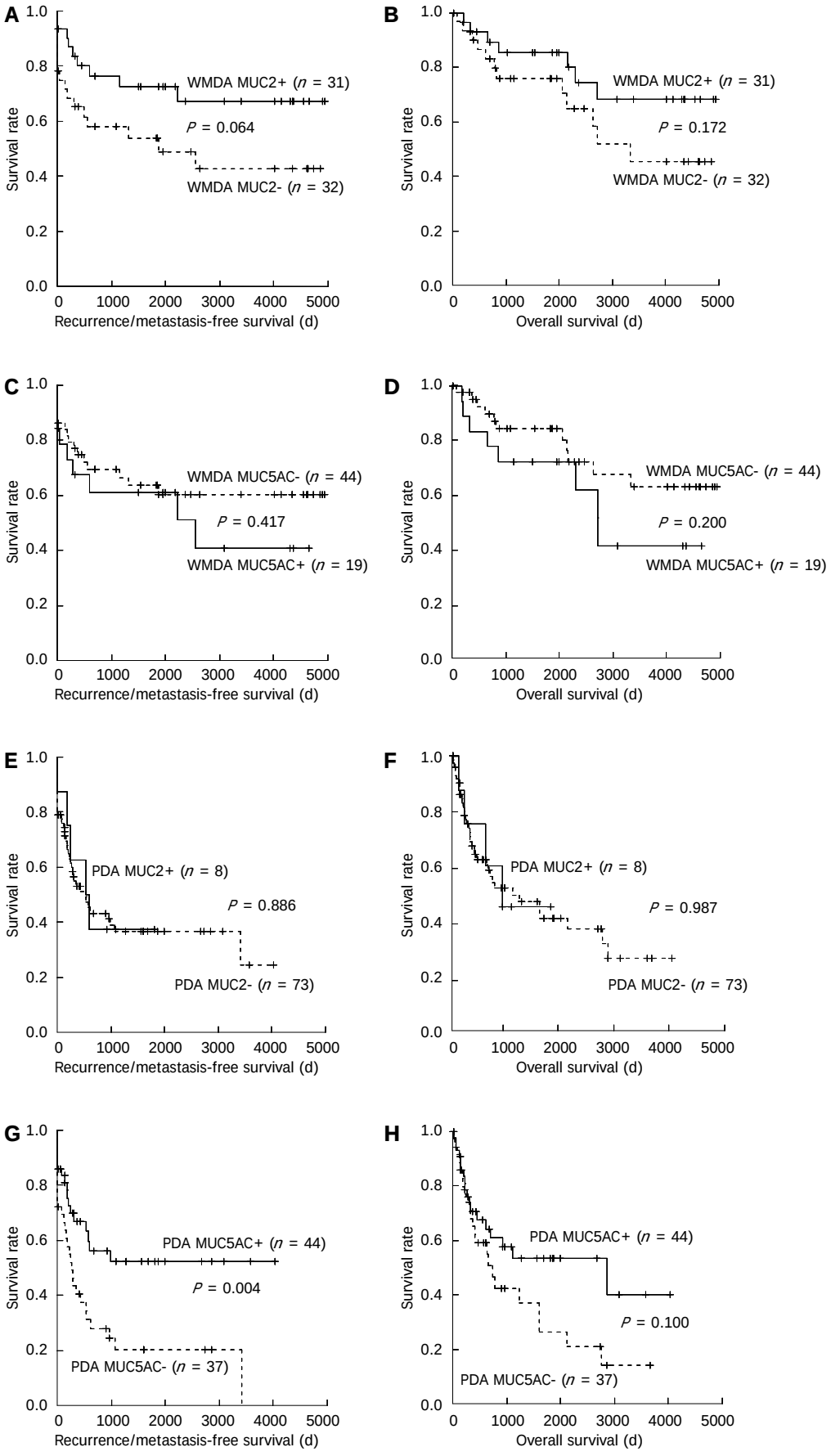
Thus, no significant associations were found between mucin expression and the prognosis of each CRC subtype by multivariate Cox analysis. However, as the data suggested marginal associations between mucin expression and prognosis, and from the need for subsequent discussion, Kaplan-Meier survival curves for associations with mucin expression were generated and assessed by log-rank test for each CRC subtype.

For this analysis of WMDA, marginally better RFS and OS with MUC2-positive tumors were found as compared with negative tumors. The prognosis of MUC5AC-positive WMDA cases tended to be worse as compared to those that were negative, although this difference was not significant (Figure 4A-D). There was also no difference with respect to MUC2 status in PDA. However, MUC5AC expression in PDA was significantly and marginally associated with a better prognosis in terms of RFS and OS, respectively (Figure 4E-H). Because MUC2 positivity was very high in MUA (77 of 81 tumors), its clinical significance in these settings was not investigated. MUC5AC expression in MUA did not affect its prognosis (Figure 4I and J).

DISCUSSION

MUC2 is normally expressed in the perinuclear cytoplasm of goblet cells in normal colonic mucosa. MUC2 is also expressed in adenomas and mucinous carcinomas[4]. MUC2 downregulation occurs in non-mucinous adenocar-cinomas that arise within adenomas, whereas cancers that are considered to develop de novo do not express MUC2[4]. Thus, MUC2 levels have been thought to be a predictor of malignant potential. However, despite a poor prognosis, higher levels of MUC2 expression were maintained in MUA and SRCC than in WMDA. This suggests the difficulty with using MUC2 levels as a differentiation marker in these subtypes.

Some investigators reported MUC2 expression in CRC in association with clinical significance, although the association between MUC2 expression and prognosis has been controversial. For example, Matsuda *et al*[9] analyzed 86 CRCs that included 82 WMDA tumors and 4 other variants and reported that the MUC2 expression level was not associated with advanced Dukes' stage and liver metastasis. Baldus *et al*[10] investigated 243 CRCs that included 213 grade Ⅰ-Ⅱ tumors and 30 grade Ⅲ tumors, which also included 22 MUA tumors, and reported that


A
Survival rate
WMDA MUC2+ (n = 31)
P = 0.064
WMDA MUC2- (n = 32)
Recurrence/metastasis-free survival (d)
B
Survival rate
WMDA MUC2+ (n = 31)
P = 0.172
WMDA MUC2- (n = 32)
Overall survival (d)
C
Survival rate
WMDA MUC5AC- (n = 44)
P = 0.417
WMDA MUC5AC+ (n = 19)
Recurrence/metastasis-free survival (d)
D
Survival rate
WMDA MUC5AC- (n = 44)
P = 0.200
WMDA MUC5AC+ (n = 19)
Overall survival (d)
E
Survival rate
PDA MUC2+ (n = 8)
P = 0.886
PDA MUC2- (n = 73)
Recurrence/metastasis-free survival (d)
F
Survival rate
PDA MUC2+ (n = 8)
P = 0.987
PDA MUC2- (n = 73)
Overall survival (d)
G
Survival rate
PDA MUC5AC+ (n = 44)
P = 0.004
PDA MUC5AC- (n = 37)
Recurrence/metastasis-free survival (d)
H
Survival rate
PDA MUC5AC+ (n = 44)
P = 0.100
PDA MUC5AC- (n = 37)
Overall survival (d)

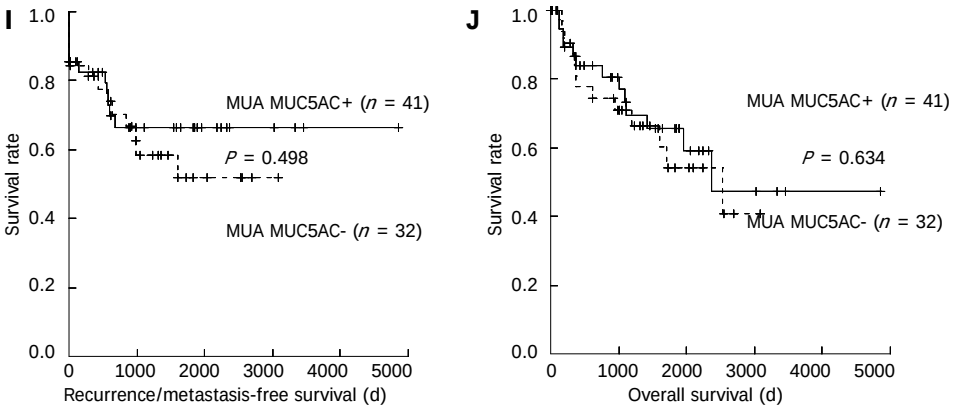


Figure 4 Survival curves. A-D: Well-to-moderately differentiated adenocarcinoma (WMDA); E-H: Poorly differentiated adenocarcinoma (PDA); I and J: Mucinous adenocarcinoma (MUA). A: Recurrence/metastasis-free; B: Overall survival curves with or without mucin (MUC) 2 expression of WMDA; C: Recurrence/metastasis-free; D: Overall survival curves with or without MUC5AC expression; E: Recurrence/metastasis-free; F: Overall survival curves with or without MUC2 expression; G: Recurrence/metastasis-free; H: Overall survival curves with or without MUC5AC expression; I: Recurrence/metastasis-free; J: Overall survival curves with or without MUC5AC expression. Curves were generated using the Kaplan-Meier method and compared by log-rank tests. *P* values were derived from comparing mucin-negative and -positive tumors.

MUC2 reactivity was not a marker for worse survival. In contrast, Kang *et al*[8] investigated 301 patients with stage Ⅱ-Ⅲ CRCs, including 200 well-to-moderately differentiated and 101 poorly differentiated cancers (also 266 nonmucinous and 35 mucinous) and reported that a loss of MUC2 expression was associated with a worse overall survival with CRC of stages Ⅱ and Ⅲ. Hanski *et al*[7] showed that a loss of MUC2 expression in CRC owing to promoter methylation was associated with liver and nodal metastasis. On the other hand, a loss of MUC2 expression in CRCs was associated with peritumoral lymphocyte infiltration[22]. Host responses, such as peritumoral lymphocyte infiltration, are known to be associated with a favorable CRC prognosis[23]. MUC2 mucin harbors the sialosyl-Tn antigen that mediates the inhibition of natural killer cell cytotoxicity[24].

However, MUC5AC expression is usually absent in normal colonic mucosa and is only occasionally found in pericancerous normally appearing colonic mucosa. Aberrant MUC5AC expression can be observed in a subset of adenomas and adenocarcinomas[11,25-27]. Microscopically, MUC5AC was detected primarily as focal staining in the cytoplasm and mucous droplets in goblet cells in the normally appearing colonic mucosa but was diffuse in the cytoplasm of cancer cells. MUC5AC expression levels are highest in adenoma but decrease with increasing degrees of dysplasia, and the positive rates for MUC5AC expression were lower in CRC than in adenoma[11,25-27]. These results suggest that MUC5AC may play a role in early carcinogenesis and its expression status can be used to classify CRC from the viewpoint of pathogenesis.

Biemer-Hüttmann *et al*[28] and Losi *et al*[29] reported associations between MUC5AC expression and MSI-H, which is linked with histological subtypes like PDA and MUA. Kocer *et al*[12] analyzed MUC5AC expression in 41 CRCs that included 33 adenocarcinomas, 5 mucinous carcinomas, and 3 neuroendocrine carcinomas. They reported that MUC5AC-negative CRCs had lower rates of disease-free status and of overall survival, but thy did not investigate associations between MSI and MUC5AC expression.

It has come to be well recognized that CRC comprises various carcinomas that originate from distinct pathogenetic pathways. Owing to this CRC heterogeneity, the significance of mucin core protein expression in CRC remains controversial. Thus, we performed these analyses for each histological CRC subtype.

In the present study, the expression profiles of MUC2 and MUC5AC in conventional adenocarcinoma (WMDA and PDA), MUA, and SRCC were similar to those in previous reports. High MUC2 expression levels in MUA and SRCC suggested distinct histogenetic pathways for these cancer cells, which maintained the feature of mucin-producing goblet cells, from conventional adenocarcinoma. In addition, the expression of the both mucin core proteins tended to decrease during the course of disease progression, from adenoma to carcinoma or from WMDA to PDA. Disease progression is usually accompanied by a decrease in cells of the goblet lineage.

These results are consistent with the hypothesis that the levels of the mucin core proteins may be a marker of malignancy potential in the adenoma-carcinoma sequence in both non-mucinous and mucinous carcinomas. We found that the prognostic significance of the mucin core proteins was different between MUC2 and MUC5AC. In our survival curve analysis, a tendency for better prognosis with MUC2-positive cases than for negative cases was observed in WMDA. A difference in prognosis was not evident from the MUC2 status in PDA.

However, MUC5AC expression in PDA was significantly associated with a better RFS and marginally associated with a better OS. MUC5AC was a significant factor for RFS by univariate Cox regression analysis. Although no significant effects of MUC5AC expression on RFS or OS were found by multivariate analysis, these results may have been because of the limited number of cases

or other unknown factors associated with this patient population. A larger study will be needed to clarify these points.

In comparison, MUC5AC expression was not a factor associated with better prognosis in WMDA and MUA. Aberrant MUC5AC expression is considered to be an early event in carcinogenesis. In addition, MUC5AC expression was significantly associated with right-sided location, absence of nodal metastasis, and a lower TNM stage and was marginally associated with older age in our PDA series. These clinical features as well as poor differentiation are characteristic of MSI-high tumors. MUC5AC expression in PDA was significantly associated with dMMR as shown by a loss of MMR protein expression (16 of 47 MUC5AC-positive cases *vs* 3 of 44 negative cases; $P = 0.003$). Unlike the report by Biemer-Hüttmann *et al*[28] stating that MUC2 expression was also associated with MSI-H, our PDA cases did not exhibit an association between the two. These results suggest that PDA also consists of heterogenous groups of cancers and that MUC5AC expression status may be one of the classification hallmarks.

To date, the mechanisms underlining aberrant MUC5AC expression in the colon have not been determined. During colon carcinogenesis, MUC5AC expression may be regarded as the re-expression of this fetal mucin. MUC5AC mucin is detected from the fourth month of gestation and is maximum during the sixth month[30]. On the other hand, the MUC5AC promoter was shown to be activated by various inflammation mediators[31]. It was also reported that tumor necrosis factor-α stimulated colon cancer HT-29 cells, which are a goblet cell line, to secrete MUC5AC mucin in a dose-dependent manner[32]. Forgue-Lafitte *et al*[33] reported that MUC5AC mucin was detectable in the mucus of ulcerative colitis patients who underwent surgery. In their series, 10 patients suffering from ulcerative colitis tested were positive for MUC5AC, which suggested that long-term chronic inflammation may induce the production of this mucin in the colonic epithelium. In addition, MUC5AC expression in the regenerating areas close to ulcerations in Crohn's disease suggests its involvement in tissue repair mechanisms[34]. CRC with MUC5AC expression may originate from precancerous lesions owing to long-standing inflammation caused by bacterial infection, inflammatory bowel disease, or other reasons.

In our study, we sometimes observed aberrant MUC5AC expression in normally-looking colonic mucosa at the interface between non-tumor and tumor tissue, where a strong anti-tumor inflammatory reaction had been observed. We speculate that this may be suggestive of the origin of a neoplasm with aberrant MUC5AC expression. Furthermore, it was previously reported that long-standing inflammation due to ulcerative colitis resulted in CRC with dMMR[35,36]. Taken together, a hypothesis of inflammation-related carcinogenesis may explain the pathogenesis of CRC from MUC5AC-positive precancerous lesions and an association between dMMR and MUC5AC expression.

In conclusion, we investigated MUC2 and MUC5AC expression status in each of the histological CRC subtypes. MUC2 levels were decreased and MUC5AC levels were increased from WMDA to PDA. MUA and SRCC maintained high MUC2 levels. The expressions of these mucins in PDA and MUA decreased during disease progression in the adenoma-carcinoma sequence. MUC5AC expression was closely associated with MMR deficiency in PDA. MUC2 and MUC5AC expression tended to be associated with a better prognosis in WMDA and PDA, respectively, although these were not statistically significant. Thus, the mucin proteins show distinct clinical significance according to the histological subtypes, and this may also suggest different pathogeneses for these tumors.

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COMMENTS

Background

Mucin (MUC)2 mucin is predominantly found in colorectal goblet cells and MUC5AC mucin is primarily expressed in gastric mucosa. Altered mucin expression may be correlated with the biological behavior of and, possibly, the prognosis for colorectal carcinoma (CRC).

Research frontiers

To date, MUC2 and MUC5AC expressions in CRC have been investigated for their association with clinicopathologic characteristics and prognosis. However, many contradictory results make it difficult to interpret the clinical significance of altered mucin expression, possibly owing to various CRC subtypes. The authors investigated mucin expression by immunohistochemistry in each of the histological CRC subtypes individually and assessed its significance.

Innovations and breakthroughs

MUC2 levels may be associated with malignant potential in conventional adenocarcinoma, whereas mucinous adenocarcinoma and signet-ring cell carcinoma retain high MUC2 levels, which suggests distinct pathogenesis. In comparison, MUC5AC expression may be an early event in tumorigenesis. MUC5AC expression in poorly differentiated adenocarcinoma (PDA) was closely associated with mismatch repair deficiency. MUC2 and MUC5AC expression tended to be associated with a better prognosis in well-to-moderately differentiated adenocarcinoma and PDA, respectively. Thus, the mucin proteins show different clinical significance according to the histological subtypes, and this may also suggest different pathogeneses of these tumors.

Applications

These results could be the basis for further studies to understand the pathogenesis of CRC. Immunohistochemical detection of MUC5AC may be useful for further subclassifications and for predicting a favorable prognosis for PDA.

Terminology

MUC2 and MUC5AC are the backbone proteins of secreted mucins. MUC2 is synthesized in goblet cells in the gastrointestinal tract and MUC5AC is normally expressed in gastric foveolar epithelium. The *MUC2* and *MUC5AC* genes encode gel-forming mucins and are located in a cluster on chromosome 11p15.5.

Peer review

This study is important and interesting. Although there are a large number of publications on the roles of MUC2 and MUC5AC expression in CRC, the results are conflicting. This study adds some refinements to these issues, such as MUC2 and MUC5AC expressions in terms of CRC patient survival, adenoma-

carcinoma sequence, and expression of MMR proteins, with regard to associations with clinicopathologic variables by offering its own evidence.

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ORIGINAL ARTICLE

Roles of BN52021 in platelet-activating factor pathway in inflammatory MS1 cells

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Abstract

AIM: To determine the effects of BN52021 on platelet-activating factor receptor (PAFR) signaling molecules under lipopolysaccharide (LPS)-induced inflammatory conditions in MS1 cells.

METHODS: MS1 cells (a mouse pancreatic islet endothelial cell line) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mmol/L glutamine and 100 μg/mL penicillin/streptomycin in 5% CO_2 at 37 ℃. After growth to confluency in media, the cells were processed for subsequent studies. The MS1 cells received 0, 0.1, 1 and 10 μg/mL LPS in this experiment. The viability/proliferation of the cells induced by LPS was observed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay. Apoptosis and necrosis of the cells under the inflammatory condition described previously were observed using Hoechst 33342-propidium iodide staining. Adenylate cyclase (AC), phospholipase A_2 (PLA_2), phospholipase Cβ (PLCβ), protein tyrosine kinase (PTK), G protein-coupled receptor kinases (GRK) and p38-mitogen-activated protein kinase (p38 MAPK) mRNA in the PAFR signaling pathway were measured by real-time polymerase chain reaction. The protein expression level of phosphorylated AC (p-AC), phosphorylated PLA_2 (p-PLA_2), phosphorylated PTK (p-PTK), phosphorylated p38 MAPK (p-p38 MAPK), PLCβ and GRK was measured using Western blotting analysis.

RESULTS: The activity of MS1 cells incubated with different concentrations of LPS for 6 h decreased significantly in the 1 μg/mL LPS group (0.49 ± 0.10 *vs* 0.67 ± 0.13, $P < 0.05$) and 10 μg/mL LPS group (0.44 ± 0.10 *vs* 0.67 ± 0.13, $P < 0.001$), but not in 0.1 μg/mL group. When the incubation time was extended to 12 h (0.33 ± 0.05, 0.32 ± 0.03 and 0.25 ± 0.03 *vs* 0.69 ± 0.01) and 24 h (0.31 ± 0.01, 0.29 ± 0.03 and 0.25 ± 0.01 *vs* 0.63 ± 0.01), MS1 cell activity decreased in all LPS concentration groups compared with the blank control ($P < 0.001$). BN52021 significantly improved the cell activity when its concentration reached 50 μmol/L compared with the group that received LPS treatment alone, which was consistent with the results obtained from fluorescence staining. The mRNAs levels of AC (4.02 ± 0.14 *vs* 1.00 ± 0.13), GRK (2.63 ± 0.03 *vs* 1.00 ± 0.12), p38 MAPK (3.87 ± 0.07 *vs* 1.00 ± 0.17), PLA_2 (3.31 ± 0.12 *vs* 1.00 ± 0.12), PLCβ (2.09 ± 0.08 *vs* 1.00 ± 0.06) and PTK (1.85 ± 0.07 *vs* 1.00 ± 0.11) were up-regulated after LPS stimulation as compared with the blank control ($P < 0.05$). The up-regulated mRNAs including AC (2.35 ± 0.13 *vs* 3.87 ± 0.08), GRK (1.17 ± 0.14 *vs* 2.65 ± 0.12), p38 MAPK (1.48 ± 0.18 *vs* 4.30 ± 0.07), PLCβ (1.69 ± 0.10 *vs* 2.41 ± 0.13) and PLA_2 (1.87 ± 0.11 *vs* 2.96 ± 0.08)

were significantly suppressed by BN52021 except for that of PTK. The level of p-AC (1.11 ± 0.12 *vs* 0.65 ± 0.08), GRK (0.83 ± 0.07 *vs* 0.50 ± 0.03), PLCβ (0.83 ± 0.16 *vs* 0.50 ± 0.10) and p-p38 MAPK (0.74 ± 0.10 *vs* 0.38 ± 0.05) was up-regulated after LPS stimulation as compared with the blank control ($P < 0.05$). The up-regulated proteins, including p-AC (0.65 ± 0.15 *vs* 1.06 ± 0.14), GRK (0.47 ± 0.10 *vs* 0.80 ± 0.06), PLCβ (0.47 ± 0.04 *vs* 0.80 ± 0.19) and p-p38 MAPK (0.30 ± 0.10 *vs* 0.97 ± 0.05), was significantly suppressed by BN52021, but p-PLA_2 and p-PTK protein level were not suppressed.

CONCLUSION: BN52021 could effectively inhibit LPS-induced inflammation by down-regulating the mRNA and protein levels of AC, GRK, p38 MAPK, PLA_2 and PLCβ in the PAFR signaling pathway.

Key words: BN52021; Platelet-activating factor receptor; Signaling pathway; Inflammation; Pancreatitis

Core tip: Microcirculatory disorder is considered to be one of the possible mechanisms of pathogenesis of severe acute pancreatitis (SAP). Platelet-activating factor (PAF) is known to mediate microcirculatory disturbance and inflammation. Although BN52021, a PAF receptor antagonist, has demonstrated significant treatment effects on SAP, its mechanism has not been elucidated in detail. In this study, we examined the signaling molecules of the PAF receptor pathway to evaluate whether BN52021 has any influence on the inflammatory effects induced by lipopolysaccharide in MS1 cells, hoping to elucidate the mechanism underlying microcirculatory disturbances in the pathogenesis of SAP *in vitro*.

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INTRODUCTION

Acute pancreatitis (AP) is an inflammatory disease that can develop into severe AP (SAP)[1]. SAP refers to AP associated with organ failure and/or local complications such as necrosis, pseudocyst or abscess, which is a disease of high morbidity and mortality with an unpredictable clinical course[2,3]. There is no clinically effective therapeutic strategy for SAP, because the pathogenesis of the disease remains largely unclarified. The possible explanations for the pathogenesis of SAP include theories of self-digestion, leukocyte overactivation, microcirculatory disorder, bacterial shifting, and secondary infection, which is a second attack by immune functional change, cell apoptosis, oxygen-free radicals, and others from different aspects[4]. Accumulated evidence has proven that microcirculatory disorders are the key pathogenesis of AP. Many complications of SAP are due to the amplifying effects of microcirculatory disruption[5-10]. The inflammation of pancreatic microvascular endothelial cells induced by lipopolysaccharide is a suitable pancreatitis model to simulate the microcirculatory disturbances *in vitro*.

Platelet-activating factor (PAF), a bioactive phospholipid synthesized and secreted by a variety of cells including pancreatic acini and microvascular endothelium cells[11], is known to mediate many physiological responses such as microcirculatory disturbance and inflammation. AP causes the release of PAF, which induces systemic effects that contribute to circulatory disturbance and multiple organ failure[1]. PAF can significantly potentiate pancreatic tissue damage, increase serum amylase and lipase levels, cause scattered hemorrhages and may serve as a primary mediator of inflammation in the pathological progress of SAP[1,7,12]. A single injection of PAF into the superior pancreaticoduodenal artery of rabbits induces dose-dependent morphologic alterations of the pancreatic tissue and increased serum amylase levels[13]. Our previous research revealed that PAF was stably expressed in the rat pancreas tissue and played an important role in inflammatory response during the procession of SAP[4,14]. PAF could produce physiological and pathological effects by binding to its cell surface receptor, PAF receptor (PAFR). Flickinger *et al*[15] revealed specific localization of PAFR in the pancreatic vascular endothelium but not in other pancreatic cell types. Recent studies have demonstrated that bacterial lipopolysaccharide (LPS) can induce an increase in the surface expression of PAF receptors[16]. Our recent study demonstrated that BN52021 exerted biological effects through inhibiting the increased PAF level and binding potential with PAFR rather than through decreasing PAFR expression in the pancreatic tissue[17]. Through binding with PAFR, PAF may, through G-protein transduction, activate phospholipase C, phospholipase A2, adenylate cyclase and tyrosine protein kinase, leading to the occurrence and development of SAP[18].

PAFR antagonists can block a series of inflammatory injuries caused by PAF, thereby improving the AP prognosis as a preventive treatment[19]. Research on such a potential therapy has helped elucidate the role of PAF in AP[20]. It was observed that BN52021 extracted from Ginkgo biloba leaves could act as a potent antagonist of PAFR[21], and BN52021 can inhibit the PAF-induced cascade effect in inflammatory reactions, exhibiting an anti-shock effect by reducing the portal vein pressure of liver cirrhosis[22,23]. In experimental pancreatitis models and clinical trials, the administration of several PAF antagonists significantly reduced the level of serum amylase, leukocyte infiltration, and improved capillary blood flow in the pancreas and distant organs, as well as the renal and respiratory functions and the survival rate. BN52021 could significantly reduce vascular permeability, pancreatic edema, hyperamylasemia, diminute superoxide dismutase activity, and inhibit lipid peroxidation in the

pancreatic tissue. These changes were accompanied by a significant reduction of acinar cell vacuolization and a remarkable inhibition of inflammatory cell infiltration in the interacinar space[24,25]. Our recent studies have also shown a therapeutic effect of BN52021 on experimental SAP[26-28], but its mechanism is not yet fully understood.

In this study, we examined signaling molecules of the PAFR pathway to evaluate whether a PAF receptor antagonist (BN52021) had any influence on the inflammatory effects induced by lipopolysaccharide in MS1 cells, hoping to elucidate the mechanism underlying the microcirculatory disturbances in the pathogenesis of SAP *in vitro*.

MATERIALS AND METHODS

Chemicals and reagents

Chemicals and reagents used in this study included BN52021, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and LPS (Sigma-Aldrich, St. Louis, MO, United States); Dulbecco's modified Eagle's medium (DMEM, Gibco/Invitrogen, Carlsbad, CA, United States); the mouse primers for the *Adcy1* [adenylate cyclase (AC)], *Pla2g4a* [phospholipase A_2 (PLA_2)], *Plcb3* [phospholipase Cβ (PLCβ)], *Ptk7* [protein tyrosine kinase (PTK)], *Adrbk1* [G protein-coupled receptor kinases (GRK)], *Mapk14* [p38-mitogen-activated protein kinase (p38 MAPK)] and *Gapdh* (glyceraldehyde 3-phosphate dehydrogenase) genes (Beijing AuGCT DNA-SYN Biotechnology Co., Ltd., Beijing, China); rabbit polyclonal antibodies of phosphorylated PTK (p-PTK) and phosphorylated AC (p-AC) (Abcam, Cambridge, MA, United States); rabbit polyclonal antibodies for GRK_2 and phosphorylated p38 MAPK (p-p38 MAPK) (Epitomics, Burlingame, CA, United States); rabbit polyclonal antibody for phosphorylated PLA_2 (p-PLA_2) (Cell Signaling Technology, Beverly, MA, United States); rabbit polyclonal antibody for PLCβ (Santa Cruz, Dallas, TX, United States); rabbit polyclonal antibody for β-actin (Abmart, Arlington, MA, United States); protein molecular weight markers, reverse transcription-polymerase chain reaction (RT-PCR) kit and quantitative PCR kit (Beijing TransGen Biotech Co., Ltd, Beijing, China); and polyvinylidene fluoride (PVDF) membranes (BD Biosciences, BD Corporation, MA, United States).

Cell culture

MS1 cell line (a mouse pancreatic islet endothelial cell line firstly established in 1994) was purchased from Shanghai Institute of Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mmol/L glutamine and 100 μg/mL penicillin/streptomycin in 5% CO_2 at 37 ℃. After grown to confluency in media, the cells were processed for subsequent studies.

MTT colorimetric assay

The viability/proliferation of the cells induced by LPS was observed using a MTT colorimetric assay as previously described[29]. MS1 cells received 0, 0.1, 1 and 10 μg/mL LPS in this experiment. Briefly, the cells were trypsinized with trypsin-ethylenediaminetetraacetic acid (EDTA), followed by incubation with DMEM in the presence of 10% FBS to inhibit trypsin activity. The cell pellets were then resuspended in DMEM with 10% FBS to a concentration of 1×10^4 cells/mL. Two hundred microliters of the cell suspension containing approximately 2000 cells was inoculated into selected wells of the 96-well plate. After the cells grew to 75% confluence, 20 μL of MTT solution was added to each well, and cultured for 4 h. Next, the medium was removed by inverting and tapping the plates, and 150 μL of dimethyl sulfoxide (DMSO) was added to each well. The spectrophotometric absorbance at 490 nm was measured by a Titertek Multiscan enzyme-linked immunosorbent assay reader. Each experiment was repeated at least three times. Every experimental condition was repeated at least in triplicate wells for each experiment.

Hoechst 33342/propidium iodide staining

The apoptosis and necrosis of the cells under the conditions described previously were observed by Hoechst 33342-propidium iodide (PI) staining[30]. MS1 cells were plated in a 6-well plate and co-incubated with media, LPS, LPS + DMSO and LPS + BN52021 when the cells achieved 90% confluence. The cells were washed twice with PBS. After the addition of 5 μL of Hoechst 33342 staining solution, the cells were stained with PI in the dark for 20-30 min at 4 ℃ and washed twice with PBS. Cells with blue and red fluorescence were examined under a fluorescence microscope.

Real-time quantitative RT-PCR

The mRNAs levels of AC, PLA_2, PLCβ, PTK, GRK and p38 MAPK were measured by real-time PCR. In detail, MS1 cells were plated in a 6-well plate and co-incubated with media, LPS, LPS + DMSO and LPS + BN52021 when the cells achieved 90% confluence. The cells were collected, and the total RNA was extracted with a Trizol RNA reagent kit according to the manufacturer's instructions. In addition, 2 μL (1 μg) of total RNA was added to the reverse transcription kit MIX system, and reverse-transcribed PCR was performed by random priming. The resulting complementary DNA amount was measured by quantitative PCR analysis using the GeneAmp 5700 Sequence Detection System and Step One Plus Real-Time PCR System (Applied Biosystems). The qPCR primer sequences are available online as indicated in Table 1. All expression data were normalized to the data for *Gapdh*. A no-template, double-distilled water control was included for each template. All samples were amplified simultaneously in triplicate in a single run. The relative quantitative gene expression was calculated as previously described and expressed as the percentage of the control level[31].

Western blotting

The protein expression level of p-AC, p-PLA_2, p-PTK,

Table 1 Specific primers for *Adcy1*, *Pla2g4a*, *Plcb3*, *Ptk7*, *Adrbk1*, *Mapk14* and *Gapdh* genes

Gene	Primer	Length (bp)	Annealing temperature (℃)
Adcy1			
Forward	5'-GACTTTGTTCTCCGAGTTG-3'	19	49
Reverse	5'-GTGCTATCCATCCGACTG-3'		
Pla2g4a			
Forward	5'-GAATAAAGGCTCTACAATGG-3'	20	49
Reverse	5'-GTTGTCGCTTTGGTACTC-3'		
Plcb3			
Forward	5'-CCTCAACTTCAACCGAGTT-3'	19	49
Reverse	5'-CAGAGTGAGGTACGGCTTG-3'		
Ptk7			
Forward	5'-CACTGCGATGTCACATTG-3'	18	49
Reverse	5'-CACTATGTTCGGGACTGG-3'		
Adrbk1			
Forward	5'-AAGCCAGCCAACATTCTC-3'	18	51
Reverse	5'-CCCTTCTGTAGGACTTCG-3'		
Mapk14			
Forward	5'-GGACCTGAACAACATCGTG-3'	19	50
Reverse	5'-CTAGGTTGCTGGGCTTTAG-3'		
Gapdh			
Forward	5'-CATCTTCCAGGAGCGAGAC-3'	19	50
Reverse	5'-GGCTAAGCAGTTGGTGGTG-3'		

Adcy1: Adenylate cyclase; *Pla2g4a*: Phospholipase A2; *Plcb3*: Phospholipase Cβ; *Ptk7*: Protein tyrosine kinase; *Adrbk1*: G protein-coupled receptor kinases; *Mapk14*: p38-mitogen-activated protein kinase; *Gapdh*: Glyceraldehyde 3-phosphate dehydrogenase.

p-p38 MAPK, PLCβ and GRK was measured using Western blotting analysis. In detail, MS1 cells were plated in a 6-well plate and co-incubated with media, LPS, LPS + DMSO and LPS + BN52021 for 24 h when the cells achieved 90% confluence. The cells were washed twice with 0.1 mol/L PBS and then lysed in RIPA lysis buffer (Tris-HCl 10 mmol/L, pH 7.4; NaCl 0.15 mmol/L; EDTA 0.5 mmol/L; phenylmethylsulfonyl fluoride 10 mmol/L; Tritonx-100 1%; dithiothreitol 40 mmol/L). The protein concentration of the lysate was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology, Beijing, China). Cell lysates containing 60 mg of protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis using 12% polyacrylamide resolving gels. After electrophoresis, the proteins were transferred onto PVDF membranes, which were then blocked with 5% nonfat dry milk in TBS-0.05% Tween 20 (TBST) for 1 h at room temperature, washed in TBST for 10 min × 3, and incubated at 4 ℃ with gentle shaking overnight with rabbit primary antibodies against the protein of interest at corresponding dilutions, followed by incubation with horseradish peroxidase conjugated to goat anti-rabbit immunoglobulin G at 1:2000 dilution, incubation with 1 mL of enhanced chemiluminescence reagent for 3 min, and exposure to the film. The optical density of the protein of interest relative to that of β-actin was analyzed using Quantity One 4.6.2.

Statistical analysis

The data are expressed as the mean ± SE. The dose and time effects of LPS on the activity of MS1 cells were evaluated with a two-way analysis of variance (ANOVA). The differences between three or more groups were evaluated by one-way ANOVA. A P value less than 0.05 (2-tailed) was considered statistically significant. All tests were performed using the statistical software package GraphPad 5.0 (GraphPad Software Inc., San Diego, CA, United States).

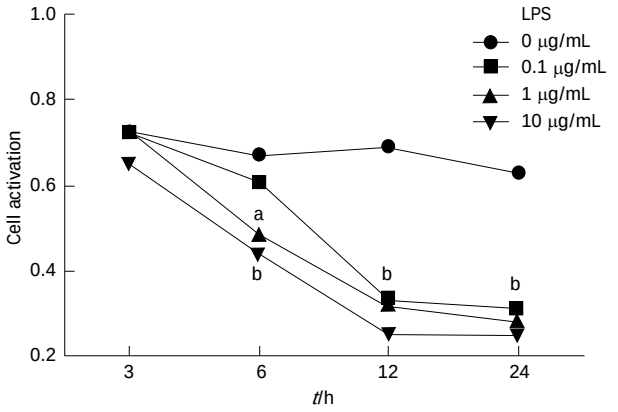

Figure 1 The optimal dose and duration of lipopolysaccharide stimulation were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. The cell survival rate was determined after incubation with 0 (saline) and 0.1, 1 and 10 μg/mL lipopolysaccharide (LPS) for 3, 6, 12 and 24 h. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the saline group.

RESULTS

Dose and time effect of LPS on MS1 cell activity

MS1 cells received 0, 0.1, 1 and 10 μg/mL LPS to mimic the inflammation condition of AP *in vitro*. The optimal dose and duration of LPS stimulation were determined using the MTT method. As shown in Figure 1, there was no significant difference in MS1 cell activity between cells co-incubated with the different concentrations of LPS and control cells 3 h after culture ($P > 0.05$), but when the incubation time was extended to 6 h, MS1 cell activity decreased significantly in the 1 μg/mL LPS group (0.49 ± 0.10 *vs* 0.67 ± 0.13, $P < 0.05$) and 10 μg/mL LPS group (0.44 ± 0.10 *vs* 0.67 ± 0.13, $P < 0.001$), but not in the 0.1 μg/mL group ($P > 0.05$) compared with the control group. When the incubation time was extended to 12 h (0.33 ± 0.05, 0.32 ± 0.03 and 0.25 ± 0.03 *vs* 0.69 ± 0.01) and 24 h (0.31 ± 0.01, 0.29 ± 0.03 and 0.25 ± 0.01 *vs* 0.63 ± 0.01), MS1 cell activity decreased in all LPS concentration groups compared with the blank control ($P < 0.001$). Therefore, we chose the concentration 10 μg/mL LPS for the 24 h stimulation as the optimal protocol in the following experiments.

Dose effect of BN52021 on LPS-induced inflammation

The dose effect of BN52021 on LPS-induced inflammation was determined using the MTT method and Hoechst 33342/PI staining. The MS1 cell activity was significantly decreased 24 h after administration of 10 μg/mL LPS compared with the control group ($P < 0.01$). Pretreat-

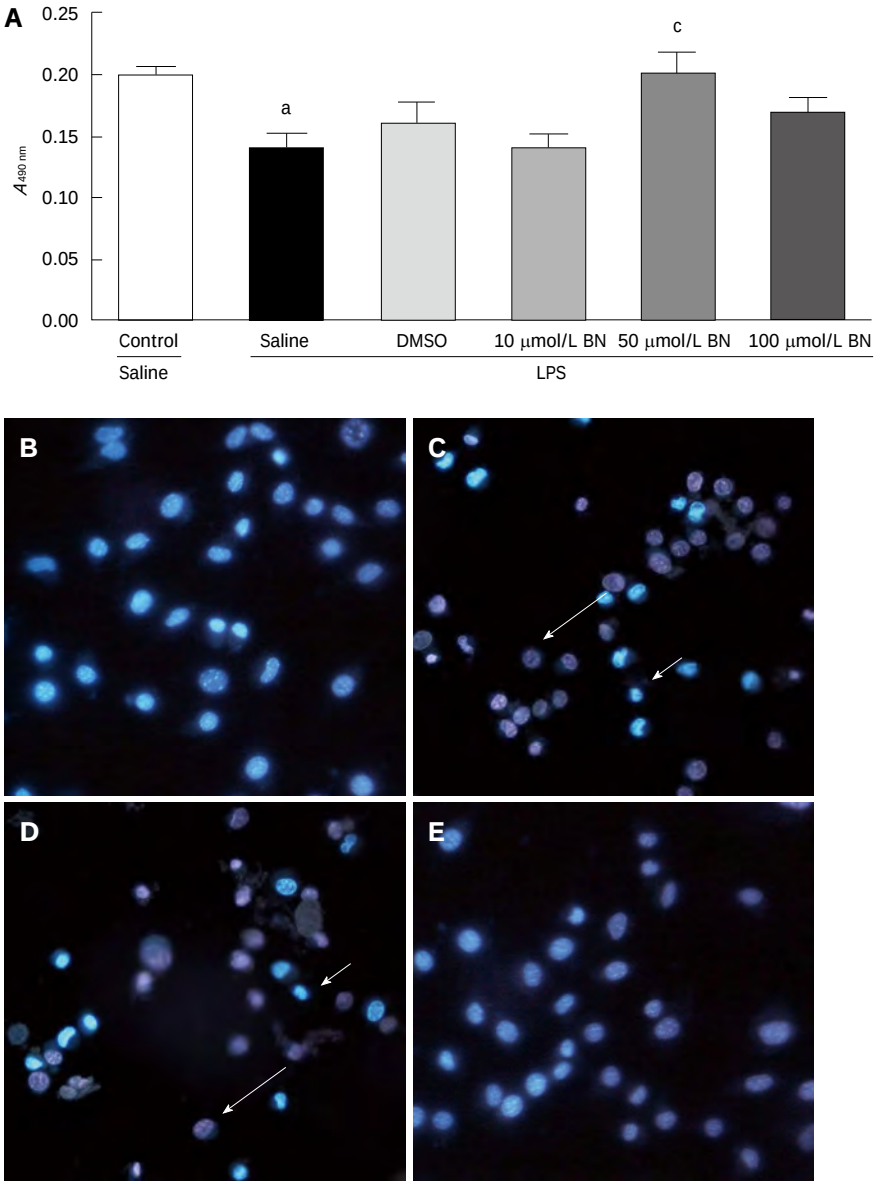

Figure 2 The dose effect of BN52021 on lipopolysaccharide-induced inflammation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method and Hoechst 33342/propidium iodide staining. MS1 cell activity at $A_{490\ nm}$ was significantly decreased 24 h after administration of 10 μg/mL lipopolysaccharide (LPS) *vs* the control group ($^aP < 0.05$). Pretreatment with BN52021 for 20 min before incubation with LPS significantly improved the MS1 cell activity at $A_{490\ nm}$ *vs* the group that received LPS treatment only when its concentration reached 50 μmol/L ($^cP < 0.05$) (A). Pretreatment with 50 μmol/L BN52021 for 20 min before incubation with LPS significantly improved MS1 cell activity *vs* the LPS + saline group, and the LPS + dimethyl sulfoxide (DMSO) group as determined Hoechst 33342/propidium iodide staining (B, C, D and E). The arrows indicate the apoptosis (short) and necrosis (long) of the cells.

ment with BN52021 20 min before incubation with LPS significantly improved the cell activity compared with the group receiving LPS only when its concentration reached 50 μmol/L, which was consistent with the results obtained by Hoechst 33342/PI staining ($P < 0.05$) (Figure 2). Therefore, the concentration of 50 μmol/L BN52021 was used for pretreatment in the following experiments.

Effect of BN52021 on PAFR signaling molecules at the mRNA level in LPS-induced inflammation

The mRNAs levels of AC (to 4.02 ± 0.14 folds), GRK (to 2.63 ± 0.03 folds), p38 MAPK (to 3.87 ± 0.07 folds), PLA_2 (to 3.31 ± 0.12 folds), PLCβ (to 2.09 ± 0.08 folds) and PTK (to 1.85 ± 0.07 folds) were up-regulated after LPS stimulation compared with the blank control ($P < 0.05$). The up-regulated mRNAs were significantly suppressed by BN52021, except for that of PTK (fold-change relative to control, 1.83 ± 0.13, $P > 0.05$), including that of AC (fold-change relative to control, down to 2.35 ± 0.13), GRK (down to 1.17 ± 0.14), p38 MAPK (down to 1.49 ± 0.18), PLCβ (down to 2.09 ± 0.08) and PLA_2 (down to 1.87 ± 0.11), as shown in Figure 3.

Effect of BN52021 on PAFR signaling molecules at the protein level in LPS-induced inflammation

The level of p-AC (fold-change relative to control, increase from 0.65 ± 0.08 to 1.11 ± 0.12), GRK (increase from 0.50 ± 0.03 to 0.83 ± 0.07), PLCβ (increase from 0.50 ± 0.10 to 0.83 ± 0.16) and p-P38 MAPK (increase from 0.38 ± 0.05 to 0.74 ± 0.10) was up-regulated after LPS stimulation compared with the blank control ($P < 0.05$). The up-regulated protein level was significantly suppressed by BN52021 for p-AC (decrease from 1.11 ± 0.12 to 0.65 ± 0.15), GRK (decrease from 0.83 ± 0.07 to 0.47 ± 0.10), PLCβ (decrease from 0.83 ± 0.16 to 0.47 ± 0.04) and p-p38 MAPK (decrease from 0.74 ± 0.10 to 0.30 ± 0.10). However, the level of p-PLA_2 and p-PTK was not significantly up-regulated after LPS stimulation and was not significantly altered by BN52021, as shown in Figure 4.

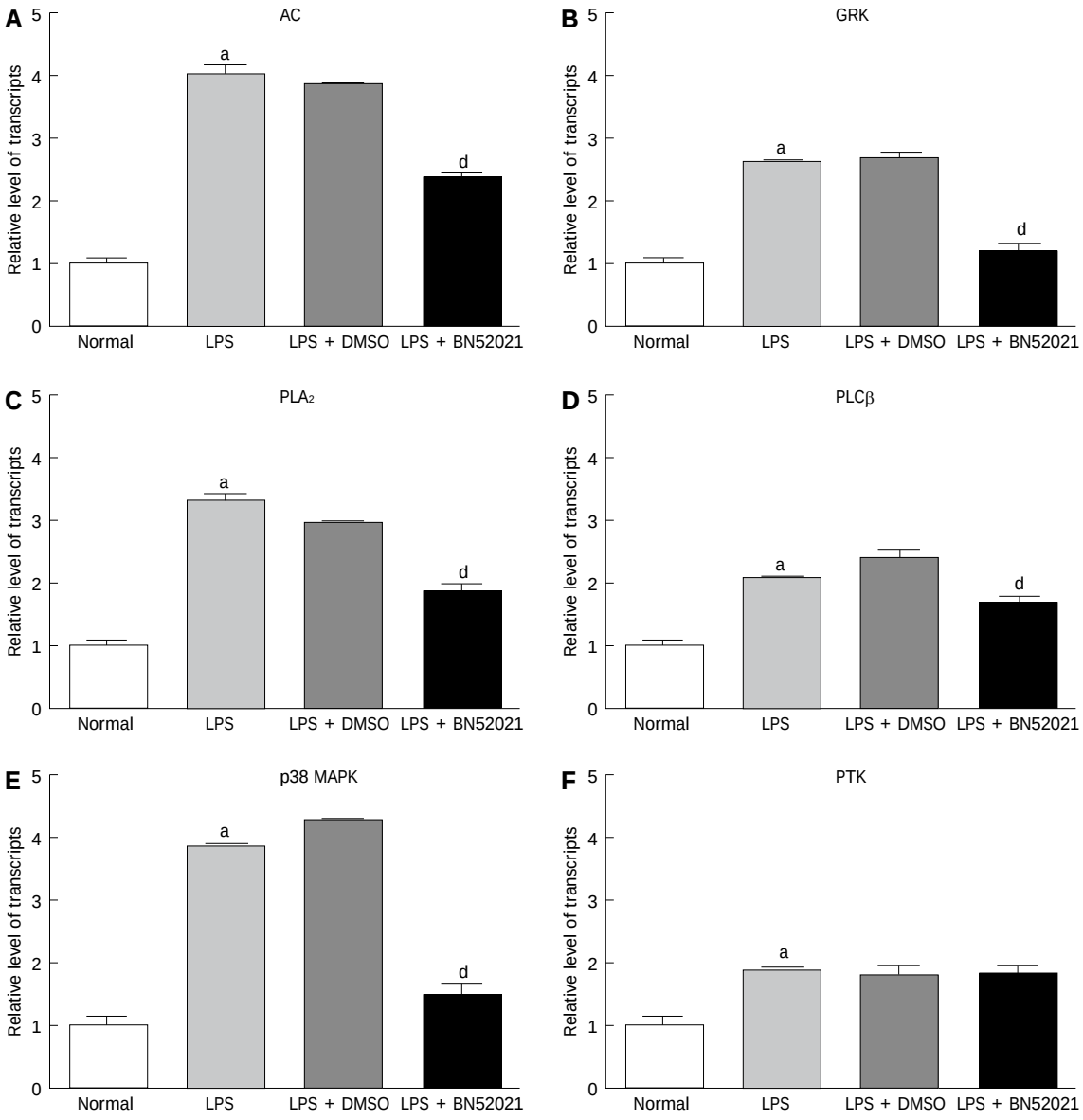

Figure 3 The effect of BN52021 on platelet-activating factor receptor signaling molecules at the mRNA level under lipopolysaccharide-induced inflammation. The mRNA level of adenylate cyclase (AC) (A), G protein-coupled receptor kinases (GRK) (B), phospholipase A_2 (PLA_2) (C), phospholipase Cβ (PLCβ) (D), p38-mitogen-activated protein kinase (p38 MAPK) (E) and protein tyrosine kinase (PTK) (F) was up-regulated after lipopolysaccharide (LPS) stimulation. The up-regulation of AC, GRK, p38 MAPK, PLCβ and PLA_2 mRNA was significantly suppressed by BN52021 except for that of PTK. $^aP < 0.05$ *vs* control; $^dP < 0.01$ *vs* the LPS + dimethyl sulfoxide (DMSO) groups.

DISCUSSION

In this study, we examined the signaling molecules of the PAFR pathway to evaluate whether the PAFR antagonist BN52021 had any influence on LPS-induced inflammation in MS1 cells. It was observed that BN52021 could sufficiently inhibit the inflammation, apoptosis and necrosis induced by LPS in pancreatic vascular endothelial cells. BN52021 could inhibit the up-regulation of signaling molecules in the PAFR pathway, which may help to explaining the mechanism underlying microcirculatory disturbance in the pathogenesis of AP.

PAF-induced microcirculatory disruption plays a key role in the pathogenesis of AP

Platelet-activating factor is a proinflammatory lipid mediator that plays a key role in many pathophysiological conditions, including asthma, ischemia, gastrointestinal ulceration, pancreatitis and multiple organ failure[32]. A number of experimental studies suggest that the pathogenesis of AP correlates with microcirculatory disorders. An experiment that constricted interlobular pancreatic arteries 2 min after intraductal infusion of sodium taurocholate indicated that microcirculatory changes are closely related to the process of AP[6]. Many complications of SAP are due to the amplifying effect of microcirculatory disruption[7]. PAF is one of the most important vasoactive mediators activated during the inflammatory response to pancreatic injury that can cause microcirculatory disorders in AP. Recent data suggest that PAF can directly modulate microvascular permeability and increase venular permeability[10]. Increased microvessel permeability

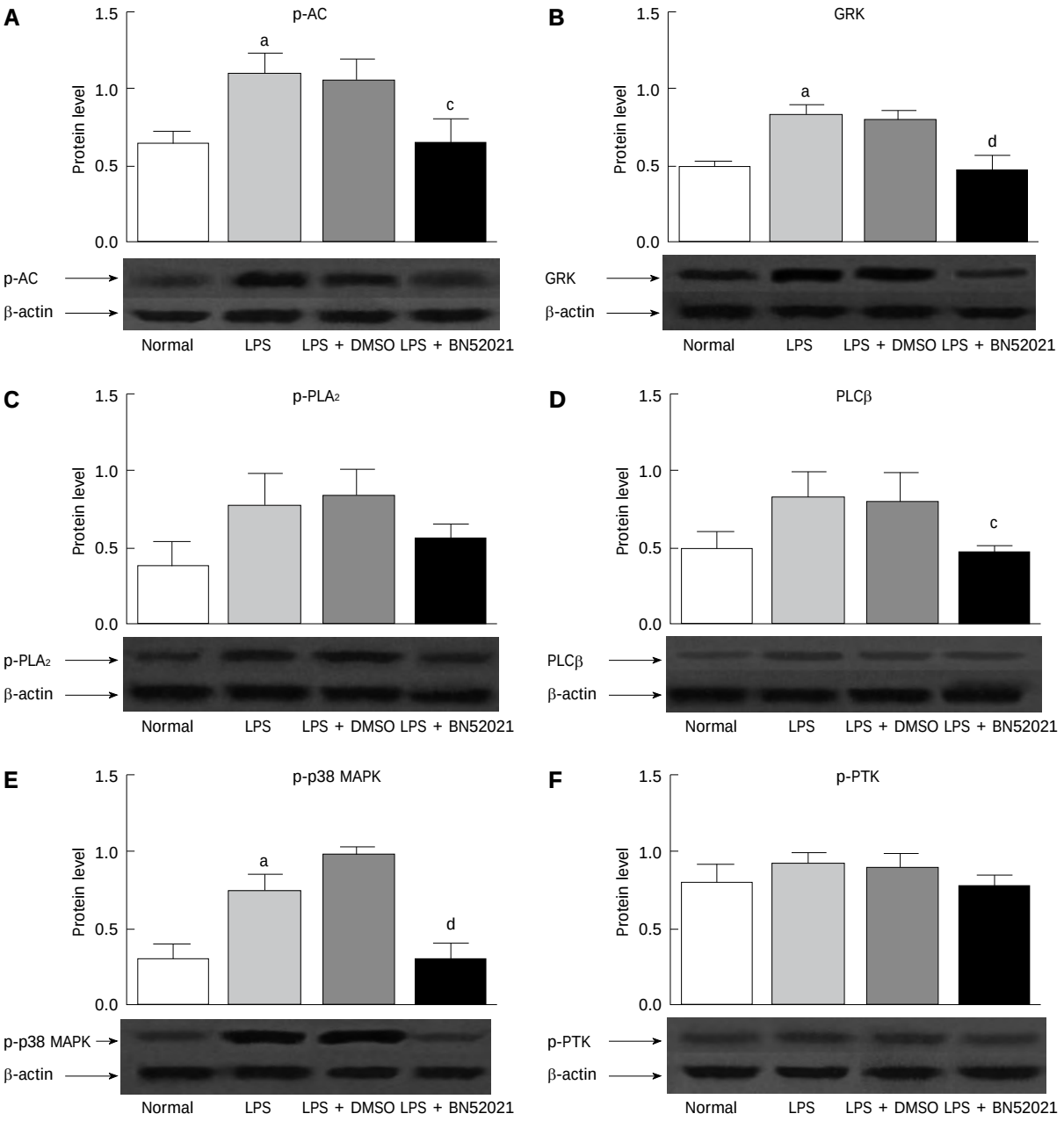

Figure 4 The effect of BN52021 on platelet-activating factor receptor signaling molecules at the protein level under lipopolysaccharide-induced inflammation. The protein level of p-adenylate cyclase (p-AC) (A), G protein-coupled receptor kinases (GRK) (B), p-phospholipase A_2 (p-PLA_2) (C), phospholipase Cβ (PLCβ) (D) and p-p38-mitogen-activated protein kinase (p-p38 MAPK) (E) was up-regulated after lipopolysaccharide (LPS) stimulation *vs* the blank control ($^aP < 0.05$). The up-regulation of p-AC, p-p38 MAPK, GRK and PLCβ protein levels was significantly suppressed by BN52021. However, p-PLA_2 and phosphorylated protein tyrosine kinase (p-PTK) protein levels were insignificantly up-regulated after LPS stimulation and were not significantly changed by BN52021 (F). $^cP < 0.05$, $^dP < 0.01$ *vs* LPS + dimethyl sulfoxide (DMSO) groups.

induced by PAF may be related directly to endothelial cell activation, adhesion molecule expression, and leukocyte activation[7,8]. Increased capillary permeability permits the sequestration of macromolecules and fluid, causing deficiency of circulating blood volume and microcirculatory disorders[7]. In addition, vasospasm and microthrombus formation due to hypercoagulability can also lead to the deterioration of pancreatic microcirculation and pancreatic necrosis[7]. The treatment of AP with PAF antagonists can significantly improve capillary blood flow in the pancreas and colon, renal and respiratory function, and the survival rate and can stabilize capillary permeability and decrease fluid loss into the third space[33,34]. As a preventive treatment, PAFR antagonists such as BN52021 can block a series of PAF-mediated inflammatory injuries, thus improving the prognosis of AP[1]. This protective effect of PAF antagonists further supports the role of PAF in microcirculatory disorders.

LPS-induced inflammation of pancreatic microvascular endothelial cells is a suitable pancreatitis model to simulate microcirculatory disturbance *in vitro*. The MS1 cell line is a mouse pancreatic islet endothelial cell line

first established in 1994. It can represent the pancreatic microvascular endothelium because previous studies[35] have verified that the pancreatic lobule is a structured and functional basic unit of pancreatic microcirculation, and insulo-acinar portal circulation represents the basic feature of the pancreatic microcirculation. Therefore, in this study, we examined the signaling molecules of the PAFR pathway in MS1 cells to evaluate whether the PAF receptor antagonist BN52021 had any influence on the LPS-induced inflammatory effect, hoping that it could help elucidate the mechanism underlying microcirculatory disturbance in the pathogenesis of SAP *in vitro*. Our results indicated that pretreatment with BN52021 for 20 min before incubation with LPS could significantly improve the MS1 cell activity compared with the group that received LPS treatment only.

PAFR signaling pathway plays a pivotal role in pancreatic proinflammatory response

In recent years, researchers have become concerned with the significance of the signal transduction pathway of PAF in the pathogenesis of AP[4,22,28], because it has been reported to induce morbidity and unacceptably high mortality[18]. However, the impact of a PAF receptor antagonist (BN52021) on the signaling molecules of the PAFR signaling pathway in pancreatic microvascular endothelial cells under the LPS-induced inflammatory condition remains unclear.

PAFR is almost ubiquitous in diverse type cells and acts not only on the local pancreas cells, including the pancreatic vascular endothelium, but also on distant organs, inducing systemic inflammatory response and multiple organ injury[15]. PAFR belongs to the G protein-coupled receptor subfamily[36]. By binding to its receptor, PAF activates the associated G protein, which, in turn, activates phosphoinositide hydrolysis by phosphoinositide specific phospholipase C, arachidonic acid release by phospholipase A_2, increases in intracellular Ca^{2+} concentration, activation of protein kinase C and PTK[37]. PAF has also been shown to activate MAPKs, including extracellular signal-regulated kinase[38-42], p38 MAPK[38,40,41], and c-Jun N-terminal kinase[43]. Deo *et al*[44] reported that PAF activated pertussis toxin-insensitive Gαq protein upon binding to its seven transmembrane receptors and adenylate cyclase, elevating cAMP levels, and thus activating protein kinase A in human umbilical vein endothelial cells. GRK plays a key role in the homologous desensitization of G protein-coupled receptor (GPCR) and GRK phosphorylate activated receptors, promoting high affinity binding of arrestins, thus precluding G protein coupling. Direct binding to active GPCRs activates GRKs so that they selectively phosphorylate only the activated form of the receptor regardless of the accessibility of the substrate peptides within it and their Ser/Thr-containing sequence[45]. Most GPCRs display a rapid loss of responsiveness in the continuing presence of chemoattractants in a process of desensitization that involves the phosphorylation of agonist-occupied GPCR by GRK[46]. The inflammation in pancreatic vascular endothelial cells induced by LPS was suppressed by BN52021. This finding might contribute to an understanding of the mechanism underlying the microcirculatory disturbances in the pathogenesis of SAP.

According to our results, the mRNA and protein levels of AC, GRK, p38 MAPK, PLA_2 and PTK were up-regulated after LPS stimulation compared with the blank control. The up-regulated AC, GRK, p38 MAPK and PLA_2 mRNA and protein levels were significantly suppressed by BN52021, suggesting that BN52021 could effectively inhibit the apoptosis and necrosis of MS1 cells under the LPS-induced inflammatory condition. The mechanism underlying the inhibition might relate to the suppression effect of BN52021 on the up-regulation of AC, GRK, p38 MAPK and PLA_2 mRNA and protein levels in the PAFR signaling pathway.

Other potential mechanisms of PAFR antagonism in AP treatment

It is known that PAFR is also able to interact with components of the bacterial wall, such as lipopolysaccharides[47] and phosphorylcholine[48]. The cell wall components exit the vasculature into the heart and brain, accumulating within endothelial cells, cardiomyocytes, and neurons in a PAFR-dependent way. The physiological consequences of the cell wall/PAFR interaction are cell specific, being noninflammatory in endothelial cells and neurons but causing a rapid loss of cardiomyocyte contractility that contributes to death. Thus, PAFR shepherds phosphorylcholine-containing bacterial components such as the cell wall into host cells from where the response ranges from quiescence to severe pathophysiology[48]. The explanation for the protective effect of BN-52021 cannot simply be attributed to the antagonism of LPS binding to PAFR or the prevention of PAF binding to its receptor. Therefore other potential mechanisms of PAFR antagonism in AP treatment must exist.

Bacterial translocation from the gastrointestinal tract to mesenteric lymph nodes and other extra intestinal organs is an important source of infection in AP. Preventing bacterial dissemination in early AP may have beneficial effects on the evolution of this disease[26,49]. PAF antagonist treatment decreases the bacterial spread to distant sites, suppresses elevation of interleukin (IL)-6 level, and has a significant effect on serum pancreatic enzymes and the histologic score of pancreatitis without reducing serum amylase and tumor necrosis factor alpha levels or ameliorating pancreatic damage in rats with AP[7,50]. In addition, BN52021 has been shown to have protective effect on slow mesenterioangial small arteriolar and venular blood flow velocity and dilated mesenterioangial small venular diameter in the early phase of AP[51]. Pretreatment with lexipafant could reduce the pancreatic endothelial barrier dysfunction and severity of pancreatitis-associated intestinal dysfunction as well as systemic concentrations of IL-1 and local leukocyte recruitment in experimental AP rats[52-54]. PAFR antagonism appears

to be involved in the maintenance of intestinal barrier integrity and the inhibition of cytokines release, such as IL-1 and IL-6[32]. Moreover, PAFR antagonists can also exert their effects by inhibiting the activity of neutrophils and depressing pulp peroxidase, competing for targets with PAF and inhibiting the activity of PAF, inhibiting increases in PAF in AP, and reducing plasma cytokines and inflammatory mediators, enzyme activity and the role of self-digestion of pancreatic tissue[1]. The involvement of the PAFR signaling pathway in these mechanisms needs to be further investigated.

The PAFR antagonist BN52021 could effectively inhibit LPS-induced inflammation, apoptosis and necrosis in pancreatic vascular endothelial cells. The mechanisms underlying the inhibition might be related to the suppression effect of BN52021 on the up-regulation of AC, GRK, p38 MAPK and PLCβ mRNA and protein levels in the PAFR signaling pathway, which may help to explain the mechanism underlying the microcirculatory disturbance in the pathogenesis of AP.

COMMENTS

Background

Microcirculatory disorder is considered to be one of the possible mechanisms of severe acute pancreatitis (SAP) pathogenesis. Platelet-activating factor (PAF), a bioactive phospholipid synthesized and secreted by a variety of cells including pancreatic acini and microvascular endothelium cells, is known to mediate many physiological responses, including microcirculatory disturbance and inflammation.

Research frontiers

Recent studies have demonstrated that PAF plays an important role in the pathological progress of SAP. Although BN52021, a PAF receptor antagonist, has demonstrated significant treatment effects against SAP, its effects on PAF receptor (PAFR) signaling molecules have not been elucidated in detail.

Innovations and breakthroughs

The authors found that BN52021 could effectively inhibit the apoptosis and necrosis of MS1 cells under lipopolysaccharide (LPS)-induced inflammatory conditions. The mechanism underlying the inhibitory effect may relate to the inhibitory effect of BN52021 on the up-regulation of adenylate cyclase, G protein-coupled receptor kinases, p38-mitogen-activated protein kinase, phospholipase A_2 and phospholipase Cβ mRNA and protein levels in the PAFR signaling pathway.

Applications

This study may contribute to a future strategy involving SAP treatment with BN52021 by investigating how PAF is induced and blocking its expression.

Terminology

PAF is a biologically active phospholipid mediator that plays its role by binding to PAFR, which is a unique G-protein-coupled seven transmembrane receptor, and the binding activates multiple intracellular signaling pathways. Ginkgolide B (code: BN52021) is one of the four Ginkgolide constituents (Ginkgolide A, B, C and J) that are present in the whole extract of Ginkgo biloba leaves.

Peer review

This article attempts to elucidate the protective role of BN-52021 against LPS-induced apoptosis and necrosis in a pancreatic islet endothelial cell line. PAF is a crucial mediator of acute pancreatitis. Therefore the inhibition of its actions by BN-52021 is interesting from a pharmaceutical point of view. Because BN-52021 is a well-established antagonist of PAFR, the authors investigated its effect on certain members of the signal transduction pathways initiated by PAFR activation. The results are novel and interesting.

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ORIGINAL ARTICLE

Polydatin attenuated food allergy *via* store-operated calcium channels in mast cell

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Abstract

AIM: To investigate the effect of polydatin (PD), a resveratrol glucoside, on mast cell degranulation and anti-allergic activity.

METHODS: After the rats were orally sensitized with ovalbumin (OVA) for 48 d and underwent PD treatment for 4 d, all the rats were stimulated by 100 mg/mL OVA for 24 h and then sacrificed for the following experiments. The small intestines from all the groups were prepared for morphology examination by hematoxylin and eosin staining. We also used a smooth muscle organ bath to evaluate the motility of the small intestines. The OVA-specific immunoglobulin E (IgE) production and interleukin-4 (IL-4) levels in serum or supernatant of intestinal mucosa homogenates were analyzed by enzyme-linked immunosorbent assay (ELISA). Using toluidine blue stain, the activation and degranulation of isolated rat peritoneal mast cells (RPMCs) were analyzed. Release of histamine from RPMCs was measured by ELISA, and regulation of PD on intracellular Ca^{2+} mobilization was investigated by probing intracellular Ca^{2+} with fluo-4 fluorescent dye, with the signal recorded and analyzed.

RESULTS: We found that intragastric treatment with PD significantly reduced loss of mucosal barrier integrity in the small intestine. However, OVA-sensitization caused significant hyperactivity in the small intestine of allergic rats, which was attenuated by PD administration by 42% (1.26 ± 0.13 g *vs* OVA 2.18 ± 0.21 g, $P < 0.01$). PD therapy also inhibited IgE production (3.95 ± 0.53 ng/mL *vs* OVA 4.53 ± 0.52 ng/mL, $P < 0.05$) by suppressing the secretion of Th2-type cytokine, IL-4, by 34% (38.58 ± 4.41 pg/mL *vs* OVA 58.15 ± 6.24 pg/mL, $P < 0.01$). The ratio of degranulated mast cells, as indicated by vehicles (at least five) around the cells, dramatically increased in the OVA group by 5.5 fold (63.50% ± 15.51% *vs* phosphate-buffered saline 11.15% ± 8.26%, $P < 0.001$) and fell by 65% after PD treatment (21.95% ± 4.37% *vs* OVA 63.50% ± 15.51%, $P < 0.001$). PD mediated attenuation of mast cell degranulation was further confirmed by decreased histamine levels in both serum (5.98 ± 0.17 *vs* OVA 6.67 ± 0.12, $P < 0.05$) and intestinal mucosa homogenates (5.83 ± 0.91 *vs* OVA 7.35 ± 0.97, $P < 0.05$). Furthermore, we demonstrated that administration with PD significantly decreased mast cell degranulation due to reduced Ca^{2+} influx through store-operated calcium channels (SOCs) (2.35 ± 0.39 *vs* OVA 3.51 ± 0.38, $P < 0.01$).

CONCLUSION: Taken together, our data indicate that PD stabilizes mast cells by suppressing intracellular Ca^{2+} mobilization, mainly through inhibiting Ca^{2+} entry *via* SOCs, thus exerting a protective role against OVA-sensitized food allergy.

Key words: Polydatin; Food allergy; Mast cells; Store-operated calcium channels; Ca^{2+}

Core tip: In the present study, we have demonstrated for the first time that polydatin has the capacity for preventing pathogenesis of food allergy, which is dependent on regulation of Ca^{2+} mobilization *via* store-operated calcium channels in mast cells.

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INTRODUCTION

Food allergy (FA) is an adverse reaction mediated by immunoglobulin E (IgE) or non-IgE antibodies[1], which involves an abnormal response by the immune system to specific proteins in foods[2]. FA has been recognized as a worldwide health problem, especially in western countries, which is due to the severity of the reactions and its dramatic increase over the past three decades[3-5]. The majority of food allergies worldwide are caused by "eight main food allergens", including peanuts, tree nuts, eggs, milk, fish, crustacean shellfish, wheat, and soy[6]. It has been suggested that 25% of infants[7], 8% of children[3-5], and 2%-5% of adults[8] suffer from FA. However, the current understanding about the etiology of food allergies remains poor, and no effective treatment is available except the preventative measure of avoiding the offending food in the diet.

Mast cells play an essential role in the development of intestinal inflammatory disorders during food allergy. Cross-linking of the high-affinity IgE receptor (FcεRI) on mast cells by allergens results in degranulation, leukotriene generation, and cytokine synthesis. Degranulated mast cells release inflammatory mediators, including histamine and Th2 cytokines[9], which cause abnormal gut contractions and intestinal mucosa damage, which in term then result in abdominal pain, cramps, vomiting, and/or diarrhea[10]. It has been known that IgE-dependent mast cell degranulation relies on intracellular Ca^{2+} signaling[11,12]. Cytoplasmic Ca^{2+} mainly comes from the stored Ca^{2+} in the endoplasmic reticulum (ER) and extracellular Ca^{2+} through store-operated calcium channels (SOCs)[13]. Therefore, modulation of Ca^{2+} mobilization is a potential therapeutic strategy for stabilizing mast cells upon FcεRI activation and potentially offering a novel treatment method for allergic diseases.

Polydatin (PD), also known as polygoni cuspidati radix, is a natural component isolated from *Polygonum cuspidatum*. It has been determined as a resveratrol glucoside with a 3,4,5-trihydroxystilben-3-*D*-mono-*D*-glucoside molecular structure. Previous studies have demonstrated that PD has a therapeutic effect on the treatment of allergic diseases. Using the passive cutaneous anaphylaxis (PCA) model in mice, Lim *et al*[14] showed that PD reduced mast cell degranulation by suppressing phosphorylation of Syk and mitogen-activated protein kinases. On the other hand, Yuan *et al*[15] found that PD alleviated PCA in mice by stabilizing mast cells *via* the inhibition of Ca^{2+} release-activated Ca^{2+} channels. However, the therapeutic effect of PD on food allergies has not yet been determined.

In this study, we established ovalbumin (OVA)-induced food allergic models and evaluated the therapeutic effect of PD on food allergy. Furthermore, we explored the effect of PD on mast cell degranulation and found that the underlying mechanism was related to Ca^{2+} mobilization. Our research presented here is the first to reveal that PD can inhibit food allergy by suppressing mast cell degranulation *via* regulation of SOCs.

MATERIALS AND METHODS

Animals

Four-week old female Brown-Norway (BN) rats were purchased from Vital River Laboratories (Beijing, China) and housed in groups of four per cage in a controlled environment with a photoperiod of 12 h light to 12 h dark and a temperature of 20 ± 2 ℃. Sanitary controls were performed for all major rodent pathogens, with the results of these tests being uniformly negative. All the animal experimental procedures were approved by the Animal Care and Use Committee of Shenzhen University and carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (publication No. 85-23, revised 1996).

Forty-eight Brown-Norway rats were randomly divided into four groups: control group (n = 12), OVA group (n = 12), OVA + PD group (n = 12), and PD group (n = 12). Each group received phosphate-buffered saline (PBS), OVA, or PD, as shown in Figure 1[16]. The control group received 1 mL PBS (0.1 mol/L) daily by gavage administration for 52 d, while the OVA group was orally treated with 1 mg OVA (1 mg/mL) for the first 48 d and 1 mL PBS (0.1 mol/L) from days 48 to 52. The OVA + PD group received PD (150 mg/mL × 1 mL daily per rat) oral treatment daily from days 49 to 52 after OVA sensitization. The PD group was not challenged by OVA. All the groups of rats were stimulated by 100 mg/mL OVA for 24 h at the end of day 52 and then sacrificed for the following experiments.

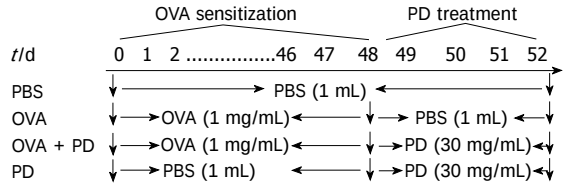

Figure 1 Protocol of ovalbumin sensitization/challenge and Formula-3 treatment. Rats were sensitized with ovalbumin (OVA) (1 mg/mL × 1 mL daily per rat) intragastrically for 48 d. For the polydatin (PD) treatment group, the rats were orally treated with PD (30 mg/mL × 1 mL daily per rat) from days 49 to 52 after OVA sensitization. All the rat groups were challenged by 100 mg/mL OVA for 24 h at the end of day 52 and then sacrificed. PBS: Phosphate-buffered saline.

Hematoxylin and eosin staining in small intestine tissues

The jejunal parts of the small intestine were isolated from the rats and embedded with paraffin. Sections (7 μm) were prepared and subjected to hematoxylin and eosin (HE) staining as previously reported[17].

Measurements of smooth muscle contractility

The tension of smooth muscle contractility was measured as previously reported[18]. Briefly, 2 cm long segments of the small intestine from the upper part of the jejunum to the lower part of the ileum were cut and mounted by hanging from triangle hooks. The hooks were connected to transducers from the upper end, and were inserted through the gut lumen from the lower end, allowing the circular muscle to contract. The tissue segments were incubated in chambers containing 20 mL Tyrode's solution (136 mmol/L NaCl, 5.4 mmol/L KCl, 1.0 mmol/L $MgCl_2$, 0.33 mmol/L NaH_2PO_4, 1.8 mmol/L NaCl, 10.0 mmol/L glucose and 5.0 mmol/L HEPES), which was kept at 37 ℃ and constantly aerated with a mixture of 95% oxygen and 5% carbon dioxide. The initial tension load was set at 1.0 g, from which the segments spontaneously relaxed over time. The segments were allowed to stabilize for 30 min before they were stimulated with 1 mg/mL OVA for 3 min. *R0* was defined as the mean basal tension, when the segments were under rest conditions. ΔR denotes (*R1-R0*), where *R1* is the contract tension when the segments were stimulated with OVA.

Toluidine blue stain

Typical mast cells in rat small intestine tissue or peritoneal lavage solution (RPLS) were stained with toluidine blue stain as previously described[19]. Briefly, 200 L RPLS was air dried on cromolyn sodium pretreated slides and then covered with several drops of staining solution (toluidine blue stain dissolved in 70% ethanol). After 90 s, the staining solutions were washed away quickly with running tap water and the stained cells were examined and counted under a light microscope (Olympus, Japan).

Enzyme-linked immunosorbent assay

The contents of interleukin-4 (IL-4) (eBioscience Inc., CA, United States) and histamine (R & D Inc., MN, United States) in RPLS and serum were assayed by commercial enzyme-linked immunosorbent assay (ELISA) kits using paired antibodies according to the manufacturer's instructions. Serum IgE levels were also checked using a commercial ELISA kit (BD Pharmingen, CA, United States), following the manufacturer's instructions.

Rat peritoneal mast cell isolation

The BN rats were sacrificed after being anaesthetized by ether inhalation in air. Rat peritoneal mast cells (RPMCs) were obtained by peritoneal lavage and purified by density gradient fractionation as described previously[20,21]. Isolated RPMCs preparations contained > 98% mast cells and at least 98% of these cells were viable, as checked by metachromatic staining in 0.05% toluidine blue.

Ca^{2+} imaging by confocal microscope

Intracellular Ca^{2+} signal was measured as described previously with minor modification[22]. RPMCs or RBL-2H3 cells were incubated with 5 μmol/L Ca^{2+} fluorescent probe fluo-4 AM (Invitrogen, CA, United States) for 30 min at room temperature. After washing with Tyrode's solution three times, the dye inside the cells was allowed to de-esterify for 30 min at 37 ℃. It has been determined that nearly 95% of the fluorescent dye was retained in the cytoplasm. Fluorescent images of Ca^{2+} were obtained using an Olympus 1000 confocal microscope with a 40 × oil immersion lens (NA 1.3) (Olympus, Japan). The fluo-4 signal was excited at 488-nm and emitted at > 505 nm. Frame-scan images were acquired at a sampling rate of 15 ms per frame and 20 s per interval.

Image data were analyzed off-line using fv10-asw.2.1 software. A selected image from each image set was used as a template for designating the region of interest (ROI) within each cell. The integrated intracellular Ca^{2+} concentration was determined by calculating $\Delta F/F0$. *F0* was defined as the mean basal fluorescence intensity of the dye recorded during the first 5-10 scanning frames, when the cells were under rest conditions. ΔF denotes (F-*F0*), where F is the temporal fluorescence intensity. The $\Delta F/F0$ values within each ROI were plotted as a function of time (typical time-courses of Ca^{2+} response to thapsigargin or DNP-BSA stimulation in single RBL-2H3 cells). The amplitude of the Ca^{2+} response within each cell was quantified as the highest $\Delta F/F0$ level reached during the measurement period, which was averaged over all cells within each group.

Statistical analysis

Data are presented as mean ± SE. When two comparisons were obtained, Student's unpaired two tailed t test was used. When multiple comparisons were obtained, the analyses consisted of one-way analysis of variance for repeated measures and Student-Newman-Keuls multiple comparison test. A value of $P < 0.05$ was considered to be statistically significant.

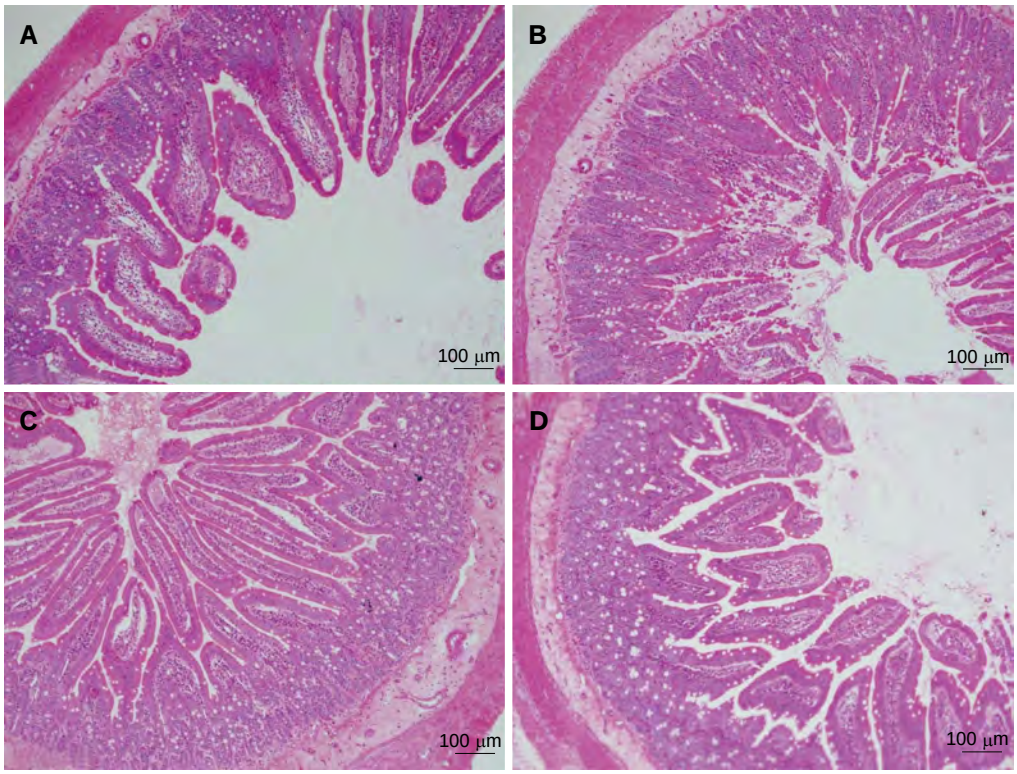

Figure 2 Polydatin attenuated tissue injury in small intestine caused by ovalbumin sensitization. A: Phosphate-buffered saline group; B: Ovalbumin (OVA) group; C: OVA + polydatin (PD) group; D: PD group. Morphology of intestinal jejunum was analyzed by hematoxylin and eosin staining. Representative images from three independent experiments are shown (magnification, × 63).

RESULTS

PD attenuated OVA-challenge caused small intestine abnormality in rats

In the present study, 1 mg OVA was used to sensitize BN rats orally and establish a food allergy model as previously described[16,23]. Loss of mucosal barrier integrity is a leading cause of food allergy[24]. Thus, we isolated jejunal fractions from the small intestine and checked tissue damage by HE staining. As shown in Figure 2, the results revealed that the intestinal mucosae were severely injured in the OVA group: the intestinal villi were eroded, and the swelling, shedding, and numbers of intestinal villi were significantly reduced. The morphological abnormality of the small intestine caused by OVA-sensitization was significantly attenuated by PD treatment.

The correlation between intestinal allergy and smooth muscle motility has been indicated by the fact that exposure to luminal allergen induces a state of proximal small intestinal hyperreactivity[25,26]. In order to evaluate the effects of PD on intestine motility, 2 cm intestine segments from each group were prepared, and the intestinal contraction tension was detected by smooth muscle organ bath. In response to 1 mg/mL OVA, intestinal segments isolated from OVA-allergic rats had a significant higher tension than the PBS group. The elevation of tissue tension caused by OVA sensitization was significantly attenuated by PD treatment by approximately 42% (Figure 3).

Treatment with PD decreased IL-4 levels and attenuated IgE production in OVA-sensitized group

The body weight of all rats in each group was monitored on days 0, 48 and 52. We found that basal body weight levels on day 0 were similar in all four groups. Compared to the PBS group, OVA sensitization significantly reduced body weight on day 48 (Figure 4A, left panel). After being treated with PD for 4 d, there was no significant difference between the PBS and OVA + PD groups (Figure 4A, left panel), which indicates that PD administration could maintain the body weight of an allergic rat at a normal level. The cytokine levels in the supernatant of the intestine mucosa were measured by ELISA. The results showed that the concentration of IL-4 in the OVA-challenged group was significantly higher than in the control group (58.15 ± 6.24 pg/mL *vs* 35.51 ± 5.48 pg/mL) (Figure 4B). Treatment with PD reduced the enhancement of IL-4 by 34%. Meanwhile, ELISA analysis showed that the concentration of OVA-specific IgE in serum was enhanced by 1.2 fold in the OVA group and PD therapy returned it to a normal level (Figure 4C).

PD reduced mast cell activation and degranulation in small intestine

Mast cell degranulation and histamine release are major factors in food allergy. The number and morphology of the mast cells in rat small intestine tissues (data not shown) or RPLS were examined by toluidine blue stain. In the OVA group, the number of mast cells was significantly increased and the cell size was much bigger, with more shrink on the cell membrane, bubbles in the cytoplasm, and degranulation vehicles around the cells (Figure 5A-D). *In vivo* administration with PD for 4 d reversed OVA-challenge-induced damage in mast cells. The ratio of degranulated mast cell, as indicated by vehicles (at least five) around the cells, dramatically increased in the OVA group by 5.5 fold and fell by 65% after PD treatment

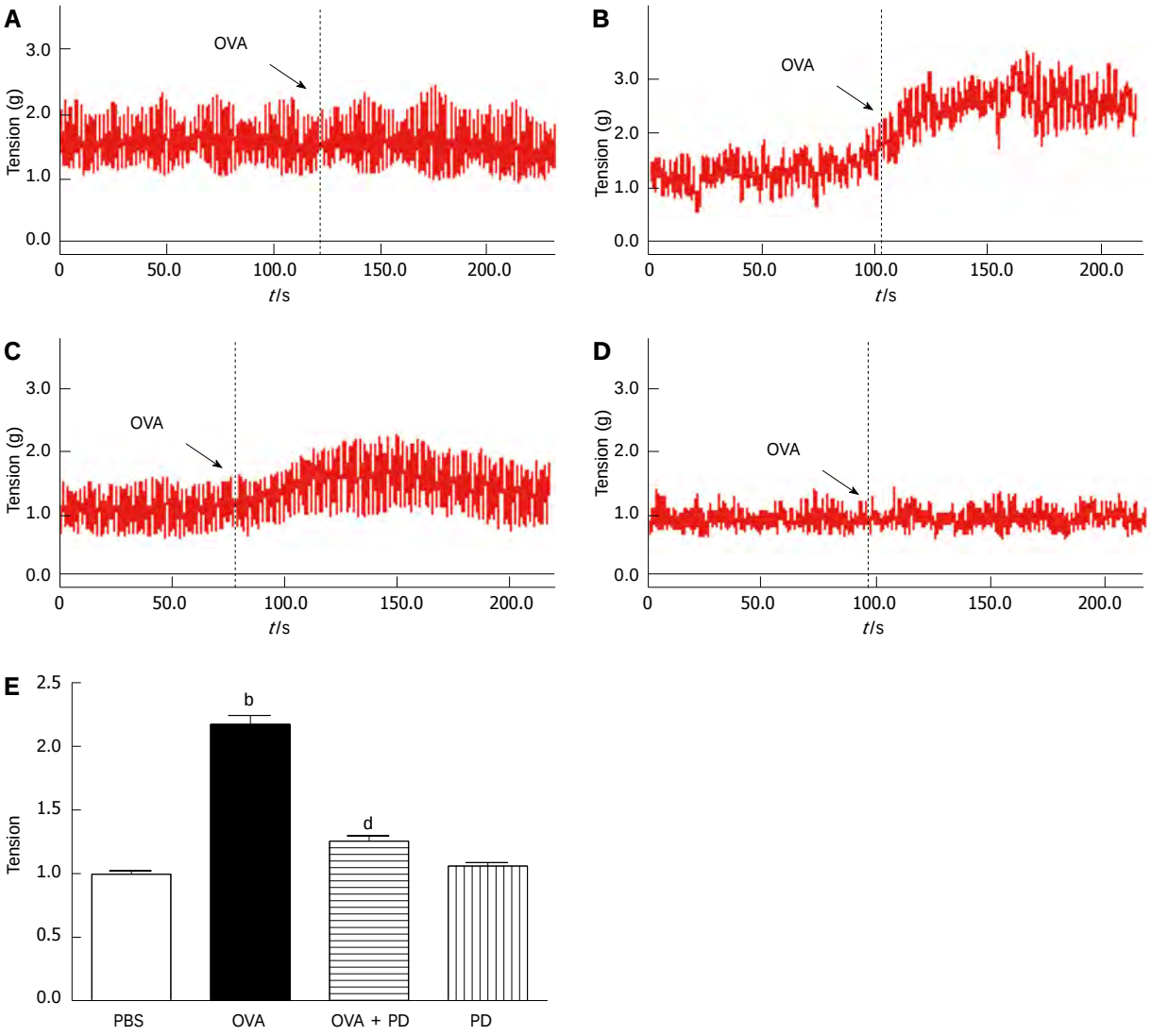

Figure 3 Polydatin attenuated small intestinal hyperreactivity in ovalbumin-allergic rats. A: Phosphate-buffered saline (PBS) group; B: Ovalbumin (OVA) group; C: OVA + polydatin (PD) group; D: PD group. n = 8, $^{b}P < 0.01$ *vs* PBS group; $^{d}P < 0.01$ *vs* OVA group. The tension of intestinal mobility was measured by smooth muscle organ bath, typical contraction curves (A-D), and the peak tension of each group (E).

(Figure 5E). PD mediated attenuation of mast cell degranulation was further confirmed by decreased histamine levels. It was found that histamine release in both serum and RPLS was significantly increased in the OVA-induced food allergic group, which was attenuated by PD therapy by approximately 11% and 20% respectively (Figure 5F).

PD inhibited mast cell degranulation by modulating Ca^{2+} mobilization through SOC channels

Rapid translocation of Ca^{2+} has been well-known to be essential for mast cell degranulation[27]. In a food allergic model, we also found that mast cell activation is related to stimulation of Ca^{2+} mobilization (data not published). To explore the underlying mechanism for the inhibitory effect of PD on mast cell degranulation, we isolated RPMCs and monitored intracellular Ca^{2+} with fluo-4 (5 mol/L). Using a standard Ca^{2+} add-back assay, in which intracellular Ca^{2+} stores were depleted by thapsigargin (TG), a sarcoplasmic/endoplasmic reticulum calcium ATPase (Ca^{2+} pump) blocker, in Ca^{2+}-free extracellular solution, after which the extracellular Ca^{2+} concentration was returned to 2 mmol/L[28]. Using this protocol, TG elicited two Ca^{2+} peaks, where the first one represented the ER Ca^{2+} release, and the second represented Ca^{2+} entry through activated SOCs. As shown in the first peaks in Figure 6A-D, when the cells were in Ca^{2+}-free solution, the TG-evoked Ca^{2+} amplitude was similar in all the groups, suggesting the amounts of Ca^{2+} released from ER are nearly the same. In the presence of 2 mmol/L extracellular Ca^{2+}, the TG-evoked Ca^{2+} influx was dramatically enhanced in OVA-sensitized RPMC by 1.5 fold, while PD treatment reduced the Ca^{2+} entry to normal level. The results indicate that PD attenuated OVA-induced Ca^{2+} influx elevation through SOCs.

DISCUSSION

The effect of resveratrol, a structural and functional

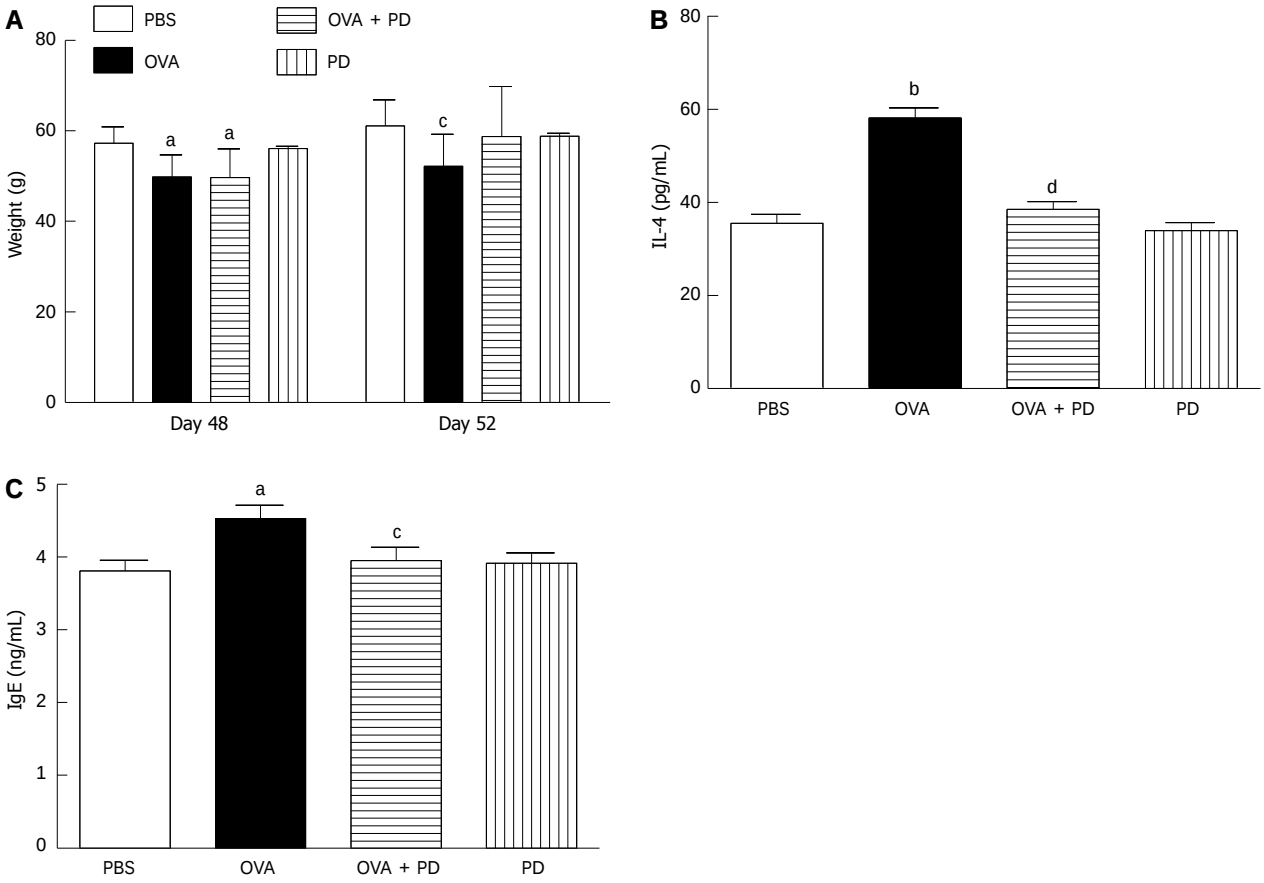

Figure 4 Polydatin suppressed interleukin-4 release and immunoglobulin E production in ovalbumin-allergic rats. A: Body weight of rats on day 48 [left panel, before polydatin (PD) treatment] or day 52 (right panel, after PD treatment) are shown; B: The cytokine levels in rat small intestine tissue or peritoneal lavage solution were analyzed by enzyme-linked immunosorbent assay; C: Statistical analysis of ovalbumin (OVA)-specific immunoglobulin E (IgE) in serum, which were collected from allergic rats administered with or without PD. $n = 8$. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* phosphate-buffered saline (PBS) group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* OVA group.

analog of PD, on the regulation of intracellular Ca^{2+} signaling has been reported by several groups, although the results vary in different cell types[29,30]. Furthermore, a previous study in our lab identified PD as a novel mast cell stabilizer in passive cutaneous anaphylaxis mice[15]. There are two major findings in the present study. Firstly, using an *in vivo* food allergic model, we demonstrated that PD has therapeutic effects against food allergy by decreasing antigen-stimulated mast cell degranulation. Secondly, it was showed that PD suppressed Ca^{2+} mobilization by inhibiting Ca^{2+} entry through SOCs, which were the major contributors to PD-induced mast cell stabilization.

Food allergy is an immunological adverse reaction caused by food, which encompasses a range of disorders including IgE-mediated anaphylaxis, food protein-induced enterocolitis syndrome, and food-induced eosinophilic gastrointestinal disorders. Allergens from eggs seem to be one of the most frequent causes of food allergic reaction reported[31]. Thus, in this study, we used OVA to sensitize rats and establish a food allergic model. The allergic animal exhibited abnormal intestinal morphology and increased smooth muscle contractility, enhanced Th2 cytokine levels (IL-4), and OVA-specific IgE concentration. Our results are in line with other published data, as IL-4 has been reported to be the hallmark Th2-type cytokine with multiple immunological functions, including directing Th2 cell differentiation, triggering Ig class switching to IgE in B cells, driving mast cell expansion in intestines[32], and inducing an exaggerated contractile response in intestinal smooth muscle[33]. Furthermore, mast cell activation and degranulation, which was due to Ca^{2+} mobilization *via* SOCs, was also demonstrated.

Mast cells are the main effector cells in the pathogenesis of multiple allergic diseases, including asthma, allergic rhinitis, gastrointestinal allergy, and cutaneous anaphylaxis. The majority of mast cell studies have addressed their predominant role in acute allergic reactions (immediate hypersensitivity) and more recently, their roles in late-phase allergic reactions[34,35]. Therefore, developing new drugs capable of stabilizing mast cells would be valuable for treating diseases attributable to type I hypersensitivity reactions. Using the RBL-2H3 mast cell line, intensive research have been focused on looking for promising drugs to inhibit mast cell activation and degranulation, among which PD showed some latent effects[15]. In the present study, following administration with PD, the mast cell-dependent food allergic rats did not have apparent anaphylactic symptoms (data not shown) and had a marked decrease in Th2 responsiveness after oral challenge with OVA. We have found that PD significantly reduced IgE

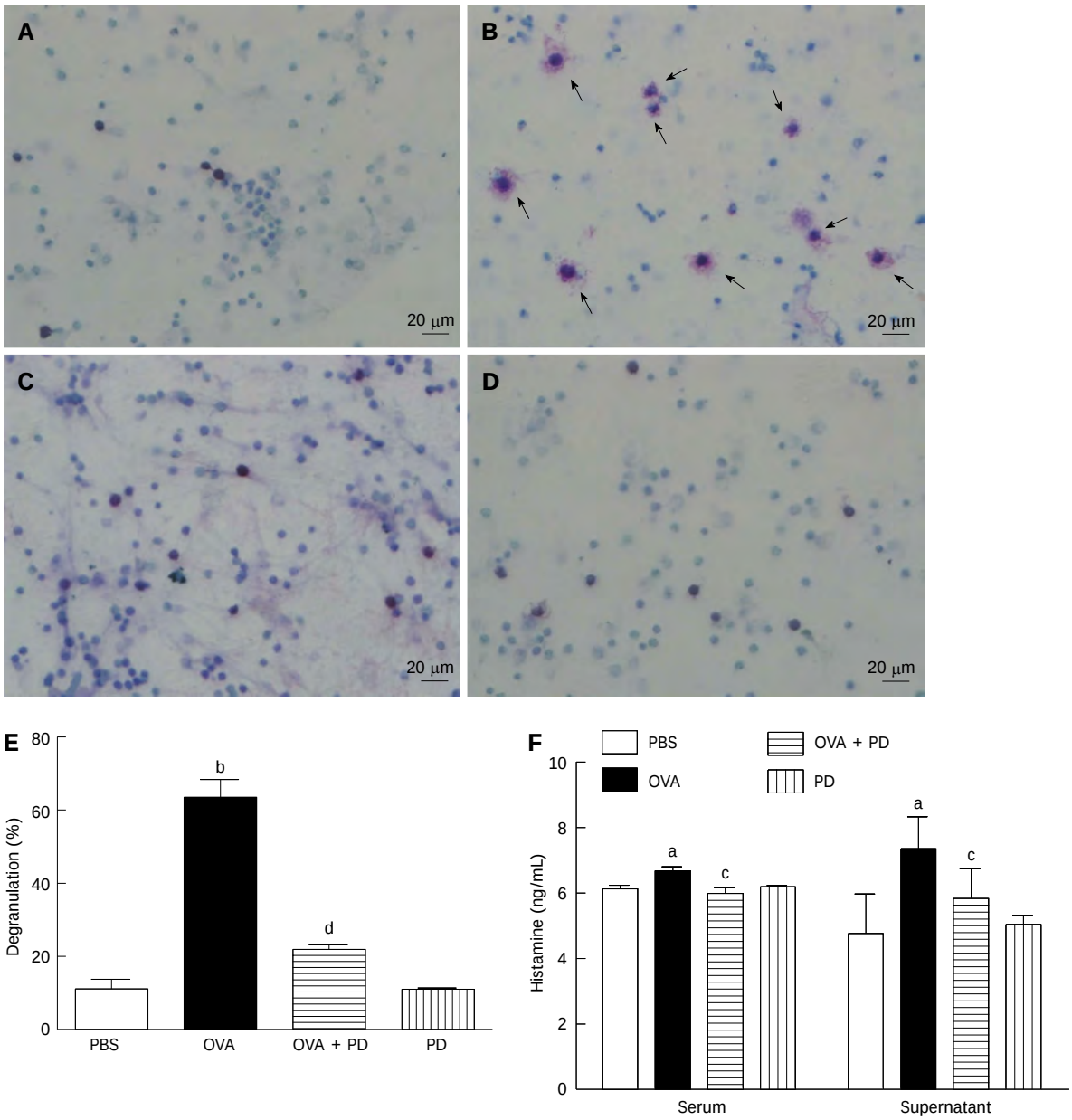

Figure 5 Polydatin therapy reduced mast cell degranulation and activation. A: Phosphate-buffered saline (PBS) group; B: Ovalbumin (OVA) group; C: OVA + polydatin (PD) group; D: PD group; E: Degranulated mast cells were counted from at least 500 total cells and the percentage of degranulation was calculated as degranulated cells against total cells; F: The release of histamine in serum (left panel), rat small intestine tissue, or peritoneal lavage solution (right panel) was measured by enzyme-linked immunosorbent assay. n = 8, $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* PBS group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* OVA group. The mast cells in rat small intestine tissue or peritoneal lavage solution were identified by toluidine blue stain. Mast cells were considered degranulated if at least 5 granules appeared outside the cell body. Arrows indicate degranulated mast cells (magnification, × 250).

production by inhibiting Th2 cytokines release. On the other hand, PD showed the potential to block mast cell degranulation by decreasing Ca^{2+} influx *via* SOCs.

The importance of calcium influx in mast cell activation and degranulation has been well recognized[36]. The degranulation of mast cells is Ca^{2+} dependent, and an increase in intracellular Ca^{2+} characterized by Ca^{2+} entry through SOCs is essential for granules release[13,27,37]. In this study, we found that PD treatment significantly attenuated FcεRI-elicited intracellular Ca^{2+} increase, indicating that PD stabilized mast cells by suppressing Ca^{2+} mobilization. Furthermore, we found that PD inhibited Ca^{2+} entry through SOCs. Multiple mechanisms are involved in the regulation of SOC activity. It has recently been discovered that two subunits, STIM1 and Orai1, play a vital role in both the signaling and the permeation mechanisms for Ca^{2+} influx through SOCs. Overexpression of STIM1 together with Orai1 caused a dramatic increase in store-operated Ca^{2+} entry in RBL cells[38]. The mechanism underlying PD-mediated inhibition of SOCs activity remains unclear.

In summary, the present study established PD as a novel mast cell stabilizer, with the capacity for preventing pathogenesis of food allergy and perhaps other mast cell-

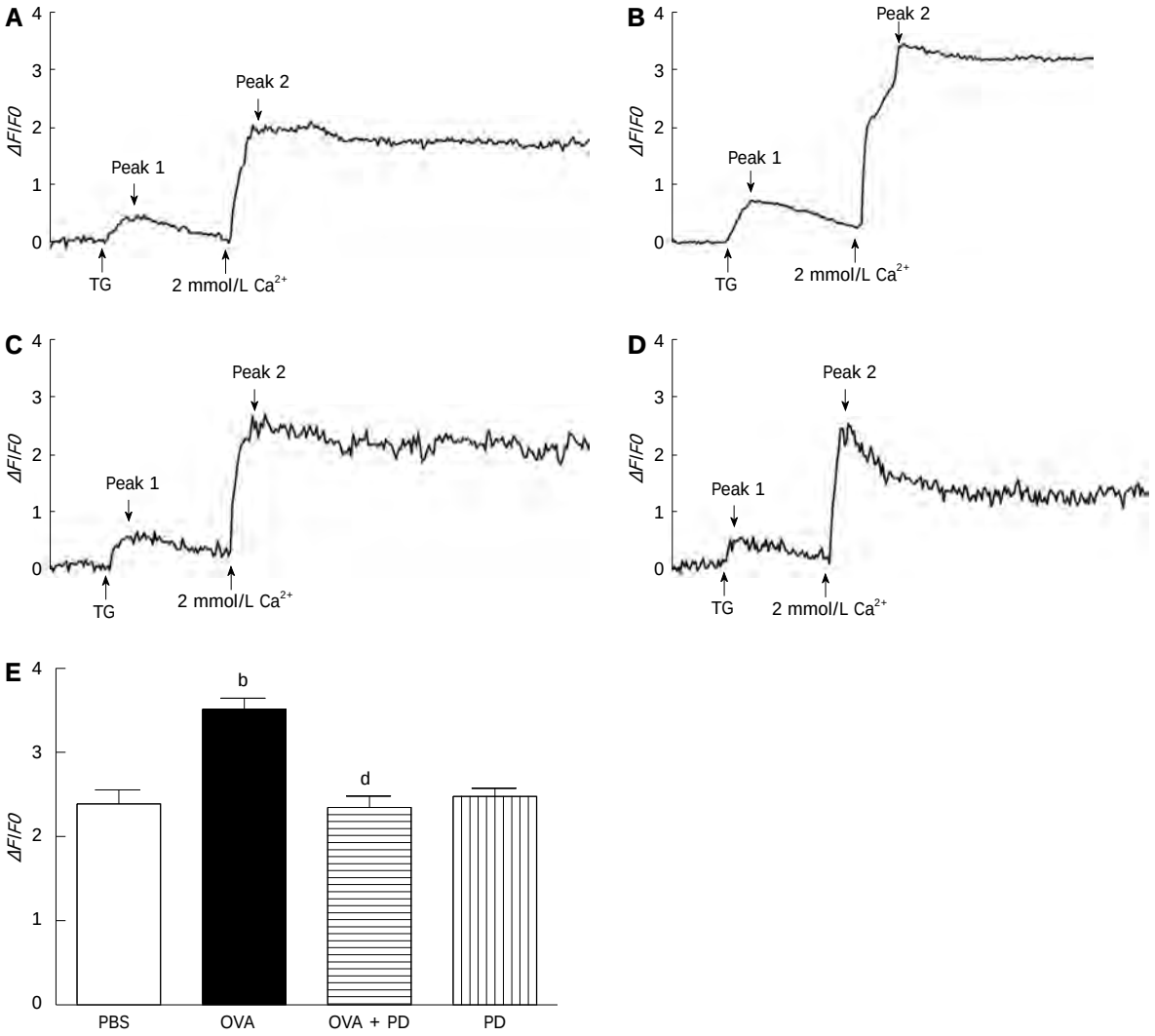

Figure 6 Polydatin reduced Ca^{2+} entry through store-operated calcium channels in food allergic mast cells. Typical responses of thapsigargin (TG)-evoked Ca^{2+} entry through store-operated calcium channels in rat peritoneal mast cells (RPMCs). A: Phosphate-buffered saline (PBS) group; B: Ovalbumin (OVA) group; C: OVA + polydatin (PD) group; D: PD group; E: Averaged peak amplitude of Ca^{2+} entry (second peak) as recorded in each group. $^{b}P < 0.01$ *vs* PBS group; $^{d}P < 0.01$ *vs* OVA group. RPMCs were isolated from Brown-Norway rat treated with or without PD. Intracellular Ca^{2+} was indicated by a fluo-4 fluorescent probe. Total cell numbers are 40-50 for each group, and the cells were from four independent experiments.

dependent allergic diseases through stabilizing mast cells. The underlying mechanism for PD-induced stabilization of mast cells is related to the inhibition of Ca^{2+} mobilization upon FcεRI activation.

COMMENTS

Background

The prevalence of food allergy has increased dramatically during the last three decades, but currently there is no satisfactory therapy except avoidance of the allergen in the diet. However, the offending foods causing an allergic effect are usually essential nutrients to human health. Therefore, to develop a new drug for food allergies is extremely important. Polydatin is a natural component isolated from *Polygonum cuspidatum*, and has been demonstrated to be effective in the treatment of allergic diseases.

Research frontiers

Polydatin is a sort of natural biological material and has been used as a medicine for many diseases. In the area of treatment of allergic diseases with polydatin, the research hotspot is how this product could stabilize mast cells and reduce allergic reactions in passive cutaneous anaphylaxis animals. The therapeutic effect of polydatin on food allergy has not yet been determined.

Innovations and breakthroughs

In previous studies in other laboratories and author's group, the extract of polydatin has been identified as a novel mast cell stabilizer in passive cutaneous anaphylaxis mice, in addition to its other new therapeutic effects, such as anticancer activity. Using an ovalbumin-sensitization mouse model, the authors are the first group to demonstrate that polydatin could attenuate food allergy by reducing mast cell degranulation. Furthermore, it was shown that polydatin suppressed Ca^{2+} mobilization by inhibiting Ca^{2+} entry through store-operated calcium channels, which were the major contributors to polydatin -induced mast cell stabilization.

Applications

The current results suggest that polydatin is a potential therapeutic drug that could be used in food allergy therapy.

Terminology

Food allergy mediated by immunoglobulin E (IgE) or non-IgE reaction, is an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food. It encompasses a range of disorders including IgE-mediated anaphylaxis, food protein-induced enterocolitis syndrome, and food-induced eosinophilic gastrointestinal disorders; Polydatin, also known as polygoni cuspidate radix, is a natural component isolated from *Po-*

lygonum cuspidatum. It has been determined as a resveratrol glucoside with a 3,4,5-trihydroxystilben-3-*D*-mono-*D*-glucoside molecular structure. It is traditionally used in South Korea, China, and Japan as a folk remedy for menoxenia, skin burns, gallstones, hepatitis, inflammation, and osteomyelitis.

Peer review

The authors investigated the effect of polydatin, a resveratrol glucoside, on mast cell degranulation and anti-allergic activity. This is a paper with some interesting value.

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BRIEF ARTICLE

A prospective study evaluating emotional disturbance in subjects undergoing defecating proctography

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Abstract

AIM: To investigate the prevalence of psychiatric illness in association with functional gastrointestinal disorders using defecating proctography (DP) and validated questionnaires.

METHODS: We prospectively evaluated 45 subjects referred for DP using hospital anxiety and depression scale (HADS), state trait anxiety inventory (STAI), patient health questionnaire 15-item somatic symptom severity scale (PHQ-15), validated questionnaires for sexual or physical abuse; post-traumatic stress disorder questionnaire (PTSD) and ROME-Ⅲ questionnaires for gastrointestinal complaints. DP results were considered negative if levator ani function was normal, rectoceles (if any) were < 4 cm and there was no evidence of intussusception, rectal prolapse, or other anatomic abnormality demonstrated. Subjects were subsequently divided into those with structural defects seen on DP (DP positive group) and those with a normal defecography study (DP negative group).

RESULTS: Forty five subjects were included in the study of which 20 subjects were classified as DP negative (44.4%). There was a striking prevalence of a history of sexual abuse in DP negative group compared to the DP positive group (n = 9, 5 respectively; P = 0.036). Further, subjects in the DP negative group scored significantly higher on the HADS anxiety (6.60 ± 1.00 *vs* 4.72 ± 0.40, P = 0.04) and depression scales (5.72 ± 1.00 *vs* 3.25 ± 0.46, P = 0.01). This correlated well with significantly higher scores on the STAI state anxiety scale (42.75 ± 3.16 *vs* 35.6 ± 2.00, P = 0.027), PHQ-15 questionnaire (13.15 ± 0.82 *vs* 10.76 ± 0.97, P = 0.038) and prevalence of PTSD (20% *vs* 4%, P = 0.045) among DP negative subjects. There was no difference between the groups in terms of STAI trait anxiety.

CONCLUSION: The findings of this prospective study demonstrate a significantly high degree of psychiatric ailments in patients with negative findings on DP who should be appropriately screened for a history of sexual abuse and symptoms of psychosocial distress.

Key words: Functional gastrointestinal disorders; Sexual abuse; Defecating proctography; Post-traumatic stress disorder questionnaire

Core tip: In this study, we used validated questionnaires in consort with defecating proctography and demonstrated that subjects undergoing defecating proctography who met ROME Ⅲ criteria for functional constipation have a high prevalence of psychiatric disorders and a significant history of sexual abuse. We also found an association between post-traumatic stress

disorder questionnaire, anxiety, history of sexual abuse and functional constipation. Taken together, these findings suggest that a very detailed history about psychiatric co-morbidities and traumatic experiences must be taken in selected patients complaining of constipation.

Kashyap AS, Kohli DR, Raizon A, Olden KW. A prospective study evaluating emotional disturbance in subjects undergoing defecating proctography. *World J Gastroenterol* 2013; 19(25): 3990-3995 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i25/3990.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i25.3990

INTRODUCTION

Patients with chronic gastrointestinal symptoms such as abdominal pain and constipation often display features suggestive of concomitant emotional disturbance[1]. Conversely, patients with a history of sexual or physical abuse have a high prevalence of gastrointestinal and genitourinary complaints[2].

There is evidence suggesting that patients with history of abuse and functional gastrointestinal disorders have a higher incidence of surgeries, lower quality of life and greater disability[3]. Given the lack of a surgically correctable etiology, surgical intervention may be inappropriate in these patients. In this context, we decided to evaluate patients with constipation, studying both anatomic and psychosocial variables using a variety of instruments.

We sought to assess the prevalence of abuse and psychiatric diagnoses in patients referred for defecating proctography (DP). Specifically, we sought to ascertain the prevalence of sexual abuse, physical abuse, anxiety, depression, somatization and post-traumatic stress disorder (PTSD) in subjects meeting criteria for functional constipation and without radiographic evidence of structural explanation for symptoms. We hypothesized that patients with no anatomic disorder of the pelvic floor evidenced by radiological parameters are likely to have a history of abuse and/or psychiatric diagnosis.

The purpose of the study was to use DP and a variety of validated questionnaires to assess the prevalence of psychiatric ailments in subjects with chronic constipation.

MATERIALS AND METHODS

Study design and setting

This prospective cohort study was undertaken at the Washington Hospital Center, a 926 bed tertiary care hospital in Washington DC. All subjects undergoing DP for the evaluation of lower gastrointestinal complaints were prospectively enrolled over 18 mo starting October 2010. Patients with obvious anatomical anomalies such as rectal prolapse, solitary rectal ulcers, fissures and fistulae were excluded.

At the time of enrollment, demographic information was recorded and each subject was assigned a unique identifying number. All other subject identifiers were removed. The study was approved by the institutional review board of Washington Hospital Center and all subjects gave written informed consent.

Immediately prior to undergoing the DP, all subjects were asked to complete a set of self-administered questionnaires.

Questionnaires

All subjects answered a total of 7 self-administered validated questionnaires that assessed a history of sexual or physical abuse, generalized anxiety disorder (state and trait anxiety), PTSD, depression and somatoform disorders. Gastrointestinal symptoms were assessed using the ROME Ⅲ criteria questionnaires that included the constipation module and irritable bowel syndrome module. All questionnaires were self-administered and met appropriate reliability and validity criteria. Further, each questionnaire was easily readable and understandable.

State-trait anxiety inventory: The state-trait anxiety inventory (STAI) is a self-administered test for evaluation of state and trait anxiety and has been used extensively in research and clinical practice. It has been translated into 30 languages and has since been extensively used and validated in the research literature[4]. For the purpose of our study the revised version of the test; "Form-Y" was used[5]. The STAI-Y is a self-administered test and takes 6-15 min to complete, depending on the subject's level of education. The S-anxiety scale (STAI Form Y-1) consists of 20 statements that evaluate how the respondent feels "right now, at this moment" using a 4 point Likert scale. The T-anxiety scale (STAI Form Y-2) consists of 20 statements that evaluate how individuals "generally feel". A score of over 40 on the STAI Form Y-1 and STAI Form Y-2 was considered diagnostic of state of anxiety and trait anxiety respectively.

Screening questionnaire for sexual and physical abuse history: This is a self-report questionnaire developed by Drossman *et al*[6] and has been validated against a detailed psychological interview[7]. The questionnaire has two sections to identify sexual abuse and physical abuse as a child or adult respectively. For the purpose of our study, no distinction was made between abuse as an adult or child.

Hospital anxiety and depression scale: The hospital anxiety and depression scale (HADS) is a self-administered questionnaire to assess generalized anxiety or depression[8] and takes approximately 2-5 min to complete. It has been extensively validated in patients with gastrointestinal disorders[9] in the in-patient[5] as well as the outpatient setting[10]. Each item is answered by the patient on a four point scale (0-3) with possible scores ranging from 0-21 for anxiety and 0-21 for depression. Any score above 11 is indicative of abnormal levels of anxiety or depres-

sion, thus a positive screen for the appropriate disorder.

Screening for somatoform disorders: We used the patient health questionnaire (PHQ-15) to screen for somatoform disorders in our subjects. The PHQ-15 is a 15 item scale addressing somatic symptoms during a 2 wk period on a scale of 0-2 with a maximum score of 30[11] and has been validated among patients with gastrointestinal complaints[12]. We compared the scores of the subjects based on the findings of the DP.

PTSD: We used the 4 item screen for PTSD in primary care developed by Prins *et al*[13]. This validated screening questionnaire[14] uses a binary yes/no response to a specific experience and a score of 3 or greater on the scale was defined as a positive case of PTSD.

ROME Ⅲ constipation module and ROME Ⅲ irritable bowel syndrome module: The ROME Ⅲ criteria were used to screen for irritable bowel syndrome (IBS) and constipation[15]. The ROME Ⅲ criteria are a system used to classify functional gastrointestinal disorders and we used validated self-administered questionnaires that are freely available for download[16]. The ROME Ⅲ criteria were used to rule out constipation predominant IBS as a cause of the patients symptoms and also to diagnose patients with true functional constipation.

Notably, all the questionnaires inquired about information which may have been potentially distressing to the subjects. Hence, one of the authors (Olden KW) who is a board-certified psychiatrist was available to the subjects in case emotional and mental distress was caused or detected by the protocol related questions. After completing the questionnaires, subjects underwent DP.

Single contrast DP

Defecating proctography was used to evaluate for anatomical defects that could explain the gastrointestinal symptoms in the subjects. Briefly, the study involved rectal administration of a radio-opaque semi-solid paste with the consistency of soft stool. The subject was then seated on a commode and made to excrete the material in a manner similar to defecation[17]. The radiological images taken during the evacuation process were interpreted by a radiologist who specialized in DP. The radiologist was blinded to the results of the psychosocial evaluation and did not interact with the subjects.

DP has been used extensively in patients with defecatory dysfunction, pelvic prolapse or puborectalis dysfunction[18-20] to visualize anatomic defects like internal or complete rectal prolapse, enterocele or rectocele. The quantification of the rectal evacuation is especially helpful in patients with pelvic floor dysfunction or dyssynergia and is recommended as a physiological means of assessing rectal dysfunction[21]. DP was considered "negative" for anatomical abnormalities if levator ani function was normal, rectoceles (if any) were < 4 cm[22] and there was no evidence of intussusception, rectal prolapse, or other anatomic abnormality demonstrated. Subjects were subsequently divided into those with structural defects seen on DP (DP positive group) and those with a normal defecography study (DP negative group). Responses to the questions were subsequently compared between the DP positive and the DP negative group.

Table 1 Demographic profile and psycho-social factors of subjects

Variables	DP positive group (n = 25)	DP negative group (n = 20)	P value
Demographics			
Males	3	5	> 0.050
Age (yr)	61.8 ± 2.8	58.1 ± 2.9	> 0.050
Assessment of psycho-social factors			
Sexual abuse	5	9	0.036
Physical abuse	1	2	> 0.050
Post traumatic stress disorder	1	4	0.045
STAI state anxiety	35.60 ± 2.00	42.75 ± 3.16	0.027
STAI trait anxiety	35.08 ± 1.76	38.06 ± 2.42	> 0.050
HADS anxiety	4.72 ± 0.40	6.60 ± 1.00	0.040
HADS depression	3.25 ± 0.46	5.72 ± 1.00	0.014
PHQ-15	10.76 ± 0.97	13.15 ± 0.82	0.038

All data are presented as mean ± SE. DP: Defecating proctography; HADS: Hospital anxiety and depression scale; STAI: State trait anxiety inventory; PHQ-15: Patient health questionnaire 15-item somatic symptom severity scale.

Statistical analysis

Unpaired Student's t test was used for analyzing demographic differences in demographic variables with continuous distribution while χ^2 test was used for analyzing categorical variables using GraphPad Prism software (v 5.0a, GraphPad Prism Inc., San Diego, CA, United States). A P value of < 0.05 was considered significant. All data are presented as mean ± SE.

RESULTS

A total of 45 patients were included in the study and completed the psychosocial evaluation prior to undergoing the DP. Thirty seven (82%) of the total subjects were females (Table 1). Forty four subjects (97.7%) were referred for DP for evaluation of constipation. One subject was referred for possible anismus (pelvic floor dyssynergia).

In 20 (44.4%) of the 45 subjects, the DP did not show any anatomical anomaly and these subjects were classified as DP negative (*i.e.*, negative for anatomical abnormalities on DP). The remaining 25 patients were classified as DP positive (*i.e.,* DP demonstrated anatomical abnormalities that could contribute to symptoms).

Anxiety and depression

Subjects in the DP negative group had strikingly high scores on the HADS anxiety and depression questionnaires (P = 0.04 and P = 0.01) compared to subjects in the DP positive group (Figure 1A and B). This correlated well with the significantly higher score on the STAI state

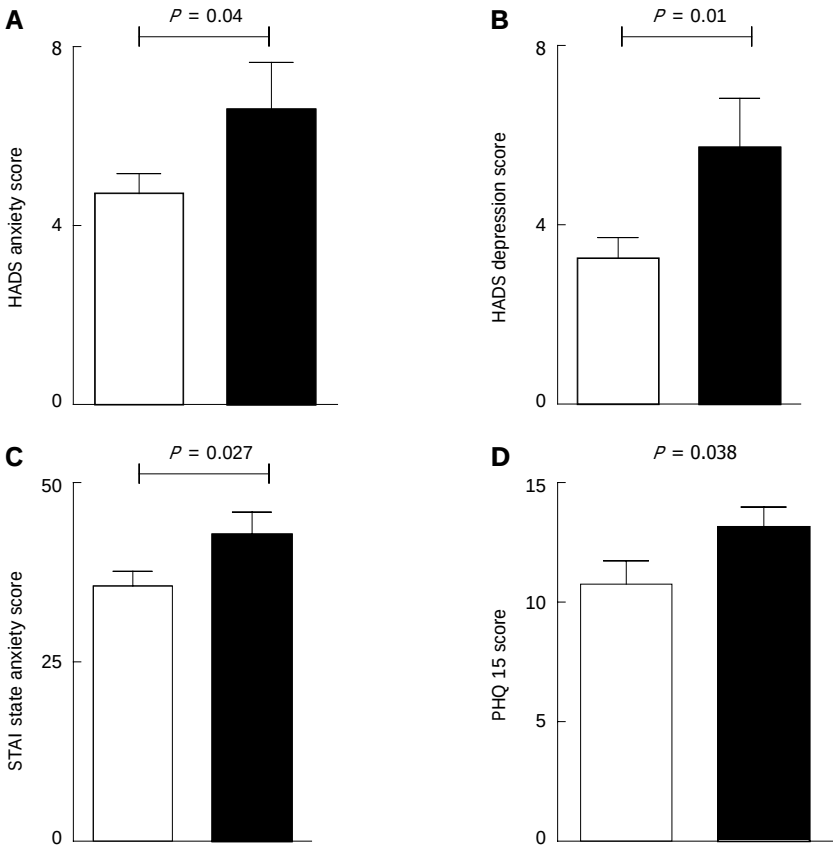

Figure 1 Subjects with no anatomical abnormalities seen on defecating proctography. Defecating proctography (DP negative; black column) have a significantly greater degree of psychiatric disorders as compared to the subjects with anatomical abnormalities on defecating proctography (DP positive; white column). A: Comparison of hospital anxiety and depression scale (HADS) anxiety; B: HADS depression; C: State trait anxiety inventory (STAI) state anxiety; D: Patient health questionnaire 15-item somatic symptom severity scale (PHQ-15) scores among DP positive and DP negative subjects using unpaired *t*-test is shown. All data are shown as mean ± SE.

anxiety questionnaire among subjects in the DP negative (P = 0.027) compared to the DP positive group (Figure 1C). Further, subjects in the DP negative group reported significantly worse PHQ-15 scores compared to the DP positive group (P = 0.038, Figure 1D).

Sexual abuse and PTSD

Notably, we found that a fair proportion of the subjects reported a history of sexual abuse. Nine of the 20 (45%) subjects in the DP negative group and 5 of the 25 (20%) subjects in the DP positive group reported a history of sexual abuse (P = 0.036, Figure 2A). This correlated well with a higher prevalence of PTSD among subjects in DP negative group (4 of 20 subjects, 20%) than the DP positive group (1 of 25 subjects, 4%; P = 0.04, Figure 2B).

Trait anxiety and physical abuse

There was no significant difference among the DP positive and DP negative groups in terms of STAI trait anxiety and prevalence of physical abuse. Further, there was no significant difference in the 2 groups in terms of prevalence of IBS or constipation assessed using the ROME Ⅲ questionnaires. Of note, all subjects included in the study had complaints of constipation and the results of the prevalence confirm the presenting complaints.

DISCUSSION

In this study, we demonstrated that subjects undergoing DP who met ROME Ⅲ criteria for functional constipation have a high prevalence of psychiatric disorders. Greater levels of state anxiety as well depression were found in the above mentioned population. We also found an association between PTSD, history of sexual abuse and functional constipation. Taken together, these findings suggest that a very detailed history about psychiatric co-morbidities and traumatic experiences must be taken in selected patients complaining of constipation.

We included subjects who were referred for DP as part of further work-up of constipation that was refractory to conservative management. It is pertinent to note that a majority of the referrals were from colo-rectal surgeons for pre-operative assessment. As the results of the study show, 44% of the patients (DP negative) did not have any surgically correctable cause and hence would not be appropriate candidates for surgical interventions.

Sexual abuse is very common among patients with functional disorders of the lower gastrointestinal tract[1] with one study showing prevalence of 40%. Further, abused patients were found to have constipation as the most common gastrointestinal complaint[23]. In our study,

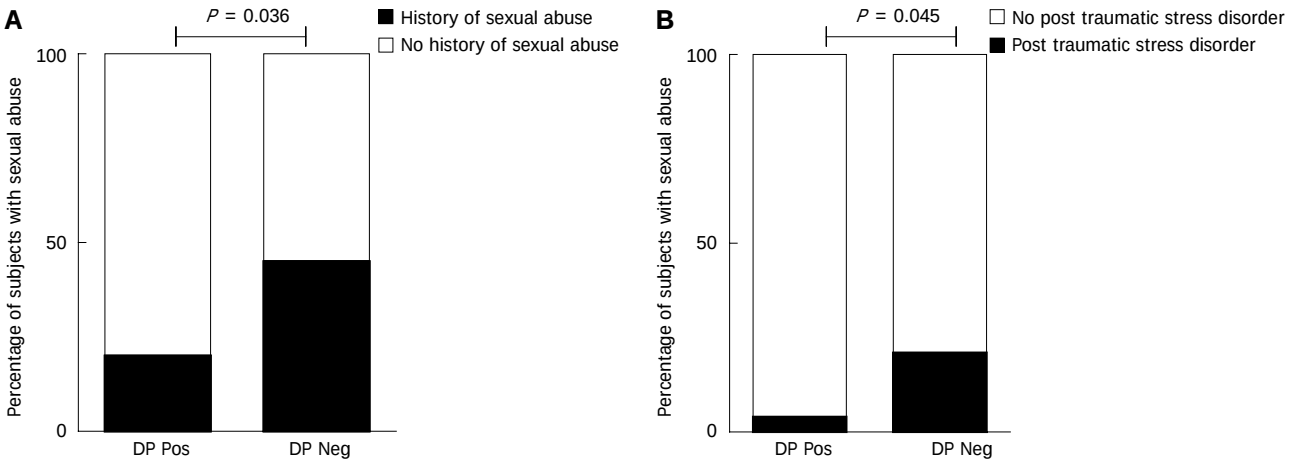

Figure 2 Subjects with no anatomical abnormalities seen on defecating proctography have a significantly greater prevalence of sexual abuse (A) and post-traumatic stress disorder (B) as compared to the subjects with anatomical abnormalities on defecating proctography. DP: Defecating proctography; Pos: Positive; Neg: Negative.

14 subjects had a history of sexual abuse of which a majority had constipation without any anatomical explanation. The difference in the prevalence of sexual abuse between the 2 groups was statistically significant. Notably, patients in the DP negative group had a significantly higher prevalence of PTSD, a condition closely associated with sexual abuse. Since a history of sexual abuse is a strong predictor of multiple surgeries and poor surgical outcomes for slow-transit constipation[24], a careful history is essential in this patient population.

There is a strong association between psychological dysfunction and gastro-intestinal disorders for which various therapeutic paradigms have been found to be effective. Cognitive behavior therapy, psychodynamic psychotherapy and pharmacological agents including anti-depressants been shown to be efficacious in managing the gastrointestinal symptoms in selected patients[1]. However, there needs to be a greater emphasis on the detection of psychological disturbance and eliciting a detailed history in these patients.

A novel aspect of our study was the use of DP for the evaluation of constipation and classifying subjects with organic (DP positive) *vs* functional (DP negative) gastrointestinal disorders. DP has been used extensively for evaluation of patients with evacuatory dysfunction[25] and is often considered the gold standard for imaging in patients with defecation disorders, most notably rectocele, enterocele, anismus and perineal descent[26]. Patients in the DP negative group would likely not benefit from surgical interventions given the functional nature of the symptoms detected by DP.

The findings of this prospective study demonstrate a significantly high degree of anxiety, depression, somatization, PTSD and sexual abuse in subjects with negative findings on DP. Our study confirms that psychological ailments can impact the lower gastrointestinal tract and is especially associated with constipation. We recommend that patients with refractory constipation and features suggestive of psychological ailment/abuse[3] should be appropriately screened for the aforementioned disorders.

COMMENTS

Background

Functional constipation is a common gastrointestinal ailment and is often associated with emotional disturbance. Defecating proctography (DP) is a radiological tool to visualize anatomical causes leading to gastrointestinal symptoms. This study assessed the prevalence of psychiatric ailments in patients with gastrointestinal symptoms who had been referred for defecating proctography.

Research frontiers

This study uses a novel approach of combining a radiological tool (DP) and multiple validated psychiatric instruments to assess the prevalence of psychiatric ailments causing gastrointestinal manifestations.

Innovations and breakthroughs

The study demonstrates that a significant proportion of subjects undergoing DP and met ROME Ⅲ criteria for functional constipation have psychiatric ailments. Further, there is an association between functional constipation and a history of sexual abuse.

Applications

It is suggested that a very detailed history about psychiatric co-morbidities and traumatic experiences must be taken in selected patients complaining of constipation. Further large scale studies are warranted to explore these associations.

Terminology

Defecating proctography is a radiological study that requires a subject to excrete a rectally administered contrast to visualize anatomical defects pertinent to rectal evacuation.

Peer review

The authors demonstrated a prospective study evaluating emotional disturbance in subjects undergoing defecating proctography. In my opinion, such a prospective study will give us more important information in this field.

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BRIEF ARTICLE

Gastroenterology training in a resource-limited setting: Zambia, Southern Africa

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Abstract

AIM: To evaluate need for and efficacy of a structured gastroenterology didactic session in expanding awareness and understanding of digestive disorders.

METHODS: A four-day symposium was developed with didactic sessions (days 1, 2) and practical endoscopy (days 3, 4). Didactic sessions included case presentations highlighting pathophysiology and management. One nurse and four practicing gastroenterologists from the United Kingdom led lectures and supervised workshops with audience participation. Practical endoscopy focused on diagnostic and therapeutic procedures and their application to diagnosis and treatment of ailments of the gastrointestinal tract. Pre- and post-workshop questionnaires were distributed to participants during didactic sessions. A pre-workshop questionnaire gauged expectations and identified objectives to be met at the symposium. Post-workshop questionnaires were administered to assess efficacy of each session. Participants graded sessions from 1 (poor) to 5 (excellent) on quality of case presentations, knowledge, clarity and mode of presentation. We assessed if time allotted to each topic was sufficient, value of sessions, impact on practice and interest in future symposiums.

RESULTS: There were 46 attendees on day 1: 41% undergraduates, 41% residents, 11% consultants and 4% unspecified. Day 2 (a Saturday) had 24 participants: 17% undergraduates, 71% residents, 9% consultants, 4% unspecified. Primary pre-workshop symposium expectation was to gain knowledge in: general gastroenterology (55.5%), practical endoscopy (13.8%), pediatric gastroenterology (5%), epidemiology of gastrointestinal disorders specific to Zambia (6%), and interaction with international speakers (6%). The post-symposium questionnaire was answered by 19 participants, of whom 95% felt specific aims were met; all would attend future conferences and recommend to others.

CONCLUSION: The beneficial effect of a structured symposium in developing countries warrants further attention as a mechanism to improve disease awareness in areas where resources are limited.

Key words: Gastroenterology training; Resource-limited country; Zambia; Specialist training; Postgraduate training; Hepatology

Core tip: The global burden of digestive diseases is increasing, yet formal training in gastroenterology is lacking in traditionally underserved areas such as the African continent. In this study we designed, implemented, and evaluated the effectiveness of a structured 4 d symposium focusing on general topics in the diagnosis and management of digestive disease. This

symposium was geared towards health care professionals and attendees reported improvement in their knowledgebase in gastrointestinal disorders. Structured symposiums are an effective and viable adjunct to medical education and their utility may be highest in regions where traditional academic medical resources are limited.

Asombang AW, Turner-Moss E, Seetharam A, Kelly P. Gastroenterology training in a resource-limited setting: Zambia, Southern Africa. *World J Gastroenterol* 2013; 19(25): 3996-4000 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i25/3996.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i25.3996

INTRODUCTION

Zambia is a Southern African nation with a population of approximately 13 million people[1,2]. The University Teaching Hospital (UTH) in Lusaka has a capacity of approximately 1600 adult and 300 pediatric beds and is the main medical training institution in Zambia. UTH houses an endoscopy unit which serves as both an inpatient and ambulatory care facility providing both emergency and routine endoscopies. The unit is equipped with a Pentax video endoscopy suite which includes gastroscopes (including pediatric scope) and colonoscopes. The unit performs approximately 1000 gastrointestinal endoscopic procedures per year. Instrument cleaning and disinfection follows international guidelines (British Society of Gastroenterology)[3]. Continuing medical education is encouraged as staff regularly attend the South African Gastroenterology conference to maintain up to date proficiency. For a combination of epidemiologic (relatively lower prevalence of biliary disease compared with industrialized nations) and economic (lack of available funding) the unit does not currently carry out endoscopic retrograde cholangiopancreatography. However, there is a significant public health burden of luminal gastrointestinal and hepatology disease, and recent attention has turned to increasing the health care communities' awareness of these disorders[4-7]. Our objective was to develop and host a formal gastrointestinal/hepatology workshop to educate the healthcare sector and also evaluate its place as a mechanism to address the growing interest in this field. We review our experience in the development of the workshop and its impact on the health professionals working in a resource-limited setting.

MATERIALS AND METHODS

We hosted a course to improve understanding of gastrointestinal disorders. The specific aims of the conference were to promote a greater understanding of: (1) the pathophysiology and management of common gastrointestinal/hepatological disorders; (2) principles of endoscopy (upper and lower) including indication, risks and benefits; (3) clinical skills in endoscopy with emphasis on management and evaluation of varices, non-portal hypertensive related gastrointestinal bleeding and colonic polyp recognition and removal; and (4) maintenance of endoscopic efficiency and patient safety.

Lectures

Didactic sessions over the first two days introduced participants to essential pathophysiology and management of prevalent disorders emphasizing a multidisciplinary approach to patient care. The sessions were open to all participants and led by a panel of experts from the United Kingdom in conjunction with staff physicians at UTH. These lectures were focused on pertinent topics including: diarrheal disease, gastrointestinal emergencies, malnutrition, esophageal disease, abdominal pain and hepatology. Sessions were conducted in a case presentation format in which clinical findings and course were reviewed to illustrate key points in pathophysiology and management.

To illustrate, a case of cholera was used as an opportunity to review the physiology of secretory (toxin-mediated) diarrhea, which in turn served as a platform to discuss the rationale of oral rehydration therapy. As another example, a session was centered on gastrointestinal bleeding using cases of peptic ulcer bleeding and variceal bleeding due to schistosomiasis to illustrate principles of emergency management and differing endoscopic approaches to both non-portal hypertensive and portal hypertensive related bleeding. We ran focused hepatology sessions which included cases that fostered discussion on the applicability of gold-standard management with limited resources (for example, availability of vaccinations and access to ultrasound). Guidelines on the management of ascites, hepatitis B and encephalopathy were developed in break-out sessions. On day 2, patients with specific gastrointestinal and hepatology ailments were interviewed in front of the audience to elaborate points for discussion. Audience participation was actively encouraged throughout and sessions were designed to facilitate interaction and comparison of management strategies.

Prior to the workshop, we administered a questionnaire to gauge attendees' expectations and identify weak areas in their knowledge base. Post-workshop questionnaires on the quality of each session asked participants to grade sessions from 1 (poor) to 5 (excellent) on the following criteria: quality of the case presentations, knowledge, clarity and mode of presentation. We also assessed if the time allotted for each topic was considered sufficient, if participants felt presented information was applicable to their stage of training, if they felt there was a need for specific guidelines for the management of presented disorder in Zambia and finally if information learned at the presented session would change their personal management approach.

Practical endoscopy

Our practical sessions complemented lecture-based dis-

Table 1 Demographics of participants *n* (%)

Variables	Preconference	Day 1	Day 2
Gender			
Male		19 (41)	13 (52)
Female		18 (39)	7 (28)
Not stated		9 (20)	5 (20)
Training/specialty			
Undergraduate	17 (47)	19 (41)	4 (17)
Postgraduate	14 (39)	19 (41)	17 (63)
Consultants	4 (11)	5 (11)	4 (9)
Other	1 (3)	3 (7)	1 (4)
Total participants	36	46	24

Table 2 Preconference questionnaire *n* (%)

Expectations	
General GI	20 (56)
Practical endoscopy (adult/children)	5 (14)
Pediatric GI	2 (5)
Epidemiology of GI disorders specific to Zambia	4 (6)
Interaction with international visitors	4 (6)
None	1 (3)
Weak areas	
Hepatology	9 (19)
GI Bleeds (including peptic ulcer disease)	7 (15)
IBD	8 (17)
GI malignancy	4 (8)
Infectious Gastroenterology (including HIV)	6 (13)
Malnutrition	1 (2)
Malabsorption (including celiac)	2 (4)
Diarrheal disease	1 (2)
Fluid management	1 (2)
Autoimmune conditions	1 (2)
Colon pathology	2 (4)
Biliary disease (pancreatic and gallbladder)	1 (2)
Pediatric GI	2 (4)
Practical endoscopy skills	3 (6)

GI: Gastrointestinal; IBD: Inflammatory bowel disease; HIV: Human immunodeficiency virus.

cussion on management in the first two days. Participants were instructed on endoscope instruments and accessories, set-up of endoscopy equipment and preparation for endoscopy including patient safety and informed consent. The nurses were involved in the hands-on experience, and obtained additional training related to the patient preparation, aftercare and maintenance of equipment in the endoscopy unit.

There were 15 participants for the live cases. The first endoscopy day was a combination of adult and pediatric cases: esophageal variceal banding, duodenal polypectomy with hemoclip application for hemostasis and appropriate biopsies in a case of gastric ulcer. The colonoscopies included hematochezia and ulcerative colitis. All cases were followed by a case and management discussion.

RESULTS

Forty-six attendees answered our questionnaires on day 1: 41% undergraduates, 41% residents, 11% consultants and 4% unspecified. Day 2 had 24 participants: 17% undergraduates, 71% residents, 9% consultants, 4% unspecified. Attendees from neighboring countries included 3 physicians from Zimbabwe, 1 from Malawi and 1 from the Democratic Republic of Congo. The organizing committee included five visiting experts in gastroenterology from the United Kingdom, who led several of the didactic sessions. Table 1 describes the demographics.

Table 3 Evaluation of each session *n* (%)

Diarrheal disease	1 (poor)	2	3	4	5 (excellent)
Case presentation	0 (0)	0 (0)	1 (2)	25 (58)	17 (40)
Knowledge	0 (0)	0 (0)	0 (0)	21 (53)	19 (48)
Clarity	0 (0)	0 (0)	7 (18)	16 (41)	16 (41)
Mode of presentation	0 (0)	0 (0)	1 (3)	19 (49)	19 (49)
GI emergencies					
Case presentation	0 (0)	1 (3)	2 (6)	20 (59)	11 (32)
Knowledge	0 (0)	0 (0)	2 (6)	21 (62)	11 (32)
Clarity	0 (0)	0 (0)	4 (11)	17 (47)	15 (42)
Mode of presentation	0 (0)	0 (0)	1 (3)	19 (51)	16 (43)
Malnutrition					
Case presentation	0 (0)	0 (0)	1 (3)	29 (73)	10 (29)
Knowledge	0 (0)	0 (0)	1 (3)	22 (55)	17 (43)
Clarity	0 (0)	0 (0)	3 (8)	11 (64)	11 (28)
Mode of presentation	0 (0)	0 (0)	2 (5)	24 (60)	14 (35)
Oesophageal diseases					
Case presentation	0 (0)	0 (0)	0 (0)	6 (30)	14 (70)
Knowledge	0 (0)	0 (0)	0 (0)	9 (45)	11 (55)
Clarity	0 (0)	0 (0)	0 (0)	11 (55)	9 (45)
Mode of presentation	0 (0)	0 (0)	0 (0)	9 (45)	11 (55)
Abdominal pain					
Case presentation	0 (0)	0 (0)	0 (0)	6 (30)	14 (70)
Knowledge	0 (0)	0 (0)	1 (5)	5 (25)	14 (70)
Clarity	0 (0)	0 (0)	1 (5)	4 (20)	15 (75)
Mode of presentation	0 (0)	0 (0)	0 (0)	5 (25)	15 (75)
Hepatology					
Case presentation	0 (0)	0 (0)	1 (5)	7 (33)	13 (62)
Knowledge	0 (0)	0 (0)	2 (10)	7 (33)	12 (57)
Clarity	0 (0)	0 (0)	1 (3)	12 (57)	8 (38)
Mode of presentation	0 (0)	0 (0)	1 (5)	7 (33)	13 (62)

Participants graded sessions from 1 (poor) to 5 (excellent) on quality of case presentations, knowledge, clarity and mode of presentation.

Primary pre-workshop symposium expectations were to gain knowledge in: general gastroenterology (55.5%), practical endoscopy (13.8%), pediatric gastrointestinal (GI) disorders (5%), epidemiology of GI disorders specific to Zambia (6%), and interaction with international speakers (6%) (Table 2). The most common areas that participants thought their knowledge was weak were: hepatology (19%), inflammatory bowel disease (17%), gastrointestinal bleeds-including peptic ulcer disease (15%) and infectious gastroenterology-including human immunodeficiency virus (HIV) (13%) and gastrointestinal malignancy (8%) (Table 2). Sessions already planned by the time these responses were received covered the majority of these areas.

The sessions were on diarrheal disease, gastrointestinal emergencies, malnutrition, esophageal diseases, abdominal pain and hepatology (Table 3). The cumulative average percentage of respondents who scored sessions either good (4/5) or excellent (5/5) for the following

criteria: case presentation 97%, knowledge 96%, clarity 93%, mode of presentation 97%. The percentage of respondents who answered "Yes" to the following questions on each topic: was time allotted to this topic sufficient? 81%; did you find this session valuable for your stage of training? 98%; do we need to develop specific management guidelines? 95%; will this session change your management? 94%.

There were some variations between sessions although the overall quality was considered high. Significant numbers of respondents said that the time allocated was insufficient for diarrhoeal disease (33%), malnutrition (24%) and esophageal disease (25%). This demonstrates an interest and need for further training in these areas. The large majority (95%) who felt specific management guidelines should be developed on these topics is also indicative of the need for further work. The post-symposium questionnaire was answered by 19 participants, of whom 95% felt specific aims were met; 90% would pay for future conferences, all would attend future conferences and recommend to others.

DISCUSSION

There is a recognized shortage of general and specialized medical doctors in Zambia and Sub-Saharan African countries[8-10]. The detailed reasons for such shortages are beyond the scope for this paper, however one of the identified strategies to curtail this problem includes training opportunities and continued medical education[9,10]. Based on 2010 statistics, the health life expectancy at birth in Zambia is 49 years[11]. The dominant gastrointestinal and hepatological clinical problems are variceal bleeding due to schistosomiasis, esophageal strictures due to caustic substance ingestion, infectious diarrhea (often HIV related), peptic ulceration, hepatitis B, and gastrointestinal cancer (Kaposi sarcoma, esophageal, gastric and colon cancer)[12,13]. There is also a considerable burden of neurogastroenterological problems including achalasia, functional dyspepsia and irritable bowel syndrome. The most common cause of esophageal bleeding in patients presenting to our endoscopy unit is esophageal varices (25%), other etiologies are duodenal ulcer (17%) and gastric ulcers (21%); less frequent but significant causes are Kaposi's sarcoma (2%) and Mallory Weiss tear (1%)[12]. It has been estimated that more than 90% of schistosomiasis cases occur within Sub-Saharan Africa[14]. The prevalence in Zambia is 77% and the two predominant forms are *Schistosoma hematobium* and *Schistosoma mansoni*[15,16]. *Schistosoma mansoni* has been implicated in intestinal and liver disarray resulting in portal hypertension, esophageal varices, gastrointestinal bleed and rarely liver failure[17-19].

The estimated prevalence of HIV infection in Zambian adults aged 15-49 years is estimated between 10.3%-19.7%, leading to an increased burden of HIV enteropathy[20,21]. With this HIV burden there is also concern for the increasing trend of hepatitis B and hepatitis C[22]. There are no Zambian liver disease management guidelines, thus current practice follows guidelines of the World Health Organization[23], American Association for the Study of Liver disease[24] and European Society for the Study of Liver[25], which are not suitable in resource-limited settings and in an area where etiopathogenesis is different.

The incidence of gastric and esophageal cancer in adults younger than 45 years is higher than in United States or United Kingdom[12]. Gastric cancer in patients under 45 years accounts for 33% of cases, whilst esophageal cancer represented 16% of endoscopically diagnosed cases[12]. Survival from digestive disease is lower in developing countries, in those within the African continent[26], thus raising awareness and developing prevention programs are important and can be enhanced through educational symposia such as described in this paper. Caustic injuries, either suicidal or accidental, are another area of concern; with patients presenting late in the course with resultant gastric outlet obstruction (55%), esophageal strictures (30%), gastric ulcerations (21%)[27].

One of the aims of the workshop was to draw in other health care workers interested in gastroenterology and hepatology but working outside UTH so as to facilitate networking and optimize standards of care for GI diseases throughout the country, and positively impact the African continent. We successfully included physicians from neighbouring countries: 3 from Zimbabwe, 1 Malawi, 1 Congo. These surrounding nations benefited from this course because they share a similar disease burden as Zambia. Continued training in the field of gastroenterology in Zambia and other resource-limited areas, is necessary to enhance understanding of pathophysiology and management, thus improving overall patient care.

ACKNOWLEDGMENTS

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COMMENTS

Background

Formal training in gastroenterology is lacking despite the huge burden of digestive disease across Africa. Given the disparity in the supply of formally-trained gastroenterologists and the ever increasing demand of citizens, the authors organized a structured four-day symposium focusing on gastrointestinal/hepatological case based presentations and introduction to endoscopy.

Research frontiers

Promotion of such educational activities should be encouraged not only to help physicians develop new perspectives on disease, but also to improve overall patient care.

Innovations and breakthroughs

In this work the authors organized the first gastroenterology symposium in Zambia, attracting students and physicians from neighbouring countries. The authors have set a foundation for similar activities in the future.

Applications

This could be replicated in other developing countries that face similar disease burdens and require improvements in undergraduate and postgraduate training.

Terminology

The symposium was an opportunity to teach, increase awareness of gastroin-

testinal and hepatological diseases whilst creating an environment for networking. This symposium addressed current knowledge and recent advances in gastrointestinal/hepatological disease.

Peer review

This article provides information and guidelines for setting up a structured symposium in a resource-limited setting. Equally important, this highlights the need for structured clinical gastroenterology/hepatology training programmes with adequate curricula that emphasize knowledge, skills, and scientific productivity.

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BRIEF ARTICLE

Cytokine profiles in patients receiving antioxidant therapy within the ANTICIPATE trial

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Abstract

AIM: To measure a broad profile of pro- and anti-inflammatory cytokines in patients with clinically proven chronic pancreatitis (CP) taking either antioxidant therapy or placebo as part of the larger ANTICIPATE study.

METHODS: Patients with chronic pancreatitis were recruited to the ANTICIPATE study following informed consent and were randomised to intervention with either antox version 1.2-based antioxidant therapy or placebo. After a separate ethics committee amendment a subgroup of 7 patients from either arm of the study were selected for additional analysis of cytokines. Cytokines were measured at baseline and after 6 mo of either antox therapy or placebo by biochip array and enzyme-linked immunosorbent assay.

RESULTS: Antioxidant therapy and placebo groups were well-matched in terms of age, gender, aetiology of CP, opiate use and disease duration. Baseline antioxidant levels were similar in patients allocated to the antioxidant group as compared to the group allocated to placebo. After 6 mo of antioxidant therapy there was significant elevation in vitamin C levels in the intervention group: 17.6 μg/mL (12.8-29.3 μg/mL) compared to 4.8 μg/mL (1.6-9.1 μg/mL) in placebo ($P < 0.001$; 95%CI: 9.0-20.2) with similar trends in selenium levels. There was no elevation in a broad array of pro- and anti-inflammatory cytokines in the antioxidant group compared to placebo [interleukin (IL)-1B, IL-4, IL-6, IL-10, tumor necrosis factor-α] either at baseline or after 6 mo of antioxidant therapy.

CONCLUSION: Cytokine levels were low at baseline and at 6 mo despite a significant elevation in plasma antioxidants. In patients with CP, with opiate-dependent abdominal pain, circulating cytokine levels are low suggesting that pain in this disease is not simply a manifestation of inflammation.

Key words: Chronic pancreatitis; Antioxidant therapy; Cytokine

Core tip: This study examines cytokine levels in a subset of patients recruited from within the ANTICIPATE randomized controlled trial of antox for painful chronic pancreatitis. At baseline, pro- and anti-inflammatory cytokine levels were within the laboratory reference range in patients allocated to the antioxidant arm and those allocated to placebo. After 6 mo of intervention with antox, there was a significant elevation in antioxidant levels in patients in the active treatment arm. This was not associated with any change in either pro- or anti-inflammatory cytokine levels. In patients with chronic pancreatitis, with opiate-dependent abdominal pain, circulating cytokine levels are low suggesting that pain in this disease is not simply a manifestation of inflammation.

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INTRODUCTION

The oxidative stress hypothesis proposed that cell injury in chronic pancreatitis (CP) was mediated at the acinar level by short-lived oxygen free radicals produced as a result of imbalance in the physiological processes producing these agents and those pathways involved in deactivating them[1]. A key component of this theory was that the methionine transsulfuration pathway which yields glutathione (important in the quenching of antioxidants) is overwhelmed in patients with CP as the detoxification of xenobiotics by cytochrome P450 led to overproduction of oxygen-derived free radicals[1]. There was evidence that the dietary intake of some patients with CP was deficient in selenium, methionine and vitamin C, key cofactors in these transsulfuration pathways[2]. This finding was supported by evidence showing that plasma/blood levels of circulating antioxidants were low in CP compared to control[3]. The logical completion of this paradigm was the development of antioxidant therapy - a pharmacological preparation containing methionine, vitamin C, vitamin E and selenium and designed to restore these critical co-factors to patients with CP[1]. Early clinical trials of antioxidant therapy failed to establish evidence of clinical efficacy and thus the treatment was not widely accepted. To address this issue, we conducted and reported the largest randomized controlled trial of antox for treatment of pain in chronic pancreatitis - the ANTICIPATE study[4]. In this, 356 patients with CP were screened for eligibility, 92 randomised and 70 completed intervention with 6 mo of antioxidant therapy or matched placebo. At the end of this period there was no difference between treatment and placebo in the primary endpoint of abdominal pain as assessed by a numerical rating scale or in secondary endpoints of pain assessed by pain diaries and quality of life assessed by validated questionnaire[4]. However, blood and plasma antioxidant levels were significantly elevated in patients in the treatment group[4]. In keeping with other clinical studies of antioxidant therapy in chronic pancreatitis with clinical endpoints there is little information on the effects of intervention on inflammatory markers.

The present study does provide unique data on cytokine profiles in patients with chronic advanced pancreatitis at their end disease stage receiving antioxidant therapy and in a matched cohort receiving placebo and provides negative results which should be regarded as important pilot data. Thus, although the principal findings were negative, the ANTICIPATE study provided a unique vehicle with which to assess the potential interaction between antioxidant therapy and cytokine markers of inflammation and fibrosis in chronic pancreatitis. To the best of our knowledge, this interaction has never previously been studied.

In chronic pancreatitis there is evidence that levels of platelet-derived growth factor-BB and transforming growth factor (TGF)-β1 are elevated and that these cytokines play an important role in pancreatic fibrosis[5]. Pancreatic stellate cells are activated by alcohol in CP and are key mediators of subsequent inflammatory changes and fibrosis with these changes being modulated by cytokines including epidermal growth factor[6,7]. Pancreatic ductal epithelium produces TGF-β which also mediates fibrosis[8]. Thus cytokines are known to be key mediators of inflammatory and fibrotic change in CP.

The aim of the present study was to examine circulating cytokine levels in a cohort of patients within the ANTICIPATE study. The principal endpoint was to assess whether there were differences between patients receiving antioxidant therapy and those receiving matched placebo.

MATERIALS AND METHODS

Study design

This is a case-control analysis of a sub-group of patients recruited from both arms of the ANTICIPATE double-blind, placebo-controlled, randomised trial of Antox version 1.2 (Pharma Nord, Morpeth, United Kingdom) in patients with painful chronic pancreatitis[4].

Setting

Tertiary care academic medical centre was eventually chosen as setting in which to implement the requirment.

Inclusion/exclusion criteria

The inclusion criteria were as for the main ANTICIPATE study and can be summarised as follows: patients with evidence of chronic pancreatitis on cross-sectional imaging together with evidence of impairment of pancreatic exocrine function as assessed by assay of faecal elastase. Patients who did not meet these criteria were excluded as were patients with evidence of malignancy. The inclusion/criteria for the main study are provided in detail elsewhere[4].

Identification and selection of study sub-group

Recruitment to ANTICIPATE commenced in February 2008 and a protocol amendment to permit additional enrolment to the present study was approved 6 mo later. Patients recruited to ANTICIPATE were allocated to receive either 6 mo intervention with antox or matched placebo in a randomised, double-blind, placebo-controlled fashion. Those patients selected to participate in this study signed an additional consent form. No additional inclusion or exclusion criteria were used. Allocation arm was unknown during the conduct of ANTICIPATE and patients were stratified at enrolment to this study by whether or not they had undergone prior pancreatic intervention (either surgical or endoscopic). Blood samples were drawn from 22 patients in the "prior intervention" stratification arm and from 15 in the "no prior intervention" stratification arm. Following the code break at the end of the clinical ANTICIPATE study, investigators were notified which patients had been allocated to active drug and which had

been allocated to placebo. At this point, a study population of 10 consecutive patients from each arm of the study was identified (total 20 patients). Allowing for loss to follow-up in 6 patients in whom blood samples for cytokine analysis were not taken after the original baseline assays a final study population of 7 patients treated with antox for 6 mo and 7 patients treated with placebo was obtained.

Assays

Full blood count (haemoglobin and white cell count) was measured at baseline and at 2, 4 and 6 mo. C-reactive protein (CRP) levels were also measured at these time points. Antioxidant levels comprising the following: selenium, vitamins C and E, β-carotene and glutathione were measured at baseline, study mid-point and at 6 mo. A range of cytokines were measured at baseline and at 6 mo as follows: pro-inflammatory cytokines interleukin (IL)-1β, IL-6, tumor necrosis factor alpha; anti-inflammatory cytokines: IL-4, IL-10; chemokines: IL-8, IL-18, monocyte chemotactic protein 1; the T cell regulatory cytokine IL-2; the angiogenic signalling protein vascular endothelial growth factor (VEGF) and epidermal growth factor an important regulator of cellular proliferation, differentiation and survival[7].

Methods of measurement

Full blood count was measured by the haematology department of the Manchester Royal Infirmary with CRP being measured in the clinical biochemistry service and these results were available to clinicians to guide on-going management during the study. Antioxidant levels were measured by the pancreatic laboratory of the Manchester Royal Infirmary. The results of these assays were available during the study. Cytokine assays were undertaken by Bio-chip Arrays and enzyme-linked immunosorbent assay by Randox laboratories, Crumlin, Northern Ireland. These were analysed as a batch at the end of the study.

Sample collection

Non haemolysed and non-lipaemic serum and plasma were used for the Biochip array. Samples were collected into leak-proof, non-absorbent plastic containers. After collection, samples were aliquoted into containers and stored at -70 ℃. Repeated freeze/thaw cycles were avoided. Samples were labelled prior to transportation on dry ice to Randox laboratory, Crumlin, Northern Ireland *via* a secure, approved courier.

Interference

The effect of bilirubin, haemoglobin, triglycerides and lipids were assessed to establish the level at which the interference caused a significant increase or decrease in assay performance. The criterion set for this was that analyte recovery (all cytokines) should not vary from base recovery by more than 10%.

Ethics committee approvals

The original full study protocol was approved by the North West Regional Ethics Committee (MREC, 07/MRE08/13) and the United Kingdom Medicines and Health products Regulatory Agency (MHRA, 2006-006958-10). This cytokine subgroup study was approved by the North West Regional Ethics Committee as a separate amendment. The master study ANTICPATE from within which these patients were recruited was registered with the International Registry of Randomized Controlled trials and allocated the number ISRCTN-21047731.

Statistical analysis

Two by two tables were analysed by Fisher's exact test and non-parametric data by Mann-Whitney *U* test using the Statsdirect software package (version 2.6.5. www statsdirect.com).

RESULTS

Demographic and biochemical profiles

As in the parent study, the two groups were well-matched in terms of age and gender distribution. Alcohol was the dominant etiologic agent and a majority in both groups were cigarette smokers (Table 1).

Antioxidant profiles at baseline and at 6 mo

Baseline levels of vitamin C, vitamin A, whole blood glutathione transferase and red cell glutathione transferase were similar between groups and were also within the reference range for population normal as reported by the Pancreatic laboratory of the Manchester Royal Infirmary (Table 2). Levels were towards the lower range of normal. Although median vitamin E, β-carotene and selenium levels were below the range for population normal in the placebo group, this difference was not significant compared to the antioxidant group at baseline.

Haemoglobin, white cell count and CRP were within normal levels in both groups.

At 6 mo (Table 3) there was significant elevation of vitamin C and selenium levels in the antioxidant group compared to baseline and also compared to placebo at 6 mo. Vitamin A and E levels were also significantly elevated in patients receiving antioxidant therapy compared to those receiving placebo at 6 mo. A similar pattern was seen for β-carotene although these values did not attain significance.

There was no difference in haemoglobin, white cell count or CRP at 6 mo between antioxidant therapy and placebo or between antioxidant therapy and baseline.

There were also no differences in opiate usage.

Cytokine profiles

There was no difference between the antioxidant group and placebo at baseline in any of the cytokines measured in this study (Table 4). Similarly, there was no difference between antioxidant and placebo at 6 mo and no difference in the antioxidant group at 6 mo compared to the same group at baseline. IL-1B, IL-2, IL-4 and IL-10 me-

Table 1 Demographic profiles

Variables	Antioxidant (n = 7)	Placebo (n = 7)	P value
Age (yr), median (range)	46 (34-79)	46 (37-60)	0.92 (Mann-Whitney U)
Gender (male:female)	5:2	4:3	0.90 (Fisher's exact)
Aetiology	Alcohol 6; idiopathic 1	Alcohol 4; idiopathic 3	0.55 (Fisher's exact)
Disease duration (yr)	4 (1-5)	3 (2-13)	0.92 (Mann-Whitney U)
Body mass index (kg/m^2)	24.2 (18.8-36.7)	22.5 (22.9-32.8)	0.62 (Mann-Whitney U)
Alcohol (g/d), median (range)	175.5 (0-396)	138.6 (0-252)	0.43 (Mann-Whitney U)
Cigarette smoker (Y:N)	6:1	5:2	0.59 (Fisher's exact)
Diabetes mellitus (Y:N)	2:5	1:6	0.62 (Fisher's exact)
Faecal elastase (μg/g)	68 (15-500)	27 (15-500)	0.27 (Mann-Whitney U)
Opiate use (mg/d)	40 (30-300)	85 (0-120)	0.30 (Mann-Whitney U)

Laboratory reference range for faecal elastase report values < 200 μg/g as representing end-stage exocrine failure. All opiate intakes are reported as morphine equivalent.

Table 2 Baseline antioxidant profiles

Variables	Antioxidant (n = 7)	Placebo (n = 7)	Laboratory reference range	Median difference	P value (MWU)	95%CI
Vitamin C (μg/mL)	7.7 (0.7-13)	5.8 (2.4-9.9)	4-20	1.6	0.53	-3-6.1
Vitamin E (mg/L)	12.4 (5.4-20.9)	5.4 (3.6-15.2)	5.7-14.9	4.4	0.12	-2.6-11.1
β-carotene (mol/L)	35.9 (8-87)	11.6 (7-233)	19-254	15	0.55	-166-71
Vitamin A (mg/L)	0.60 (0.30-0.68)	0.40 (0.20-0.57)	0.4-1.2	0.16	0.07	-0.03-0.37
Selenium (μg/L)	82 (27-110)	49 (27-97)	83-152	27	0.22	-14-53
WGSH (μmol/L)	1361 (1229-1682)	1336 (1149-1585)	1078-1753	62.5	0.73	-118-290
WGSH/Hb (μmol/g)	9.2 (7.8-11.4)	9.7 (8.9-10.0)	7.5-12.2	-0.3	0.70	-1.4-1.5
WCC (10^9/L)	7.7 (6.4-10.3)	10 (5-15.9)	4-11	-1.4	0.38	-4.4-2.2
Hb (g/dL)	14.9 (13.4-15.8)	13.7 (12.4-16)	13-18	1	0.33	-0.6-2.1
CRP (mg/L)	3 (3-29)	7 (3-29)	0.3-5	-1	0.27	-7-3

WGSH: Whole blood glutathione; WGSH/Hb: Glutathione corrected for haemoglobin concentration; WCC: White cell count; Hb: Haemoglobin; CRP: C-reactive protein; MWU: Mann-Whitney U.

Table 3 Antioxidant profiles at 6 mo compared to baseline

Variables	Antioxidant (n = 7)	Placebo (n = 7)	P value (antioxidant vs baseline)	95%CI	P value (antioxidant vs placebo)	95%CI
Vitamin C (μg/mL)	17.6 (12.8-29.3)	4.8 (1.6-9.1)	0.001	-18.8-6.5	< 0.001	9.0-20.2
Vitamin E (mg/L)	17.8 (11.7-25.0)	5.0 (4.0-4.6)	0.160	-1.8-12.3	0.004	4.4-14.3
β-carotene (mol/L)	155.5 (23-478)	38.1 (8-204)	0.150	-189-35	0.244	-20-190
Vitamin A (mg/L)	0.5 (0.42-0.72)	0.3 (0.25-0.64)	0.910	-0.19-0.14	0.010	0.05-0.34
Selenium (μg/L)	109 (95-133)	48 (40-92)	0.007	-75-14	< 0.001	41-85
WCC (10^9/L)	6.7 (4.9-10.8)	7.4 (6-10.8)	0.330	-1.3-3.2	0.600	-3.3-1.9
Hb (g/dL)	14.2 (13.5-16.0)	12.7 (12.3-16.0)	0.510	-1-1.6	0.150	-1.3-2.6
CRP (mg/L)	3 (3-4)	6 (3-10)	0.190	0-3	0.070	-7-0
Opiate usage	20	55	0.210	-40-79	0.630	-56-60

WGSH: Whole blood glutathione; WGSH/Hb: Glutathione corrected for haemoglobin concentration; WCC: White cell count; Hb: Haemoglobin; CRP: C-reactive protein.

dian values were below the lower limit of the laboratory reference range at all sample points although individual patient sample values registered within the reference range. IL-6 and IL-8 values were within the reference range but towards the lower end at all sample points. VEGF showed higher values in the placebo group at both baseline and at 6 mo although this difference was not significant.

DISCUSSION

To the best of our knowledge, this study is the first to examine circulating cytokine levels in patients with chronic pancreatitis receiving antioxidant therapy and to compare these values to controls (also with CP) receiving matched placebo. When interpreting these findings, several important methodological sources of error should be emphasised. First, this is a small study with only 7 patients in each group. Thus it should be borne in mind that negative findings could represent a type Ⅱ error. A second source of error is the possibility of technical compromise in assay methodology as samples were transferred for analysis. As the majority of readings were low, could deterioration in sample quality have affected the assays?

Table 4 Cytokine levels in patients receiving antioxidant therapy compared to placebo

Cytokine	Laboratory range	Antioxidant therapy baseline (pg/mL)	Placebo baseline (pg/mL)	*P* value	95%CI	Antioxidant therapy at 6 mo (pg/mL)	Placebo at 6 mo (pg/mL)	*P* value	95%CI
IL-1β[1]	1.6-250	< 1.6	< 1.6			< 1.6	< 1.6		
IL-2	4.8-3000	2.6 (0-4.8)	3.1 (0-3.5)	0.83	-1.1-1.7	2.6 (0.0-4.8)	2.9 (0.0-3.2)	0.97	-2.6-2.3
IL-4	6.6-900	2.3 (2.2-6.6)	2.5 (2.1-6.6)	0.84	-3.7-3.9	2.5 (2.2-6.6)	2.8 (2.1-6.6)	0.81	-1.3-3.7
IL-6	1.2-900	1.9 (0.8-3.5)	1.8 (0.7-8.9)	0.99	-2.6-1.4	1.6 (1.0-2.3)	1.4 (0.9-7.6)	0.78	-3.9-0.7
IL-8	4.9-3000	10.1 (8.1-23.7)	8.5 (7.5-19.8)	0.46	-5.2-8.4	11.6 (8.1-19.3)	10.8 (6.6-18.6)	0.54	-2.7-18.7
IL-10[2]	1.8-1000	< 0.6	< 0.6			< 0.6	< 0.6		
TNFα	4.4-1500	3.5 (2.4-5.8)	3.5 (2.2-4.9)	0.71	-1.1-1.6	3.8 (2.4-4.7)	3.4 (2.5-4.5)	0.40	-0.7-1.1
IL-18	0-3000	388.6 (245.7-818.9)	457.9 (317.5-775.7)	0.71	-228-165	365.5 (211.5-879.8)	435.7 (349.1-570.1)	0.40	-238-299
VEGF	14.6-3000	116.2 (49.8-191.2)	243.1 (39.5-305.5)	0.32	-187-51	93.7 (35.1-173.9)	184.4 (34.1-299.9)	0.22	-206-34.7
EGF	2.9-900	28.4 (9.8-137.7)	23.4 (11.9-75.6)	0.99	-30.0-47.6	9.4 (3.1-65.2)	34.4 (2.3-139.8)	0.09	-66.8-5.5
MCP-1	13.2-1500	335.9 (267.1-423)	298.8 (229.5-685.1)	0.81	-134-106	296.5 (235.0-426.7)	326.4 (265.5-468.6)	0.38	-127-49

[1]All patients had interleukin (IL)-1β levels below the lower threshold of detection. IL-2: Antioxidant group at 6 mo *vs* antioxidant group at baseline, *P* = 0.64 (95%CI: -1.5-2.5); IL-4: Antioxidant group at 6 mo *vs* antioxidant group at baseline, *P* = 0.89 (95%CI: -3.7-3.7); IL-6: Antioxidant group at 6 mo *vs* antioxidant group at baseline, *P* = 0.25 (95%CI: -0.4-1.7); IL-8: Antioxidant group at 6 mo *vs* antioxidant group at baseline, *P* = 0.69 (95%CI: -17.8-4.9); [2]All patients had interleukin 10 below the lower threshold of detection. Transforming growth factor type β1 (TGFβ1): Antioxidant group at 6 mo *vs* antioxidant group at baseline, *P* = 0.46 (95%CI: -39-74); tumor necrosis factor α (TNFα): Antioxidant group at 6 mo *vs* antioxidant group at baseline, *P* = 0.97 (95%CI: -1.0-1.4); vascular endothelial growth factor (VEGF): Antioxidant group at 6 mo *vs* antioxidant group at baseline, *P* = 0.54 (95%CI: -43.8- 81.0); epidermal growth factor (EGF): Antioxidant group at 6 mo *vs* antioxidant group at baseline, *P* = 0.05 (95%CI: -0.22-59.8); monocyte chemotactic protein-1 (MCP-1): Antioxidant group at 6 mo *vs* antioxidant group at baseline, *P* = 0.05 (95%CI: -50.1-100.9).

Whilst this possibility cannot definitively be excluded, the commercial laboratory which undertook these assays works closely with the clinical biochemistry department of the Manchester Royal Infirmary and regularly undertakes analysis of externally drawn samples. Sample extraction, storage and transfer were in full compliance with established protocols. Further, laboratory markers of the inflammatory response measured in-hospital such as the white cell count and CRP were also normal providing indirect support. A third caveat is that cytokine levels measured in blood may not necessarily reflect their activity at the pancreatic parenchymal level. For example, Noh and colleagues demonstrated that IL-8 concentrations are elevated (compared to non-disease controls) in pancreatic juice collected by duodenoscopy[9].

Accepting these limitations, the present study does provide unique data on cytokine profiles in patients with chronic pancreatitis receiving antioxidant therapy and in a matched cohort receiving placebo and provides negative results which should be regarded as important pilot data. The first finding of interest is that at baseline, despite having radiological evidence of chronic pancreatitis, impairment of pancreatic exocrine function and a substantial requirement for opiate analgesia there was no elevation of circulatory pro- or anti-inflammatory cytokine levels. This is finding sits well with current paradigms of chronic pancreatitis which suggest that pain is not simply a product of inflammation and that it involves a complex interaction between inflammatory mediators and neural structures with alterations in nociception[10,11]. For example, fractalkine is a cell surface membrane-spanning adhesion molecule that can be cleaved to produce a soluble neuromodulatory chemokine which increases neuropathic pain through glial activation with expression correlating with the severity of pancreatic neuritis, fibrosis, intrapancreatic nerve fibre density and pain in chronic pancreatitis[12]. Fractalkine may be a better disease-specific chemokine in chronic pancreatitis although its relation to disease stage and response to therapy have yet to be elucidated[13].

In relation to cytokine profiles in chronic pancreatitis reported in other studies, the levels of IL-18 in our study are similar to those reported by Schneider and colleagues[14]. In terms of genotype, patients with alcohol-aetiology dominant, sporadic chronic pancreatitis do not have an increased frequency of functional polymorphisms in the *TGF-β1* gene, in the *IL-10* gene or in the intron 1 of the interferon-gamma gene[15].

In this study there was no relation between antioxidant therapy and cytokine levels. Thus, the significant elevations in plasma levels of antioxidants seen in the treatment group (and also in the main ANTICIPATE study and in other studies of antioxidant therapy) do not appear to interact with circulating cytokines. The low levels of cytokines probably reflect the results of sampling of an out-patient based population with clinically quiescent disease and in particular without evidence of a systemic inflammatory response.

In conclusion, this study has measured antioxidant profiles in patients with chronic pancreatitis receiving antioxidant therapy and compared these to patients receiving matched placebo. Cytokine levels were low at baseline and at 6 mo despite a significant elevation in plasma antioxidants. The study also demonstrates that circulating cytokine levels are low suggesting that pain in this disease is not simply a manifestation of ongoing inflammation. It could be the result of the inflammation tissue damage caused long time ago.

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review of the final manuscript.

COMMENTS

Background

This study undertakes a subgroup analysis comparing pro- and anti-inflammatory cytokine levels in a sub-group of patients receiving either antioxidant therapy for chronic pancreatitis in the form of Antox (Pharmanord, Morpeth, United Kingdom) or matched placebo.

Research frontiers

The novel aspect of this study is that it is believed to be the first to examine pro- and anti-inflammatory cytokine levels in patients receiving antioxidant therapy for chronic pancreatitis and to compare these levels to those in patients receiving matched placebo.

Innovations and breakthroughs

The results show that pro-inflammatory cytokine levels were not elevated. This is potentially an important finding in that it shows that in patients with chronic pancreatitis, with established pain, inflammatory cytokine levels are not elevated.

Applications

The findings are preliminary and need to be reproduced in a larger validation dataset before more general acceptance.

Peer review

It is a very interesting paper. Considering that this paper employs patients from the ANTICIPATE study, it is desirable that the authors give the registration number of the main trial.

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BRIEF ARTICLE

Adipokines and C-reactive protein in relation to bone mineralization in pediatric nonalcoholic fatty liver disease

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Abstract

AIM: To investigate bone mineral density (BMD) in obese children with and without nonalcoholic fatty liver disease (NAFLD); and the association between BMD and serum adipokines, and high-sensitivity C-reactive protein (HSCRP).

METHODS: A case-control study was performed. Cases were 44 obese children with NAFLD. The diagnosis of NAFLD was based on magnetic resonance imaging (MRI) with high hepatic fat fraction (≥ 5%). Other causes of chronic liver disease were ruled out. Controls were selected from obese children with normal levels of aminotransferases, and without MRI evidence of fatty liver as well as of other causes of chronic liver diseases. Controls were matched (1- to 1-basis) with the cases on age, gender, pubertal stage and as closely as possible on body mass index-SD score. All participants underwent clinical examination, laboratory tests, and whole body (WB) and lumbar spine (LS) BMD by dual energy X-ray absorptiometry. BMD *Z*-scores were calculated using race and gender specific LMS curves.

RESULTS: Obese children with NAFLD had a significantly lower LS BMD *Z*-score than those without NAFLD [mean, 0.55 (95%CI: 0.23-0.86) *vs* 1.29 (95%CI: 0.95-1.63); $P < 0.01$]. WB BMD *Z*-score was also decreased in obese children with NAFLD compared to obese children with no NAFLD, though borderline significance was observed [1.55 (95%CI: 1.23-1.87) *vs* 1.95 (95%CI: 1.67-2.10); $P = 0.06$]. Children with NAFLD had significantly higher HSCRP, lower adiponectin, but similar leptin levels. Thirty five of the 44 children with MRI-diagnosed NAFLD underwent liver biopsy. Among the children with biopsy-proven NAFLD, 20 (57%) had nonalcoholic steatohepatitis (NASH), while 15 (43%) no NASH. Compared to children without NASH, those with NASH had a significantly lower LS BMD *Z*-score [mean, 0.27 (95%CI: -0.17-0.71) *vs* 0.75 (95%CI: 0.13-1.39); $P < 0.05$] as well as a significantly lower WB BMD *Z*-score [1.38 (95%CI: 0.89-1.17) *vs* 1.93 (95%CI: 1.32-2.36); $P < 0.05$]. In multiple regression analysis, NASH (standardized β coefficient, -0.272; $P < 0.01$) and HSCRP (standardized β coefficient, -0.192; $P < 0.05$) were significantly and independently associated with LS BMD *Z*-score. Similar results were obtained when NAFLD (instead of NASH) was included in the model. WB BMD *Z*-scores were significantly and independently associated with NASH (standardized β coefficient, -0.248; $P < 0.05$) and fat mass (standardized β coefficient, -0.224; $P < 0.05$).

CONCLUSION: This study reveals that NAFLD is associated with low BMD in obese children, and that systemic, low-grade inflammation may accelerate loss of bone mass in patients with NAFLD.

Key words: Bone mineralization; Dual energy X-ray absorptiometry; Adipokines; C-reactive protein; Nonalcoholic fatty liver disease; Children

Core tip: Understanding the mechanisms underlying the relationship between nonalcoholic fatty liver disease (NAFLD) and low bone mineral density (BMD) is important to prevent poor bone mineralization in obese children. We showed that obese children with NAFLD have decreased BMD compared to obese children without liver involvement independently of adiposity, and that children with more severe histology have worse mineral status than children with more mild abnormalities. We also found a significant independent association of high sensitivity C-reactive protein with BMD scores, supporting the role of an inflammatory state which may accelerate loss of bone mass in patients with NAFLD.

Pacifico L, Bezzi M, Lombardo CV, Romaggioli S, Ferraro F, Bascetta S, Chiesa C. Adipokines and C-reactive protein in relation to bone mineralization in pediatric nonalcoholic fatty liver disease. *World J Gastroenterol* 2013; 19(25): 4007-4014 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i25/4007.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i25.4007

INTRODUCTION

Concurrent with the increasing rates of childhood obesity, nonalcoholic fatty liver disease (NAFLD) has emerged as the leading cause of chronic liver disease in pediatric populations worldwide[1,2]. NAFLD comprises a disease spectrum ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), with varying degrees of inflammation and fibrosis, progressing to end-stage liver disease with cirrhosis and hepatocellular carcinoma[3]. NAFLD is strongly associated with obesity, insulin resistance, hypertension, and dyslipidemia, and is now regarded as the liver manifestation of the metabolic syndrome (MetS)[4]. Recently it has been suggested that NAFLD can be a cause of low bone mineral density (BMD) in obese children and adolescents[5-7]. However, the mechanisms explaining this relationship are not completely understood[8]. Obesity-induced low-grade systemic inflammation, a key component in the pathogenesis of insulin resistance and NAFLD, may negatively influence bone health[9,10]. Expanded and inflamed visceral adipose tissue releases a wide array of molecules potentially involved in the development of insulin resistance, including free fatty acids, tumor necrosis factor (TNF)-α, and other proinflammatory cytokines[11-14]. In the presence of increased free fatty acid flux and chronic, low-grade inflammation, the liver is both the target of and a contributor to systemic inflammatory changes[15]. Indeed, in a number of case-control studies, circulating levels of several inflammatory markers [*i.e.*, C-reactive protein (CRP), interleukin (IL)-6, monocyte chemotactic protein 1 and TNF-α], procoagulant factors, and oxidative stress markers were found to be highest in patients with NASH, intermediate in those with simple steatosis, and lowest in control subjects without steatosis, and the differences were independent of obesity and other potentially confounding factors[16].

Adipose tissue also produces adipokines, which are pleiotropic molecules that not only regulate food intake and energy metabolism but also are implicated in the complex interactions between fat and bone[17,18]. Leptin, produced in bone marrow adipocytes and osteoblastic cells, regulates appetite and weight, osteoblast proliferation and differentiation *in vitro*[19-21], and osteoclasts[19,22,23]. Its receptor is expressed in osteoblasts[19,24]. Adiponectin, exclusively expressed by adipocytes, is inversely related to visceral fat mass and body mass index (BMI)[25] and regulates metabolism and inflammatory pathways[26]. Adiponectin affects osteoblast directly and osteoclast indirectly. It stimulates the proliferation and differentiation of human osteoblasts *via* the p38 mitogen-activated protein kinase signaling pathway[27]. In contrast, adiponectin indirectly influences osteoclasts by stimulating the receptor activator of nuclear factor-κB ligand (RANKL) and inhibiting osteoprotegerin production in osteoblasts[28]. Some studies have shown a negative association between adiponectin and BMD, independent of fat mass or BMI[29].

The aims of this study were to evaluate: (1) BMD in obese children with and without NAFLD; and (2) the association between BMD and the serum adipokines, leptin and adiponectin, and a circulating marker of systemic inflammation, high-sensitivity C-reactive protein (HSCRP), using multiple regression.

MATERIALS AND METHODS

Study design and patients

A case-control study was performed. Cases were Caucasian obese children (BMI above the 95th percentile for age and gender) seen at the Hepatology outpatient Clinic of the Department of Pediatrics, Sapienza University of Rome, Italy. The diagnosis of NAFLD was based on magnetic resonance imaging (MRI) with high hepatic fat fraction (HFF $\geqslant$ 5%). Other causes of chronic liver disease, including hepatic virus infections (hepatitis A-E and G, cytomegalovirus, and Epstein-Barr virus), autoimmune hepatitis, metabolic liver disease, α-1-antitrypsin deficiency, cystic fibrosis, Wilson's disease, hemochromatosis, and celiac disease were ruled out with appropriate tests. Exclusion criteria were also smoking habits, and history of type 1 or 2 diabetes, renal disease, total parenteral nutrition, use of hepatotoxic medications, and chronic alcohol intake. Finally, children were excluded for conditions that could have adversely influenced BMD including glucocorticoid therapy, hypothyroidism, Cushing's disease; history of long bone fractures; indwelling hardware; and abnormality of the skeleton or spine[30,31].

Controls were selected from Caucasian obese children with normal levels of aminotransferases, and without

MRI evidence of fatty liver (HFF < 5%) as well as of other causes of chronic liver diseases (see above). Controls were also excluded if they had smoking habits, history of type 1 or 2 diabetes, renal disease, chronic alcohol intake, and any condition known to influence BMD[30,31]. Controls were then matched (1- to 1-basis) with the cases on age, gender, pubertal stage and as closely as possible on BMI-SD score (SDS).

The research protocol was approved by the Hospital Ethics Committee, and informed consent was obtained from subjects' parents before assessment.

Clinical and laboratory data

All participants underwent physical examination including measurements of weight, standing height, BMI and determination of the stage of puberty, and laboratory tests. The pubertal stage was categorized into two groups (prepubertal: boys with pubic hair and gonadal stage I , and girls with pubic hair stage and breast stage I ; pubertal: boys with pubic hair and gonadal stage ≥ II and girls with pubic hair stage and breast stage ≥ II). The degree of obesity was quantified using Cole's least mean-square method, which normalizes the skewed distribution of BMI and expresses BMI as SDS[32]. Blood samples were taken, after an overnight fast, for estimation of glucose, insulin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), HSCRP, leptin, and adiponectin.

Analyses of glucose, insulin, ALT, AST, and HSCRP were conducted by COBAS 6000 (Roche Diagnostics). Insulin concentrations were measured on cobas e 601 module (Electrochemiluminescence Technology, Roche Diagnostics), while the remaining analytes on cobas e 501 clinical chemistry module (Photometric Technology), according to the instructions of the manufacturer. The degree of insulin resistance was determined by a homeostasis model assessment of insulin resistance (HOMA-IR)[33]. Scores were calculated as the product of the fasting serum insulin level (μU/mL) and the fasting serum glucose level (mmol/L), divided by 22.5. A RIA was used to measure human (total) leptin (DRG Diagnostica, Marburg, Germany; detection limit, 0.5 ng/mL; inter- and intra-assay CVs, 3.0%-6.2% and 3.4%-8.3%, respectively), and adiponectin (DRG Diagnostica, Marburg, Germany; detection limit, 1 ng/mL; inter- and intra-assay CVs, 6.9%-9.2% and 1.8%-6.2%, respectively).

MRI for liver fat quantification

The amount of hepatic fat content (% HFF) was measured by MRI using the two-point Dixon method as modified by Fishbein[34], as previously described and validated[35]. MRI results were interpreted by an experienced radiologist who was blinded to clinical and laboratory findings.

Lumbar spine and whole body dual energy X-ray absorptiometry scans

Anteroposterior lumbar spine (L_1-L_4), and whole body scans were obtained from all cases and controls using a Hologic QDR-4500W (Waltham, MA, United States) in the fan beam mode with a multidetector system. All subjects were measured on the same machine. The measurements were performed by using standard positioning techniques. Quality control was performed daily using the Hologic anthropomorphic spine, and weekly with the whole body phantom. In our department, the precision error for BMD measurements is less than 1% for the spine phantom, and less than 2.5% for the whole body phantom. The data were analyzed using the software version 11.2. Spine scans were analyzed with low-density software[36]. BMD *Z*-scores for whole body (WB) and for lumbar spine (LS) were calculated using race and gender specific LMS curves[37]. Whole body DXA results (BMD, fat mass and lean mass) shown in this study represent values excluding the skull[38].

Liver biopsy

The clinical indication for biopsy was either to assess the presence of NASH and degree of fibrosis or other likely independent or competing liver diseases. Percutaneous needle liver biopsy was performed as previously described[35]. The main histologic features of NAFLD were scored according to the scoring system developed by the NASH Clinical Research Network (CRN)[39]. Features of steatosis, lobular inflammation, and hepatocyte ballooning were combined to obtain the NAFLD activity score. As recommended by a recent NASH CRN article[40], a microscopic diagnosis, based on overall injury pattern (*i.e.*, steatosis, hepatocyte ballooning, and inflammation), as well as the presence of additional lesions (*e.g.*, zonality of lesions, portal inflammation, and fibrosis), has been assigned to each case[41]. Accordingly, biopsies were subdivided into not-NASH and definite NASH subcategories[41].

Statistical analysis

Statistical analyses were performed using the SPSS package. The data are expressed either as frequencies or as means with 95%CI. Insulin, leptin and adiponectin levels were distributed with a long tail to the right (positive skew), but their logarithms were approximately normally distributed. Mean differences in anthropometric, laboratory and body composition variables between subjects were assessed by using the *t* test. Linear regression analysis was used to identify variables associated with BMD. Then, a stepwise multiple linear regression analysis (including all variables significantly associated with BMD) was used to determine the independent variables associated with BMD. A *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

Study subjects

Forty four obese children with MRI-diagnosed NAFLD were matched to 44 obese children without evidence of liver disease. By study design cases and controls were matched for age, gender, pubertal stage and BMI-SDS. The mean age of cases and controls was 12.5 (SD 1.8) years. Both cases and controls included 20 girls and 24

Table 1 Characteristics of obese children by liver status

Variables	NAFLD (n = 44)	Non-NAFLD (n = 44)	P value
Lean mass, kg	25.8 (24.0-30.0)	26.5 (24.0-29.0)	NS
Fat mass, kg	18.7 (17.0-21.0)	16.8 (15.1-19.0)	NS
Percentage body fat	40.2% (39.0%-41.0%)	38.0% (36.0%-40.0%)	NS
Aspartate amino-transferase, U/L	34 (30-38)	24 (22-26)	< 0.0010
Alanine amino-transferase, U/L	45 (35-55)	20 (18-22)	< 0.0001
Glucose, mmol/L	4.89 (4.69-5.10)	4.88 (4.77-5.02)	NS
Insulin, μU/mL	31.2 (21.9-40.6)	20.1 (16.2-24.1)	< 0.0100
HOMA-IR values	4.27 (3.40-5.10)	3.45 (2.97-4.01)	< 0.0100
Leptin, μg/L	19.5 (15.8-23.1)	20.8 (18.2-23.4)	NS
Adiponectin, μg/L	9.0 (7.3–11.0)	12.9 (10.6–15.4)	< 0.0500
HSCRP, μg/L	3310 (2785-3836)	2165 (1710-2620)	< 0.0100
Hepatic fat fraction (%)	17.0 (11.8-22.3)	1.6 (1.0-3.1)	< 0.0001

Results are expressed as n (%), mean (95%CI), or geometric mean (95%CI) for log-transformed variables. NS: Not significant; HOMA-IR: Homeostasis model assessment of insulin resistance; HSCRP: High-sensitivity C-reactive protein; NAFLD: Nonalcoholic fatty liver disease.

boys, and five prepubertal children. The mean BMI-SDS of cases and controls was 2.19 (SD 0.16) and 2.17 (SD 0.16), respectively. The clinical and laboratory characteristics for cases and controls are shown in Table 1. There were no differences between children with and without NAFLD with respect to lean and fat mass. Compared to the non-NAFLD group, children with NAFLD had significantly higher ALT, AST, insulin concentrations, HOMA-IR values, and HSCRP levels, but lower adiponectin concentrations. There were no significant differences between the two groups with respect to glucose as well as leptin.

Histological findings in children with NAFLD

Liver biopsy was obtained in 35 of the 44 children with MRI-diagnosed NAFLD, with parental refusal in 9 cases. The 35 children did not differ from those having only liver MRI with respect to age, gender, body composition, metabolic parameters, and bone measures.

Among patients with biopsy-proven NAFLD, 20 (57%) had definite NASH, while 15 (43%) no NASH. No statistically significant differences in body composition as well as in laboratory parameters such as glucose, insulin, leptin, adiponectin levels, and HOMA-IR values were found between children with NASH and those with simple steatosis. AST [mean, 41 U/L (95%CI: 34-48) *vs* 26 U/L (95%CI: 22-29); $P < 0.001$)], ALT [mean, 58 U/L (95%CI: 41-75) *vs* 30 U/L (95%CI: 20-45); $P < 0.001$)] as well as HFF [mean, 24.8% (95%CI: 19.5-30.2) *vs* 15.7% (95%CI: 5.6-28.8); $P < 0.001$)] were significantly higher in patients with NASH compared to children without NASH. HSCRP was also higher [mean, 4055 μg/L (95%CI: 2690-5419) *vs* 2870 μg/L (95%CI: 1794-3936); $P = 0.07$], although did not reach statistically significance.

Bone measures

Obese children with NAFLD had a significantly lower LS BMD Z-score than those without NAFLD [mean, 0.55 (95%CI: 0.23-0.86) *vs* 1.29 (95%CI: 0.95-1.63); $P < 0.01$] (Figure 1A). WB BMD Z-score was also decreased in obese children with NAFLD compared to obese children with no NAFLD, though borderline significance was observed [1.55 (95%CI: 1.23-1.87) *vs* 1.95 (95%CI: 1.67-2.10); $P = 0.06$] (Figure 1B). Among children with biopsy-proven NAFLD, those with NASH had a significantly lower LS BMD Z-score than children without NASH [mean, 0.27 (95%CI: -0.17-0.71) *vs* 0.75 (95%CI: 0.13-1.39); $P < 0.05$] (Figure 1C). Moreover, children with NASH had a significantly lower WB BMD Z-score than children without NASH [1.38 (95%CI: 0.89-1.17) *vs* 1.93 (95%CI: 1.32-2.36); $P < 0.05$] (Figure 1D).

Table 2 Multivariate analysis of the variables associated with lumbar spine and whole body bone mineral density Z-score in obese children

Variables	Standardized coefficient[1]	P value
LS BMD Z-score		
NAFLD	-0.230	< 0.01
HSCRP, μg/L	-0.195	< 0.05
WB BMD Z-score		
NAFLD	-0.218	< 0.05
Fat mass, kg	-0.225	< 0.05

[1]Included in the model were age, gender, pubertal stage, nonalcoholic fatty liver disease (NAFLD), and all variables significantly associated with lumbar spine or whole body bone mineral density (BMD) Z-score in univariate analysis [*i.e.*, high-sensitivity C-reactive protein (HSCRP) and leptin levels or fat mass].

In univariate analysis, LS BMD Z-score correlated negatively with NAFLD (standardized β coefficient, -0.202; $P < 0.01$) and HSCRP (standardized β coefficient, -0.212; $P < 0.05$). In contrast, leptin was positively associated with lumbar BMD (standardized β coefficient, -0.204; $P < 0.05$). No correlation was found between LS BMD Z-score and insulin as well as HOMA-IR. Likewise, neither BMI-SDS nor lean mass nor fat mass were correlated with LS BMD Z-score. After including in the model all the significant variables as well as age, gender, pubertal status, NAFLD (standardized β coefficient, -0.230; $P < 0.01$) and HSCRP (standardized β coefficient, -0.195; $P < 0.05$) remained significantly and independently associated with LS BMD Z-score (Table 2).

WB BMD Z-score was negatively associated with NAFLD (standardized β coefficient, -0.207; $P < 0.05$), fat mass (standardized β coefficient, -0.222; $P < 0.05$), and HSCRP (standardized β coefficient, -0.216; $P < 0.05$). No correlation was found between WB BMD Z-score and insulin as well as HOMA-IR. Likewise, neither BMI-SDS nor lean mass were correlated with WB BMD Z-score. After including in the model all the significant variables as well as age, gender, pubertal status, NAFLD (standardized β coefficient, -0.218; $P < 0.05$) and fat mass (standardized β coefficient, -0.225; $P < 0.05$) remained significantly and independently associated with WB BMD Z-score (Table 2).

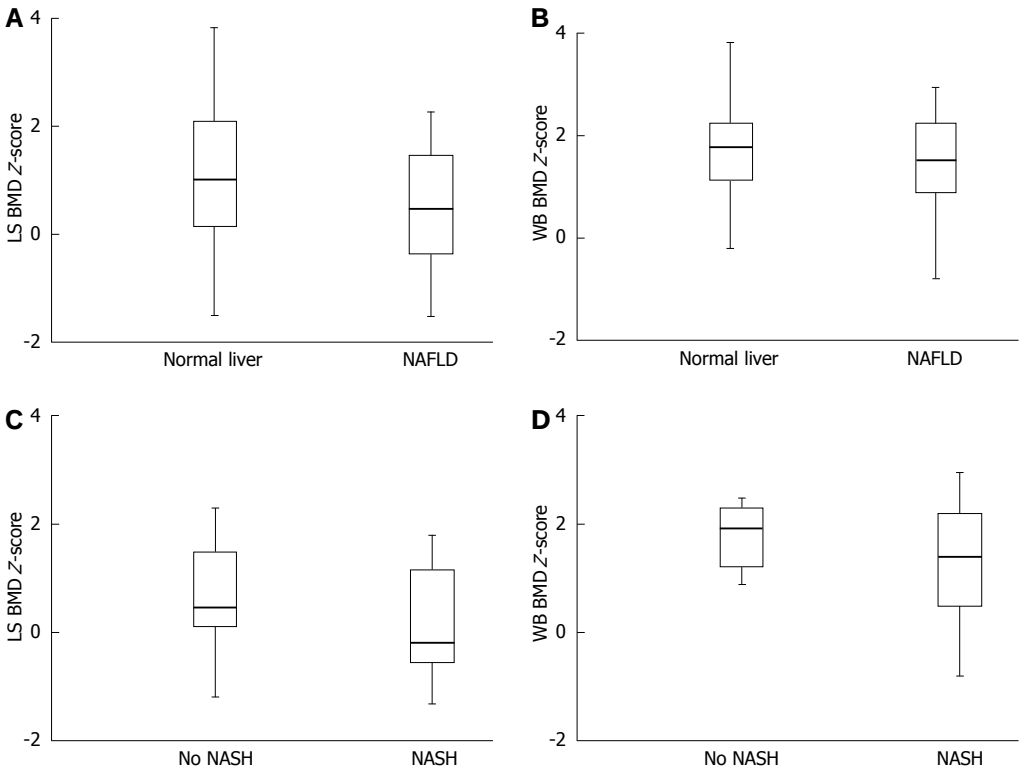

Figure 1 Bone measures. A: Lumbar spine bone mineral density *Z*-score (LS BMD *Z*-score) for obese children with and without nonalcoholic fatty liver disease (NAFLD). Box-plots give the median value (bold), 25th and 75th percentiles (lower and upper limits of the box), and lower and upper adjacent values (whiskers); B: Whole body bone mineral density *Z*-score (WB BMD *Z*-score) for obese children with and without NAFLD. Box-plots give the median value (bold), 25th and 75th percentiles (lower and upper limits of the box), and lower and upper adjacent values (whiskers); C: LS BMD *Z*-score for obese children with biopsy-proven NAFLD subdivided into those with and without nonalcoholic steatohepatitis (NASH). Box-plots give the median value (bold), 25th and 75th percentiles (lower and upper limits of the box), and lower and upper adjacent values (whiskers); D: WB BMD *Z*-score for obese children with biopsy-proven NAFLD subdivided into those with and without NASH. Box-plots give the median value (bold), 25th and 75th percentiles (lower and upper limits of the box), and lower and upper adjacent values (whiskers).

DISCUSSION

In this study, we showed that obese children with NAFLD had decreased LS BMD and WB BMD compared to obese children without liver involvement independently of adiposity, and that children with more severe histology had worse bone mineral status than children with more mild abnormalities. Furthermore, we found a significant independent association of HSCRP with BMD scores, supporting the role of an inflammatory state which may accelerate loss of bone mass in patients with NAFLD.

Growing evidence suggests the presence of a complex interplay between the skeleton and numerous homeostatic processes, including energy balance, insulin resistance, obesity and MetS[8]. Recent years have also witnessed an increased awareness of the clinical and epidemiological association between NAFLD and bone health, both in terms of reduced BMD and an increased risk of osteoporosis[8]. To our knowledge, such an association has been so far independently reported by five studies in both children and adults[5-7,42,43].

With respect to studies in adults, Moon *et al*[42] showed that in postmenopausal women ultrasound-diagnosed NAFLD was significantly associated with low lumbar BMD and this significance was maintained after adjusting for the concerned variables including age, BMI, ALT, smoking status, and alcohol consumption, and even after taking the presence of MetS into account. However, in premenopausal women, there was no such relationship. Yet, in the study by Purnak *et al*[43] involving 102 adult patients with ultrasound-diagnosed NAFLD and 54 healthy controls, there were no statistically significant differences in BMD measurements between the two groups. However, in a subgroup of patients with NAFLD, the presence of elevated serum ALT and HSCRP levels, which were suggestive of NASH, was associated with lower BMD.

With respect to studies in children, Pirgon *et al*[5] reported a negative association between BMD and insulin resistance in obese adolescents both with (n = 42) and without (n = 40) ultrasound-diagnosed NAFLD, although the obese adolescents with NAFLD had lower spine BMD *Z*-scores than their non-NAFLD counterparts. The Authors suggested that NAFLD could exert a negative impact on BMD in obese adolescents, probably *via* an increased insulin resistance. In the study by Pardee *et al*[6], poor bone mineralization was common among the 38 obese children with biopsy-proven NAFLD, but not among the 38 obese children without evidence of liver disease. Cases and controls were matched for age, gender, race, ethnicity, height and weight. Among children with NAFLD, 17 (45%) had BMD *Z*-scores $\leqslant$ -2.0, compared to none of the

controls ($P < 0.0001$). Importantly, among those children with NAFLD, children with NASH had a significantly ($P < 0.05$) lower BMD *Z*-score (-2.37) than children with NAFLD who did not have NASH (-1.58)[6]. These differences persisted after controlling for total per cent body fat. In the study by Campos *et al*[7], a 1-year interdisciplinary weight loss therapy was able to promote changes in the metabolic profile of 40 obese adolescents with ($n = 18$) or without ($n = 22$) ultrasound-diagnosed NAFLD, including a decrease in the BMI, body fat, visceral and subcutaneous fat, insulin concentration, HOMA-IR, and an increase in lean mass. At baseline, NAFLD group presented statistically lower values of bone mineral content (BMC); however, after one year of interdisciplinary therapy, there was an increase of BMC, reaching similar values of non-NAFLD group. Campos *et al*[7] suggest the importance of this kind of intervention to regulate bone mineral metabolism as result of an increased BMC and improved inflammatory state. Together, these studies indicate that NAFLD, in particular NASH, is associated with poor bone health.

Obesity and bone mineralization in children remains a topic of great interest, as data are conflicting regarding whether obesity in this age group is detrimental or protective to bone. Previous studies have suggested that body weight might improve bone mineralization in overweight adolescents by increasing the mechanical load on weight-bearing bones[44,45]. In terms of which component(s) of body weight underlie this association, the association between bone and lean mass has been found to be strongest[46]. Some studies have also suggested that fat mass may stimulate bone accrual in growing children, but these results have remained inconsistent showing both positive[47,48] and negative associations[49-51]. In multiple regression analysis, we found that fat mass had a negative association with WB BMD *Z*-score, while none of the anthropometric variables had an effect on LS BMD *Z*-scores. The basis for the negative effect of fat on WB BMD *Z*-score observed in the present study is unknown. We found that serum adipokines such as leptin and adiponectin were not significantly correlated with BMD *Z*-scores. In that vein, a recent systematic review of the literature concerning the influence of adipokines on BMD, rarely identified leptin as an independent predictor of BMD when BMI or fat mass parameters were included in the multivariate regression models[29]. Yet, in that systematic review, results were discordant for adiponectin[29]. Some studies showed a negative association between adiponectin and BMD, independent of fat mass or BMI[29]. Nevertheless, other studies did not find such associations[29]. There are possible explanations for this apparent discrepancy. Many variables, such as estrogen levels, proinflammatory cytokines, and preanalytical variability of adipokine dosage may interfere with adiponectin and bone.

Systemic inflammation is well known to contribute to low BMD in several diseases states[52-54]. CRP is a sensitive systemic marker of inflammation and tissue damage[55]. It is only produced by hepatocytes, predominantly under transcriptional control by IL-6, although other sites of local CRP synthesis and possible secretion have been suggested. Raised CRP levels are associated with many features of insulin resistance or MetS[56]. This may reflect, in part, the fact that adipocytes are the source of a substantial portion of IL-6 production[57]. On the other hand, inflammatory cytokines up-regulate the RANKL, leading to increased bone resorption and reduced BMD[58]. Some studies have suggested that an elevated CRP is associated with osteoporosis and non-traumatic fractures[9,10]. Our study suggests that HSCRP level is independently associated with LS BMD *Z*-scores in obese children with NAFLD. This finding is consistent with the hypothesis of a tight interplay between low-grade inflammation and bone turnover, even in patients with NAFLD.

COMMENTS

Background

In parallel with epidemic obesity, nonalcoholic fatty liver disease (NAFLD) has emerged as the leading cause of chronic liver disease in both pediatric and adult patients worldwide. Liver disease can be cause of low bone mineral density (BMD). However, the mechanisms explaining this relationship are still not completely understood.

Research frontiers

A better understanding of the factors that may influence bone mineral status in NAFLD may open a new frontier to fight two highly prevalent conditions like NAFLD and osteoporosis.

Innovations and breakthroughs

Recent years have witnessed an increased awareness of the clinical and epidemiological association between NAFLD and bone health, both in terms of reduced BMD and an increased risk of osteoporosis. Given the high prevalence of NAFLD and the adverse consequences of low BMD in childhood, understanding the mechanisms underlying the relationship between NAFLD and low BMD is important to prevent poor bone mineralization in this potentially vulnerable population. In this study, authors showed that obese children with NAFLD have decreased BMD compared to obese children without liver involvement independently of adiposity, and that children with more severe histology have worse mineral status than children with more mild abnormalities. They also found a significant independent association of high sensitivity C-reactive protein with BMD scores, supporting the role of an inflammatory state which may accelerate loss of bone mass in patients with NAFLD.

Applications

The presence of systemic inflammation may have important implications for the long-term skeletal health of children with NAFLD, and particularly those with nonalcoholic steatohepatitis (NASH).

Terminology

NAFLD comprises a disease spectrum ranging from simple fatty liver to NASH, with varying degrees of inflammation and fibrosis, progressing to end-stage liver disease with cirrhosis and hepatocellular carcinoma. Bone density (or BMD) is a medical term normally referring to the amount of mineral matter per square centimeter of bones. Bone density (or BMD) is used in clinical medicine as an indirect indicator of osteoporosis and fracture risk.

Peer review

In this paper, authors compared lumbar spine (LS) and whole body (WB) BMD measured by dual energy X-ray absorptiometry scans between 44 pediatric patients with magnetic resonance imaging diagnosed NAFLD and controls matched 1:1 for age, gender, and pubertal stage and body mass. They found that LS-BMD *Z* score was lower in NAFLD than in controls; Thirty three NAFLD patients were biopsied; LS and WB BMD *Z* score were lower in NASH than in non-NASH children. At multivariate analysis LS-BMD was independently associated with NASH and C-reactive protein levels. They conclude that NAFLD is associated with low BMD in obese children, and systemic low grade inflammation may play a role in such a relationship.

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BRIEF ARTICLE

Unusual histopathological findings in appendectomy specimens from patients with suspected acute appendicitis

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Abstract

AIM: To investigate the prevalence and implications of unusual histopathological findings in appendectomy specimens from patients with suspected acute appendicitis.

METHODS: The demographic and histopathological data of 1621 patients (≥ 16 years-old) who underwent appendectomy to treat an initial diagnosis of acute appendicitis between January 1999 and November 2011 were retrospectively assessed. Microscopic findings were used to classify the patients under six categories: appendix vermiformis, phlegmonous appendicitis, gangrenous appendicitis, perforated appendicitis, supurative appendicitis, and unusual histopathologic findings. The demographic and clinicopathologic characteristics of patients with unusual histopathologic findings were evaluated in detail, and re-analysis of archived resected appendix specimens was carried out.

RESULTS: A total of 912 males and 709 females, from 16 to 94 years old, were included in the study and comprised 789 cases of suppurative appendicitis, 370 cases of appendix vermiformis, 243 cases of perforated gangrenous appendicitis, 53 cases of flegmaneous appendicitis, 32 cases of gangrenous appendicitis, and 134 (8.3%) cases of unusual histopathological findings. The unusual histopathological findings included fibrous obliteration (n = 62), enterobius vermicularis (n = 31), eosinophilic infiltration (n = 10), mucinous cystadenoma (n = 8), carcinoid tumor (n = 6), granulomatous inflammation (n = 5), adenocarcinoma (n = 4; one of them mucinous), and mucocele (n = 3), adenomatous polyp (n = 1), taenia sup (n = 1), ascaris lumbricoides (n = 1), appendiceal diverticula (n = 1), and B cell non-hodgkin lymphoma (n = 1). None of the 11 patients with subsequent diagnosis of tumor were suspected of cancer prior to the appendectomy.

CONCLUSION: Even when the macroscopic appearance of appendectomy specimens is normal, histopathological assessment will allow early diagnosis of many unusual diseases.

Key words: Appendicitis; Appendectomy specimens; Histopathology; Unusual findings; Appendiceal malignancy

Core tip: Appendectomy is one of the most frequently performed surgical procedures worldwide. Although most of the resected appendectomy specimens show typical histopathologic findings, some (< 2%) show unusual histopathologic findings. The most common of these unusual features are primary or secondary appendiceal malignancies, mucocele, enterobisis, schistosomiasis, ascariasis, tuberculosis, amobiasis, and entometriosis. While some of the patients with unusual histopatholic findings require close follow-up and/or additional surgical treatment, others also necessitate antimicrobial therapy. Infectious appendicitis is respon-

sible for a significant majority of the most commonly observed unusual features, especially in cases from developing nations in geographic regions with tropical and sub-tropical climates. Therefore, regardless of the underlying etiology, the results from histopathological examination of the resected appendectomy specimen may help guide the subsequent management of cases to prevent serious appendicular diseases.

Yilmaz M, Akbulut S, Kutluturk K, Sahin N, Arabaci E, Ara C, Yilmaz S. Unusual histopathological findings in appendectomy specimens from patients with suspected acute appendicitis. *World J Gastroenterol* 2013; 19(25): 4015-4022 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i25/4015.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i25.4015

INTRODUCTION

Appendicitis remains one of the most common acute conditions of the abdomen, and suspected cases are frequently treated with emergency appendectomy[1]. The complete organ excision not only allows for definitive diagnosis but also significantly reduces the risk of life-threatening complications, such as perforation, plastron and sepsis. However, the surgical procedure itself is very invasive, representing additional risks to the patient's morbidity and mortality as well as remarkable costs to the healthcare providers. Epidemiologic studies have revealed that the incidence of acute appendicitis roughly parallels that of lymphoid development, with the peak incidence occurring between the ages of 10 and 30 years old. The most important causative factor of acute appendicitis appears to be development of luminal obstruction. In addition, several factors have been implicated as causative etiologies of this underlying feature, and show an age-related trend[1-5]. For example, lymphoid hyperplasia is the most common factor identified in patients under 20 years old, while fecalith plugs are the most common factor identified in the elderly. Apart from these usual factors, numerous other less frequent (and thus "unusual") factors have been identified as having caused the clinical symptoms that indicated the suspicion of acute appendicitis with or without histopathologic evidence for acute appendicitis[1,3,4]. The primary objective of this study was to assess the incidence and implications of unusual histopathological findings detected in appendectomy specimens from patients who received surgery to address an initial diagnosis of acute appendicitis.

MATERIALS AND METHODS

In this retrospective study, the electronic records of the Inonu University Medical Faculty Department of Surgery were searched to identify all patients who underwent appendectomy to treat an initial diagnosis of acute appendicitis between January 1999 and November 2011. The recorded demographic and histopathological data extracted for each patient included age, sex, appendectomy surgery date, and macroscopic and microscopic properties of appendix vermicularis. Patients who had received the appendectomy incidental to other surgeries, such as colorectal or gynecological cancer surgery or trauma surgery, were excluded from study enrollment. In addition, pediatric patients younger than 16 years old were also excluded from study enrollment. Four researchers working independently collected the demographic and pathologic data of all patients fitting the inclusion criteria in excel spreadsheets, which were then adjudicated and analyzed by the group.

Using the microscopic findings of each patient's appendectomy specimen that were recorded in the pathology report, the patients were classified into one of six categories: (1) appendix vermiformis; (2) phlegmonous appendicitis; (3) gangrenous appendicitis; (4) perforated appendicitis; (5) suppurative appendicitis; and (6) unusual histopathologic findings. The archived appendectomy specimens (pathology blocks and microscopic slides) were retrieved for the 134 patients in group 6 and were re-evaluated by two experienced pathologists. The patient data for each demographic or histopathologic characteristic were summarized as mean ± SD, and incidence of a characteristic within a particular group was calculated as percentage of the entire study population.

RESULTS

General characteristics of patients undergoing appendectomy for suspected acute appendicitis

A total of 1621 patients underwent appendectomy to treat an initial diagnosis of acute appendicitis. The mean age of these patients was 36.7 ± 17.4 years (range: 16-94 years) and the male-to-female ratio was nearly equal (912: 709) but with a slight male bias (56.3% males). According to the histopathological findings, 789 patients had suppurative appendicitis, 370 had appendix vermiformis, 243 had perforated gangrenous appendicitis, 134 had unusual histopathological findings, 53 had flegmaneous appendicitis, and 32 had gangrenous appendicitis. Overall, the majorities (67.3%) of the patients were ≤ 40 years old, and 13.5% were ≥ 61 years old. There was also an age bias towards patients ≤ 40 years old for those in the negative appendicitis group (64.6% of the 370 patients), with only 17.5% in that group being ≥ 61 years old. Clinicopathologic characteristics of the 1621 patients who underwent appendectomy for clinical signs of acute appendicitis are summarized in Table 1.

Characteristics of patients who showed unusual histopathologic findings in appendectomy specimens

One-hundred-and-thirty-four (8.3%) of the patients who received appendectomy to treat the initial diagnosis of acute appendicitis had unusual histopathological findings in their appendectomy specimens. This group of patients had a mean age of 48.4 ± 19.5 years old, and the male-to-

Table 1 Clinicopathologic characteristics of the 1621 patients who underwent appendectomy *n* (%)

Patient characteristics	Results
Patients	1621
Sex	
Male	912 (56.3)
Female	709 (43.7)
Age in years, mean (range)	
Overall	36.7 ± 17.4 (16-94)
Male	36.2 ± 17.5 (16-89)
Female	37.3 ± 17.3 (16-94)
Distribution of patients according to age range (yr)	
16-20	275
21-30	526
31-40	290
41-50	165
51-60	146
61-70	134
⩾ 71	85
Histopathologic findings	
Suppurative appendicitis	789 (48.7)
Appendix vermiformis	370 (22.8)
Gangrenous appendicitis-perforated	243 (15.0)
Unusual histopathologic findings	134 (8.3)
Phlegmonous appendicitis	53
Gangrenous appendicitis	32
Age distribution of the 370 patients with negative appendectomy (yr)	
16-20	67
21-30	111
31-40	61
41-50	35
51-60	32
61-70	43
⩾ 71	21

female ratio was relatively equal (60:74) but with a slight female bias (55.2% females). The mean age of the males (50.9 ± 19.3 years old; range: 16-87 years) was slightly higher than that of the females (46.4 ± 19.5 years old; range: 16-94 years). Unlike any of the five other pathology groups, the group with unusual histopathological findings had a majority (60.4%) of patients > 40 years old.

The histopathologic findings of these 134 patients with unusual histopathological findings included fibrous obliteration (*n* = 62; Figure 1A), enterobius vermicularis (*n* = 31; Figure 1B), eosinophilic infiltration (*n* = 10), mucinous cystadenoma (*n* = 8; Figure 1C), carcinoid tumor (*n* = 6; Figure 1D), granulomatous infiltration (*n* = 5; Figure 1E), adenocarcinoma (*n* = 4; Figure 1F-G), mucocele (*n* = 3; Figure 1H), adenomatous polyp (*n* = 1), taenia sup (*n* = 1; Figure 1I), ascaris lumbricoides (*n* = 1), appendiceal diverticula (*n* = 1), and B cell non-Hodgkins lymphoma (NHL) (*n* = 1; Figure 1J).

Ninety-six of the 134 total patients with unusual histopathologic findings showed no evidence of inflammatory cell infiltration. However, 21 of these 96 cases had additional inflammation-related findings, including lymphoid hyperplasia (*n* = 18) and ovarian cyst rupture (*n* = 3). The remaining 38 of the 134 total patients did show evidence of inflammatory cell infiltration to varying degrees. Among those patients, five were histologically confirmed as having perforated appendicitis and three as having gangrenous appendicitis. Five of 134 patients showed evidence of classical granulomas and multinucleated giant cells formed by epitheloid histiocytes. Staining with erlich ziehl-nielsen and periodic acid-schiff revealed a complete absence of microorganisms; thus all cases were reported as granulomatous appendicitis. Taenia sup eggs were detected in one specimen, although the adult form of the parasite was not detected and the case was not specified as *Taenia saginata* or *Taenia solium*. Clinicopathologic features of the 134 appendectomized patients with unusual histopathological findings are summarized in Table 2.

Malignancy was detected in the appendectomy specimens of 11 of the 134 patients of this group. The mean age of these patients was 49.1 ± 16.7 years old (range: 21-74 years), and the majority was female (4:7). There was no suspicion of cancer prior to the appendectomy surgery for any of these patients. Histopathological findings, however, indicated carcinoid tumor (*n* = 6), adenocarcinoma (*n* = 4) and B cell NHL (*n* = 1). Standard appendectomy was carried out in five of the six patients with carcinoid tumor, and the tumor diameters of these cases ranged from 5-25 mm; only the sixth case underwent subsequent right hemicolectomy procedure following the cancer diagnosis. Detailed tumor data could be retrieved for only one of the four patients with adenocarcinoma (age range: 51-74 years) but all of these patients underwent subsequent right hemicolectomy following the cancer diagnosis. The one patient diagnosed with B cell NHL was referred to the Medical Oncology Department following the appendectomy surgery, and medical records indicate that the patient was in remission at the 1-year follow-up. Detailed characteristics of the 11 appendectomized patients with histologically-diagnosed appendicular malignancy are summarized in Table 3.

DISCUSSION

Acute appendicitis manifests upon inflammation of the inner lining of the appendix vermiformis, which can spread to other parts of the organ. This condition may be brought on by several different physiopathological processes, but luminal obstruction is considered the most important triggering factor of the underlying inflammation[1-5]. Although lymphoid hyperplasia and fecaliths are the most frequently observed etiologies of luminal obstruction, other, less frequent factors have been observed in patients with symptoms of acute appendicitis. According to the literature, the most common of these unusual factors are mucinous cystadenoma or mucocele[6-9], carcinoid tumor[9-12], granulomatous diseases[13-15], enterobiasis[1,5,16,17], taeniasis[3,18-20], ascariasis[4,21], diverticulitis[22-25], primary or secondary adenocarcinoma[26-30], lymphoma[26,31,32], and neurogenic appendicopathy[33,34]. In addition, the study by Akbulut *et al*[1] reported cases associated with eosinophilic granuloma, amebiasis, actinomycosis, schistosomiasis, balantidiasis, tuberculosis,

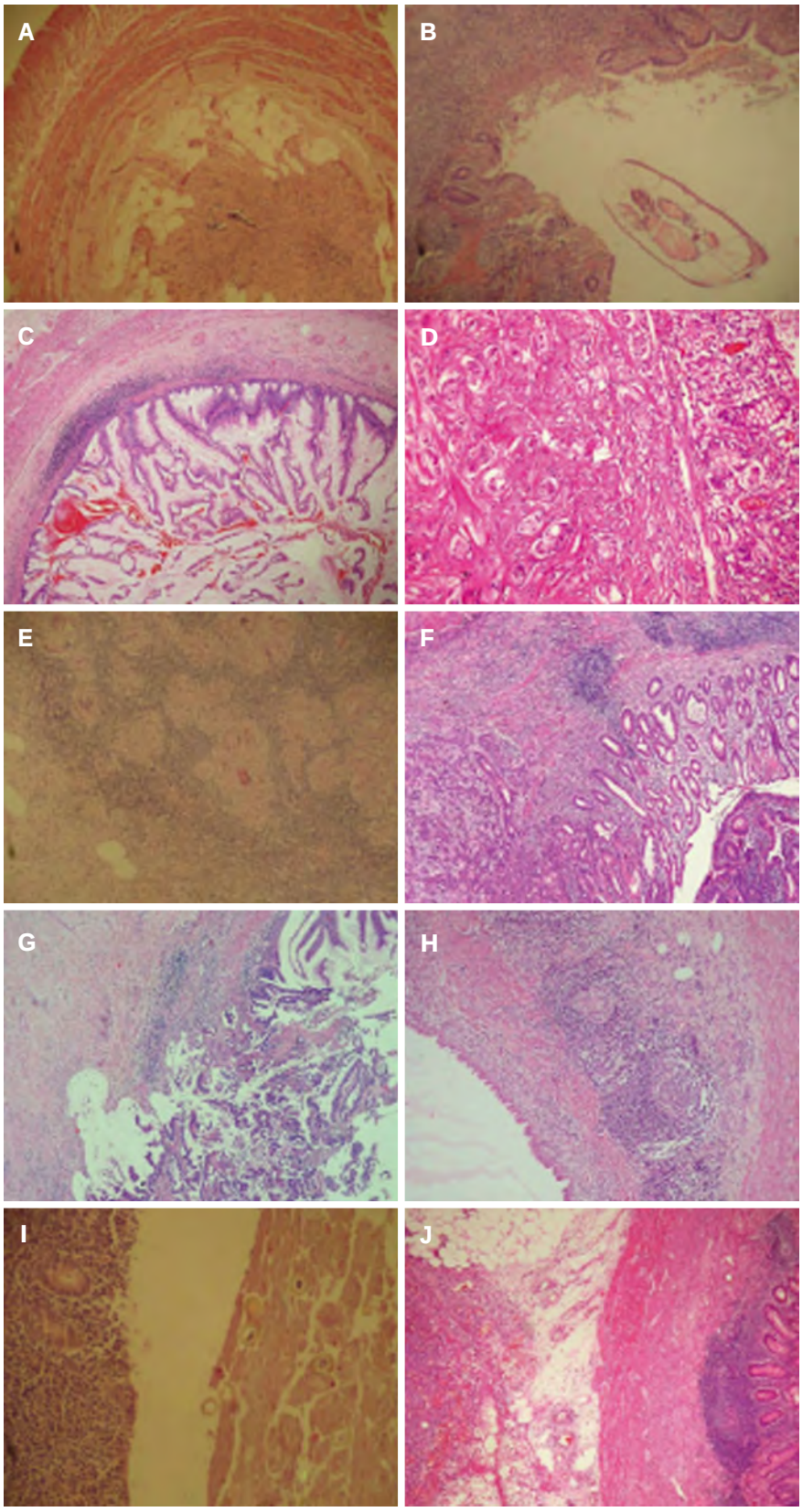

Figure 1 **Unusual histopathologic findings.** A: Appendix vermiformis showing fibrous obliteration [hematoxylin and eosin (HE) × 40]; B: View of the enterobius vermiformis in the lumen of appendix vermiformis (HE × 100); C: Mucinous cystadenoma showing proliferation of neoplastic adenomatous epithelium, which exhibits low-grade dysplasia (HE × 100); D: Carcinoid tumor of the appendix showing rounded nests and tubules of tumor cells with uniform nuclei (HE × 200); E: Granulomatous inflammation. Submucosal granuloma with central necrosis (HE × 40); F: Moderately differentiated adenocarcinoma showing infiltration of the mucosa and submucosa of the appendiceal wall (HE × 100); G: Adenocarcinoma of the appendix showing associated mucocele on the top right side (HE × 100); H: Mucocele showing a unilocular dilated appendiceal wall lined with flattened epithelial cells (HE × 100); I: Eggs of Taenia sup are present in the lumen of appendix vermiformis (HE × 100); J: Serosa of the appendiceal wall showing diffuse large B cell lymphoma infiltration (HE × 40).

Table 2 Detailed characteristics of 134 patients with unusual histopathological findings

Patient characteristic	Results
Patients	134
Sex, *n* (%)	
Male	60 (44.8)
Female	74 (55.2)
Age in years, mean ± SD (range)	
Overall	48.4 ± 19.5 (16-94)
Male	50.9 ± 19.3 (16-87)
Female	46.4 ± 19.5 (16-94)
Histopathologic findings, *n*	
Fibrous obliteration	62
Enterobius vermicularis	31
Eosinophilic infiltration	10
Mucinous cystadenoma	8
Carcinoid tumor	6
Granulomatous inflammation	5
Adenocarcinoma	3
Mucinous adenocarcinoma	1
Mucocele	3
Adenomatous polyp	1
Taenia saginata	1
Ascaris lumbricoides	1
Non-Hodgkin's lymphoma (B cell)	1
Appendicular diverticulitis	1
Age distribution of 134 patients with unusual findings (yr)	
16-20	8
21-30	30
31-40	15
41-50	16
51-60	22
61-70	22
⩾ 71	21

adenovirus, melanosis, neurofibroma, endometriosis, adenomatous or hyperplastic polyps, villous or tubulovillous adenoma, gastrointestinal stromal tumor, leukemia, and foreign body reactions.

Appendiceal tumors, which have been reported in < 3% of all appendectomy specimens, are rarely associated with manifestation of clinical symptomology. Thus, this condition is most often recognized incidentally, either during an abdominal operation or general pathological examination of a resected appendix specimen. The most frequently diagnosed type of appendiceal primary malignant lesion is the carcinoid tumor. Although it accounts for about 60% of all appendiceal tumors, its incidence in patients undergoing appendectomy is only 0.30%-2.27%. Most of the carcinoid tumors are located at the tip of the appendix and are < 1 cm in diameter. Fortunately, malignancy and metastasis of these tumors are very rare, and usually only involve tumors that exceed 1 cm. Therefore, simple appendectomy is considered sufficient management for these tumors. The risk of metastasis jumps up to 85%, however, once the tumor size reaches 2 cm or larger, in which case a formal right hemicolectomy is recommended[10-12]. In our patient series, the incidence of appendiceal carcinoid (0.37%) was similar to that in the overall literature.

Primary adenocarcinoma of the appendix is an extraordinarily rare tumor, with overall incidence in the literature between 0.01% and 0.20%. However, this tumor is most likely to occur in persons between 50 and 55 years old. Adenocarcinomas generally show aggressive behavior, the pattern of which has been likened to colonic adenocarcinomas. Therefore, appendicular adenocarcinomas are often treated by oncologic resection with right hemicolectomy[10,26,29]. In our patient series, only four patients presented with this tumor type, giving an incidence of 0.25% that is similar to that in the overall literature. In addition, these patients were within the age range of 51 and 74 years old (mean ± SD, 64.0 ± 8.3 years).

Appendiceal mucinous adenocarcinoma, also known as mucinous cystadenocarcinoma, is another rare condition of the appendix. This tumor type, however, is most often associated with a second malignancy of the gastrointestinal tract and the most common manifestation is symptoms of acute appendicitis. Like the other appendix-related cancers, diagnosis of mucinous adenocarcinoma is usually only made upon the subsequent pathological evaluation of a resected appendiceal specimen[27,28].

Mucocele is a condition in which mucoid material accumulates in the intraluminal region of the appendix, eventually causing obstructive dilatation of the organ. However, the occlusion of the appendiceal lumen may also be caused by endometriosis or carcinoid tumors[8,9]. The overall incidence of this condition in the literature ranges from 0.2% to 0.7%. Currently, four histologic types of appendiceal mucocele are recognized, and these include (in order of incidence): Mucinous cystadenoma, mucosal hyperplasia, mucinous cystadenocarcinoma, and retention cyst[1,6,7]. Up to one-half of mucocele cases are asymptomatic and the condition is incidentally diagnosed by histological examination of tissues from appendectomy, or sometimes during a laparotomy surgery. Appendectomy is the standard of care for mucinous cystadenoma, whereas a cystadenocarcinoma requires a right hemicolectomy[1,6,7].

The gastrointestinal tract is the most common site for extranodal lymphomas, accounting for 30%-45% of all extranodal cases. The incidence of primary appendiceal lymphoma is extremely low, and has been estimated at between 0.015% and 0.05%[26,31,32]. Cases of appendiceal lymphoma most often occur in young adults, between the ages of 20 and 40 years old. The usual manifestation of symptoms of acute appendicitis explain its diagnosis most frequently occurring following appendectomy and upon histopathologic analysis of the resected organ. Unfortunately, the rarity of the disease has impeded establishment of evidence-based guidelines for treatment.

The incidence of neurogenic appendicopathy is estimated to be about 30%. Although this process is often described as fibrous obliteration, recent studies have demonstrated that the occlusive proliferation is predominantly neurogenic in some cases. As of yet, the pathogenesis of this condition remains to be fully elucidated, but some studies have indicated that it may actually be secondary to hyperplasia of neuroendocrine cells. Differential diagnosis between appendiceal neuroma and acute

Table 3 Detailed characteristics of the 11 patients with appendicular malignancy

No.	Age (yr)	Sex	Primary tumor type	Tumor size (mm)	Pleomorphism	Mitosis (HPF)	Necrosis	Parietal spread	Surgical approach
1	55	M	B-NHL	CD20(+), CD79a(+)					Appendectomy
2	64	F	Adenoca	40				Muscularis propria	Appendectomy-right hemicolectomy
3	67	M	Adenoca	15					Appendectomy-right hemicolectomy
4	74	F	Adenoca	50				Serosa	Appendectomy-right hemicolectomy
5	51	M	Adenoca	50				Serosa	Appendectomy-right hemicolectomy
6	41	F	Carcinoid	25	Minimal	1/10	No	Mesoappendix	Appendectomy-right hemicolectomy
7	28	F	Carcinoid	5	Minimal	1/10	No	Submucosa	Appendectomy
8	28	F	Carcinoid	8	Minimal	0/10	No	Mesoappendix	Appendectomy
9	60	F	Carcinoid	10	Moderate	2/10	No	Mesoappendix	Appendectomy
10	21	M	Carcinoid	12	Moderate	2/10	No	Mesoappendix	Appendectomy
11	52	F	Carcinoid	13	Moderate	1/10	No	Muscularis propria	Appendectomy

HPF: High power field; M: Male; F: Female; B-NHL: B cell non-Hodgkins lymphoma.

appendicitis is relatively subjective and depends upon a patient's clinical history, symptomology, and findings from laboratory and physical examination. Accordingly, most appendiceal neuromas are incidentally indicated by histological evidence of fibrous obliteration in appendix specimens of otherwise asymptomatic patients[1,33,34]. In the current patient series, the incidence of fibrous obliteration was only 3.7%, which is lower than in the overall literature.

Enterobius vermicularis, commonly known as the pinworm, is a widespread parasitic infection that is estimated to affect up to 200 million people worldwide. The association of pinworm infection and appendicitis was first made in the late 19th century. While the reported incidence of pinworm infections in appendectomy specimens from patients with presumed appendicitis has ranged from 0.2% to 41.8%, inflammation is often associated with pinworm infection in the appendix[1,5,16,17]. In the current patient series, the incidence of pinworms in the appendectomy specimens was 1.9%, which is similar to the overall literature.

Taeniasis manifests upon intestinal infection with helminths. The first sign of infection is usually a segment of the parasite that appears in the stool. Taenia sup infection of the appendix, in particular, is so rare that the situation invites a case report. In general, cases of taeniasis do not necessitate identification of the specific species in order to initiate appropriate treatment, and a single dose of praziquantel can efficiently clear the infection[3,18-20].

Ascaris lumbricoides is one of the most common human helminthic pathogens infecting humans worldwide; however, epidemiologic studies have revealed that the highest prevalence of ascariasis occurs in tropical and semitropical countries. In the human host, the worm can establish residence in the gastrointestinal region from the stomach to the ileocecal valve, but up to 99% of the cases reported have worms localized to the jejunum and proximal ileum. Infections involving the appendix are only rarely seen. The ability of a roundworm to migrate to the appendix, thereby causing appendicitis, is controversial. The physical and physiological effects of such a migration may indeed simulate other physiopathogenic processes that promote appendicitis, but are believed less likely to be the direct cause of it[1,4,21].

Granulomatous appendicitis is another rare condition that may be discovered incidentally in a patient with a clinical presentation of acute appendicitis. The reported incidence in Western countries has ranged from 0.14% to 0.30%, and is higher (1.3%-2.3%) in underdeveloped countries[13,14]. The criteria for diagnosis are similar to those of other diseases of the gastrointestinal tract, and include granulomatous inflamation, transmural lymphoid aggregates, and fissuring-type ulcers. Various infectious agents (such as *Yersinia* spp., *Mycobacterium tuberculosis*, and *Schistosoma* spp.) and non-infectious factors (such as Crohn's disease and sarcoidosis) have been implicated as causative factors of this condition[1,14-16]. In the current series of patients, granulomatous inflammation was observed in only 0.3%. As tuberculosis is endemic in the region where our hospital is located, all of the patients had been tested accordingly; yet, no findings related to tuberculosis were encountered.

Appendiceal diverticulum is another very rare clinical entity, and the incidence is reported between 0.004% and 2.1%. The diverticula may occur as singlets or in multiples, but generally involve the distal third of the appendix, on its mesenteric side, and their size is usually < 0.5 cm. Cases of appendiceal diverticula are routinely classified as either congenital or acquired. While the congenital form (considered a true diverticulum) is extremely rare, the acquired form (a pseudodiverticulum consisting of mucosa and submucosa herniated through vascular clefts in the muscular layer) are encountered much more often. Four clinical variations of either form of this condition have been described, and include the appendiceal diverticula without inflammation, acute appendicitis with diverticula, acute appendiceal diverticulitis with acute appendicitis, and isolated acute diverticulitis. However, all

four forms are generally asymptomatic, with the related complications of perforation and inflammation causing the abdominal pain that mimics acute appendicitis[22-25].

Considering the overall case reports in the literature and the case series presented herein, it is clear that even when the macroscopic appearance of a resected appendix is normal, histopathological assessment of specimens will allow early diagnosis of malign and infectious appendiceal diseases.

COMMENTS

Background

Appendicitis is one of the most common acute surgical conditions of the abdominal cavity. While this clinicopathologic condition may manifest from several underlying etiologies, luminal obstruction is the essential triggering factor for development of the inflammatory process. Although lymphoid hyperplasia and fecaliths are the most common cause of luminal obstruction, other less commonly observed factors, such as infectious and malignant appendiceal diseases, may also underlie this pathogenic condition.

Research frontiers

According to the literature, the most common of unusual histopathologic findings are mucinous cystadenoma or mucocele, carcinoid tumor, granulomatous diseases, enterobiasis, taeniasis, ascariasis, diverticulitis, primary or secondary adenocarcinoma, lymphoma, and neurogenic appendicopathy, eosinophilic granuloma, amebiasis, actinomycosis, schistosomiasis, balantidiasis, tuberculosis, adenovirus, melanosis, neurofibroma, endometriosis, adenomatous or hyperplastic polyps, villous or tubulovillous adenoma, gastrointestinal stromal tumor, leukemia, and foreign body reactions. In this study, the authors conducted and investigation of the incidence and implications of unusual histopathological findings detected in resected appendectomy specimens obtained from patients who underwent surgery for suspected acute appendicitis.

Innovations and breakthroughs

The authors emphasize and strongly recommend that all appendectomy specimens be examined by histopathological analysis, even if specimens have a normal gross appearance.

Peer review

This is a quite well-done manuscript of appropriate interest and with images of reasonable quality.

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BRIEF ARTICLE

CYP1A1, *GSTM1*, *GSTT1* and *NQO1* polymorphisms and colorectal adenomas in Japanese men

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Abstract

AIM: To investigate the role of functional genetic polymorphisms of metabolic enzymes of tobacco carcinogens in the development of colorectal adenomas.

METHODS: The study subjects were 455 patients with colorectal adenomas and 1052 controls with no polyps who underwent total colonoscopy in a preretirement health examination at two Self Defense Forces hospitals. The genetic polymorphisms studied were *CYP1A1*2A* (rs 4646903), *CYP1A1*2C* (rs 1048943), *GSTM1* (null or non-null genotype), *GSTT1* (null or non-null genotype) and *NQO1* C609T (rs 1800566). Genotypes were determined by the polymerase chain reaction (PCR)-restriction fragment length polymorphism or PCR method using genomic DNA extracted from the buffy coat. Cigarette smoking and other lifestyle factors were ascertained by a self-administered questionnaire. The associations of the polymorphisms with colorectal adenomas were examined by means of OR and 95%CI, which were derived from logistic regression analysis. Statistical adjustment was made for smoking, alcohol use, body mass index and other factors. The gene-gene interaction and effect modification of smoking were evaluated by the likelihood ratio test.

RESULTS: None of the five polymorphisms showed a significant association with colorectal adenomas, nor was the combination of *GSTM1* and *GSTT1*. A borderline significant interaction was observed for the combination of *CYP1A1*2C* and *NQO1* (P = 0.051). The OR associated with *CYP1A1*2C* was significantly lower than unity among individuals with the *NQO1* 609CC genotype. The adjusted OR for the combination of the *CYP1A1*2C* allele and *NQO1* 609CC genotype was 0.61 (95%CI: 0.42-0.91). Although the interaction was not statistically significant (P = 0.24), the OR for individuals carrying the *CYP1A1*2C* allele and *GSTT1* null genotype decreased significantly compared with those who had neither *CYP1A1*2C* allele nor *GSTT1* null genotype (adjusted OR: 0.69, 95%CI: 0.49-0.97). Smoking did not modify the associations of the individual polymorphisms with colorectal adenomas. There was no measurable effect modification of smoking even regarding the combination of the genetic polymorphisms of the phase Ⅰ and phase Ⅱ enzymes.

CONCLUSION: Combination of the *CYP1A1*2C* and *NQO1* 609CC genotypes was associated with a decreased risk of colorectal adenomas regardless of smoking status.

Key words: Colorectal adenoma; Smoking; Polymorphism; *CYP1A1*; *GSTM1*; *GSTT1*; *NQO1*

Core tip: The study investigated the associations of *CYP1A1*2A*, *CYP1A1*2C*, *GSTM1*, *GSTT1* and *NQO1* C609T polymorphisms with colorectal adenomas among 455 cases of colorectal adenomas and 1052 controls with no polyps. None of the five polymorphisms showed a significant association with colorectal adenomas, nor was the combination of *GSTM1* and *GSTT1*. A borderline significant interaction was observed for the combination of *CYP1A1*2C* and *NQO1*. Combination of the *CYP1A1*2C* and *NQO1* 609CC genotypes was associated with a decreased risk of colorectal adenomas regardless of smoking status.

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INTRODUCTION

Colorectal cancer is one of the most common cancers, accounting for approximately 10% of incident cancer cases worldwide[1]. Colorectal adenoma is a well-established precursor lesion of colorectal cancer[2,3]. Cigarette smoking has been related to increased risk of colorectal adenomas, whereas the association between smoking and colorectal cancer risk is rather inconsistent and much weaker[4-6]. Despite the consistent association between smoking and colorectal adenomas, biological mechanisms for the association remain unknown. Tobacco smoke contains various types of carcinogens such as polycyclic aromatic hydrocarbons, heterocyclic amines, aromatic amines and *N*-nitrosamines, which are activated by phase I enzymes and/or detoxified by phase II enzymes, and thus functional genetic polymorphisms of the metabolic enzymes are of interest in colorectal carcinogenesis[7].

CYP1A1 is a phase I enzyme responsible for bioactivation of tobacco carcinogens. Two functional polymorphisms are known in the *CYP1A1* gene[8,9]. One is the 3698T>C substitution (*CYP1A1*2A*, rs 4646903) that creates an *Msp*I restriction site in the 3'-flanking region, and the other is the 2454A>G substitution (*CYP1A1*2C*, rs 1048943) that results in an amino acid change (Ile462Val) in exon 7. The *CYP1A1*2A* and *CYP1A1*2C* alleles are linked to higher inducibility of the enzyme, and have been associated with an increased risk of lung cancer and less consistently of other tobacco-related cancers[8,9]. Several studies reported an increased risk of colorectal cancer associated with *CYP1A1*2A*[10] and *CYP1A1*2C*[11], while others showed no association of either *CYP1A1*2A* or *CYP1A1*2C* with colorectal cancer[12-15] or adenomas[16]. Isoforms of the glutathione S-transferase (GST) are involved in detoxification of chemical carcinogens and environmental toxic compounds[17,18]. *GSTM1* and *GSTT1* polymorphisms have been studied most intensively in relation to tobacco-related cancers. The *GSTM1* and *GSTT1* null genotypes result in a complete loss of enzyme function[17,18]. A meta-analysis suggested an increased risk of colorectal cancer associated with the null genotype of *GSTT1*, but not of *GSTM1*[19], while another meta-analysis showed no association of either the *GSTM1* or *GSTT1* null genotype with colorectal cancer or adenomas[20]. Some recent studies have shown an increased risk of colorectal cancer associated with the *GSTM1* and *GSTT1* null genotypes in combination[21,22], but others failed to show such an increase in the risk of colorectal cancer[14,15] or adenomas[23].

NAD(P)H-quinone oxidoreductase 1 (NQO1) is involved in detoxification through two electron reductions of quinones to hydroquinones, while NQO1 can also activate procarcinogens in tobacco smoke[24]. The 609C>T polymorphism (rs 1800566) that causes an amino acid substitution (Pro187Ser) results in loss of NQO1 activity[24]. A meta-analysis reported that *NQO1* 609C>T was associated with a small increase in the risk of colorectal cancer in Caucasians[25], but a recent large Japanese study failed to corroborate such an association[15]. Homozygosity of the *NQO1* 609T allele was shown to be positively associated with colorectal adenomas[26]. Heavy smokers carrying both the *CYP1A1*2C* and *NQO1* 609T variant alleles showed a substantial increase in the risk of adenomas[26].

To clarify the role of *CYP1A1*, *GSTM1*, *GSTT1* and *NQO1* polymorphisms in colorectal carcinogenesis with reference to smoking, we examined the associations of these polymorphisms with colorectal adenomas and the effect of smoking on the associations between the polymorphisms and colorectal adenomas. A particular emphasis was placed on the combination of genetic polymorphisms of phase I and phase II enzymes, because the literature is sparse on the influence of gene-gene interactions on the risk of colorectal cancer and adenomas.

MATERIALS AND METHODS

Subjects

Study subjects were male officials in the Self Defense Forces who received a preretirement health examination at the Self Defense Forces Fukuoka Hospital or Kumamoto Hospital during the period from January 1997 to March 2001. The preretirement health examination is a nationwide program that offers a comprehensive medical examination including colonoscopy to persons retiring from the Self Defense Forces. Details of the preretirement health examination have been described elsewhere[27,28]. The subjects were Japanese in ethnicity. A 7-mL fasting venous blood sample was donated for the purpose of medical research with written informed consent. The study was approved by the Ethics Committee of Kyushu University Faculty of Medical Sciences.

The present study included 455 cases of histologically confirmed colorectal adenoma and 1052 controls with no

polyps who underwent total colonoscopy. In a consecutive series of 2454 men, five refused to participate in the study and we excluded 77 who did not undergo colonoscopy. Further exclusions were 242 men with a history of colectomy (n = 17), colorectal polypectomy (n = 212), malignant neoplasm (n = 27) or inflammatory bowel disease (n = 1). For the remaining 2135 men, colonoscopic findings were classified as polyp (n = 938, 43.9%), colorectal cancer (n = 1, 0.0%), non-polyp benign lesion (n = 123, 5.8%) and normal (n = 1073, 50.3%). Of the 938 men with colorectal polyps, 461 were found to have adenoma without *in situ* or invasive carcinoma. The controls comprised 1067 men who underwent a complete colonoscopy among the 1196 men with normal colonoscopy or non-polyp benign lesions. DNA was not available for 21 men (6 cases and 15 controls), and 455 cases and 1052 controls remained in the analysis.

Lifestyle questionnaire

Smoking habits, alcohol consumption, physical activity and other lifestyle factors were ascertained by a self-administered questionnaire, with a supplemental interview for unanswered questions given prior to colonoscopy. Details of the lifestyle questions have been described elsewhere[27,28]. Lifetime exposure to cigarette smoking was expressed by cigarette-years, which were calculated as the product of total years of smoking and the average number of cigarettes per day. Cigarette smoking was classified into 0, 1-399, 400-799 and ≥ 800 cigarette-years. Daily intake of ethanol was estimated for current alcohol drinkers based on consumption frequencies and amounts of five types of alcoholic beverages on average in the past year. Alcohol use was categorized into never, past and current use with a consumption of < 30, 30-59 or ≥ 60 mL of ethanol per day. Body mass index was categorized into four levels (< 22.5, 22.5-24.9, 25.0-27.4 and ≥ 27.5 kg/m^2). The categories for alcohol use and body mass index were arbitrary, but in accordance with those used in the previous studies[27,28]. Leisure-time physical activity was expressed as the sum of products of intensity score (metabolic equivalent) and amount of time for at most three types of regular exercise, and was categorized by quartiles in the control group.

Genotyping

DNA was extracted from the buffy coat by use of a commercial kit (Qiagen GmbH, Hilden, Germany). Genotyping was carried out by the polymerase chain reaction (PCR)-restriction fragment length polymorphism or PCR method, with agarose-gel electrophoresis and visualization by ethidium bromide. The PCR was performed in a mixture of 10 μL containing 1 μL template DNA with a concentration of 50-150 ng/μL. The PCR for *CYP1A1*2A* polymorphism was done using the primers described by Sivaraman *et al*[10], and the 340-bp PCR product was digested with *Msp*I, which resulted in fragments of 200 and 140 bp for the *CYP1A1*2A* allele. The *CYP1A1*2C* polymorphism was genotyped using the primers previously specified[29], with digestion by restriction enzyme *Hin*cII. The 187-bp product was cleaved into three fragments (120, 48 and 19 bp) in the presence of the *CYP1A1*2C* allele, and otherwise into two fragments (139 and 48 bp). *GSTM1* and *GSTT1* polymorphisms were determined by the multiplex PCR using the primers for *GSTM1*, *GSTT1* and albumin as described previously[30]. Genotyping for *NQO1* 609C>T was performed as described previously[31]. The 230-bp PCR product was digested with *Hin*fI, resulting in fragments of 195 and 35 bp for the 609C allele and fragments of 151, 44 and 35 bp for the 609T allele. The assay was repeated at most three times when the PCR was unsuccessful or when the migration pattern on the gel was aberrant.

Statistical analysis

Deviation of genotype frequency from the Hardy-Weinberg equilibrium was tested by χ^2 test with one degree of freedom using the Stata version 10 (Stata Corporation, College Station, TX, United States). The associations between the polymorphisms and colorectal adenomas were assessed by means of OR and 95%CI, which were derived from logistic regression analysis. Statistical adjustment was made for age (continuous variable), hospital (dichotomous variable), rank in the Self Defense Forces (low, middle and high), cigarette smoking, alcohol use, body mass index, physical activity and parental colorectal cancer. The gene-gene interaction and effect modification of smoking were evaluated by the likelihood ratio test. In the analysis of the effect modification of smoking, smoking status was categorized into < 400 and ≥ 400 cigarette-years, *i.e.*, < 20 and ≥ 20 pack-years, because an increased risk of adenomas associated with smoking was discernible only in the latter categories (see below). Statistical significance was declared if two-sided P was < 0.05. Statistical analysis was performed with SAS version 9.2 (SAS Institute, Cary, NC, United States).

RESULTS

Selected characteristics of the cases and controls are summarized in Table 1. The age range was 50-57 years for the cases and 47-59 years for the controls. The cases had a greater body mass index and a lower physical activity in leisure time than the controls. Heavy smoking and high alcohol consumption were more frequent in the cases than in the controls.

Among the controls, the frequencies of the *CYP1A1*2A*, *CYP1A1*2C* and *NQO1 609T* alleles were 0.39, 0.23 and 0.38, respectively, and genotype frequencies of the three polymorphisms were all in agreement with the Hardy-Weinberg equilibrium (P = 0.62 for *CYP1A1*2A*; P = 0.32 for *CYP1A1*2C*; and P = 0.76 for *NQO1* C609T). The *CYP1A1*2A* and *CYP1A1*2C* polymorphisms were in complete linkage disequilibrium except for two cases; the deviation of these two was probably due to error in genotyping.

None of the five polymorphisms showed a significant association with colorectal adenomas, nor was the combination of *GSTM1* and *GSTT1* (Table 2). The gene-gene interaction was examined for the combina-

Table 1 Selected characteristics of the study subjects

Characteristics	Cases (n = 455)	Controls (n = 1052)
Age (yr), mean ± SD	52.4 ± 0.8	52.4 ± 0.9
Body mass index (kg/m^2), mean ± SD	24.1 ± 2.8	23.7 ± 2.5
MET-h/wk, median (IQR)[1]	12 (3-24)	14 (5-24)
Smoking (cigarette-yr)		
0	20.90%	33.70%
1-399	14.10%	18.80%
400-799	45.50%	33.70%
⩾ 800	19.60%	13.70%
Alcohol use (mL/d)		
Never	11.20%	13.80%
Past	2.90%	3.10%
< 30	21.10%	30.70%
30-59	34.10%	28.70%
⩾ 60	30.80%	23.70%

[1]Leisure-time physical activity. IQR: Interquartile range; MET: Metabolic equivalent.

Table 2 Associations between genetic polymorphisms and colorectal adenomas *n* (%)

Genotype	Cases	Controls	Crude OR (95%CI)	Adjusted[3] OR (95%CI)
*CYP1A1*2A*				
0[1]	174 (38.2)	388 (36.9)	1.00 (referent)	1.00 (referent)
1	219 (48.1)	508 (48.3)	0.96 (0.76-1.22)	0.97 (0.76-1.24)
2	62 (13.6)	156 (14.8)	0.89 (0.63-1.25)	0.86 (0.60-1.23)
*CYP1A1*2C*				
0[1]	281 (61.8)	611 (58.1)	1.00 (referent)	1.00 (referent)
1	152 (33.4)	389 (37.0)	0.85 (0.67-1.07)	0.81 (0.64-1.03)
2	22 (4.8)	52 (4.9)	0.92 (0.55-1.54)	0.94 (0.55-1.60)
GSTM1				
Non-null	200 (44.0)	506 (48.1)	1.00 (referent)	1.00 (referent)
Null	255 (56.0)	546 (51.9)	1.18 (0.95-1.47)	1.19 (0.94-1.49)
GSTT1				
Non-null	258 (56.7)	552 (52.5)	1.00 (referent)	1.00 (referent)
Null	197 (43.3)	500 (47.5)	0.84 (0.68-1.05)	0.87 (0.70-1.10)
GSTM1 + *GSTT1*				
0[2]	118 (25.9)	273 (26.0)	1.00 (referent)	1.00 (referent)
1	222 (48.8)	512 (48.7)	1.00 (0.77-1.31)	1.04 (0.79-1.37)
2 (both null)	115 (25.3)	267 (25.4)	1.00 (0.73-1.35)	1.04 (0.76-1.42)
NQO1 C609T				
CC	161 (35.4)	412 (39.2)	1.00 (referent)	1.00 (referent)
CT	220 (48.4)	489 (46.5)	1.15 (0.90-1.47)	1.18 (0.92-1.52)
TT	74 (16.3)	151 (14.4)	1.25 (0.90-1.75)	1.31 (0.93-1.84)

[1]Number of variant alleles; [2]Number of null genotypes; [3]Adjusted for age, hospital, rank in the Self-Defense Forces, body mass index, cigarette smoking, alcohol use, leisure-time physical activity and parental history of colorectal cancer.

tions of the *CYP1A1* polymorphisms and the *GST* or *NQO1* polymorphism (Table 3). As regards *CYP1A1*2A*, *CYP1A1*2C* and *NQO1*, the homozygous variant genotype was combined with the heterozygous genotype because variant homozygotes were relatively few. A borderline significant interaction was observed for the combination of *CYP1A1*2C* and *NQO1* (P = 0.051). The OR associated with *CYP1A1*2C* was significantly lower than unity among individuals with the *NQO1* 609CC genotype. Although the interaction was far from statistical significance, the OR for individuals carrying the *CYP1A1*2C* allele and *GSTT1* null genotype significantly decreased compared with those who had neither the *CYP1A1*2C* allele nor the *GSTT1* null genotype.

Table 3 Associations between combinations of genetic polymorphisms and colorectal adenomas

Genotype 1	Genotype 2	n[1]	OR (95%CI)[2]	Interaction (P)
*CYP1A1*2A*	*GSTM1*			0.94
0[3]	Non-null	75/182	1.00 (referent)	
0	Null	99/206	1.20 (0.83-1.74)	
⩾ 1	Non-null	125/324	0.96 (0.67-1.35)	
⩾ 1	Null	156/340	1.12 (0.80-1.58)	
*CYP1A1*2A*	*GSTT1*			0.08
0[3]	Non-null	92/210	1.00 (referent)	
0	Null	82/178	1.14 (0.79-1.65)	
⩾ 1	Non-null	166/342	1.15 (0.83-1.58)	
⩾ 1	Null	115/322	0.85 (0.61-1.19)	
*CYP1A1*2A*	*GSTM1* + *GSTT1*			0.44
0[3]	0[4]	41/102	1.00 (referent)	
0	1	85/188	1.19 (0.75-1.88)	
0	2 (both null)	48/98	1.35 (0.81-2.27)	
⩾ 1	0	77/171	1.17 (0.74-1.86)	
⩾ 1	1	137/324	1.12 (0.73-1.72)	
⩾ 1	2 (both null)	67/169	1.03 (0.64-1.66)	
*CYP1A1*2A*	*NQO1* C609T			0.09
0[3]	CC	63/133	1.00 (referent)	
0	CT + TT	111/255	0.92 (0.63-1.36)	
⩾ 1	CC	98/279	0.73 (0.49-1.07)	
⩾ 1	CT + TT	183/385	1.02 (0.71-1.47)	
*CYP1A1*2C*	*GSTM1*			0.77
0[3]	Non-null	127/294	1.00 (referent)	
0	Null	154/317	1.16 (0.86-1.55)	
⩾ 1	Non-null	73/212	0.79 (0.56-1.12)	
⩾ 1	Null	101/229	0.98 (0.71-1.36)	
*CYP1A1*2C*	*GSTT1*			0.24
0[3]	Non-null	154/321	1.00 (referent)	
0	Null	127/290	0.97 (0.73-1.31)	
⩾ 1	Non-null	104/231	0.93 (0.68-1.27)	
⩾ 1	Null	70/210	0.69 (0.49-0.97)	
*CYP1A1*2C*	*GSTM1* + *GSTT1*			0.37
0[3]	4	74/156	1.00 (referent)	
0	1	133/303	0.96 (0.67-1.37)	
0	2 (both null)	74/152	1.13 (0.75-1.69)	
⩾ 1	0	44/117	0.79 (0.50-1.25)	
⩾ 1	1	89/209	0.92 (0.63-1.35)	
⩾ 1	2 (both null)	41/115	0.72 (0.45-1.15)	
*CYP1A1*2C*	*NQO1* C609T			0.05
0[3]	CC	102/220	1.00 (referent)	
0	CT + TT	179/391	0.99 (0.73-1.34)	
⩾ 1	CC	59/192	0.61 (0.42-0.91)	
⩾ 1	CT + TT	115/249	0.98 (0.71-1.37)	

[1]Number of cases/controls; [2]Adjusted for age, hospital, rank in the Self-Defense Forces, body mass index, cigarette smoking, alcohol use, leisure-time physical activity and parental history of colorectal cancer; [3]Number of variant alleles; [4]Number of null genotypes.

Smoking was positively associated with colorectal adenomas; the multivariate-adjusted ORs for the smoking categories of 0, 1-399, 400-799 and ⩾ 800 cigarette-years were 1.00 (referent), 1.18 (95%CI: 0.82-1.71), 2.11 (95%CI: 1.58-2.82) and 2.11 (95%CI: 1.47-3.03), respectively. However, smoking did not modify the associations of the *CYP1A1*, *GSTM1*, *GSTT1* and *NQO1* polymorphisms with colorectal adenomas (Table 4). The ORs associated with heavy smoking were consistently increased,

Table 4 Associations between genetic polymorphisms and colorectal adenomas with stratification by smoking category

Genotype	< 20 pack-years		⩾ 20 pack-years		Interaction (*P*)
	n[1]	OR (95%CI)[2]	*n*[1]	OR (95%CI)[2]	
*CYP1A1*2A*					
0[3]	59/207	1.00 (referent)	115/181	2.22 (1.52-3.24)	0.45
⩾ 1	100/346	1.05 (0.73-1.53)	181/318	1.95 (1.37-2.76)	
*CYP1A1*2C*					
0[3]	106/329	1.00 (referent)	175/282	1.90 (1.41-2.56)	0.59
⩾ 1	53/224	0.76 (0.52-1.11)	121/217	1.65 (1.20-2.27)	
GSTM1					
Non-null	69/264	1.00 (referent)	131/242	1.99 (1.41-2.82)	0.96
Null	90/289	1.19 (0.83-1.71)	165/257	2.35 (1.68-3.29)	
GSTT1					
Non-null	89/268	1.00 (referent)	169/284	1.74 (1.27-2.38)	0.26
Null	70/285	0.74 (0.52-1.06)	127/215	1.69 (1.21-2.36)	
GSTM1 + *GSTT1*					
0[4]	39/134	1.00 (referent)	79/139	1.87 (1.18-2.96)	0.63
1	80/264	1.03 (0.67-1.61)	142/248	1.91 (1.26-2.91)	
2 (both null)	40/155	0.89 (0.54-1.48)	75/112	2.14 (1.34-3.43)	
NQO1 C609T					
CC	52/221	1.00 (referent)	109/191	2.23 (1.51-3.29)	0.46
CT + TT	107/332	1.35 (0.93-1.97)	187/308	2.51 (1.75-3.60)	

[1]Number of cases/controls; [2]Adjusted for age, hospital, rank in the Self-Defense Forces, body mass index, cigarette smoking, alcohol use, leisure-time physical activity and parental history of colorectal cancer; [3]Number of variant alleles; [4]Number of null genotypes.

regardless of genotype of the polymorphism. There was no measurable effect modification of smoking even regarding the combination of the genetic polymorphisms of the phase I and phase II enzymes (Table 5). The OR was lowest for the combination of the *CYP1A1*2C* and *NQO1* 609CC genotypes among the four composite genotypes in each stratum of smoking. The OR varied according to the combination of *CYP1A1*2C* and the composite genotypes of *GSTM1* and *GSTT1* within each stratum of smoking. The OR for the combination of the *CYP1A1*2C* allele and the non-null genotypes of both *GSTM1* and *GSTT1* was significantly lower than unity in the category of < 20 pack-years, and the OR increased significantly among heavy smokers without the *CYP1A1*2C* allele who had null genotypes for both *GSTM1* and *GSTT1*.

DISCUSSION

According to recent meta-analyses[32,33], the *CYP1A1*2C* polymorphism, but not *CYP1A1*2A* polymorphism, was significantly associated with a modest increase in the risk of colorectal cancer. Neither *CYP1A1*2A* nor *CYP1A1*2C* polymorphism was related to colorectal adenomas individually in the present study. The findings are consistent with the previous observation regarding colorectal adenomas[16,26]. Further investigation is needed to clarify whether the associations with the *CYP1A1*2C* polymorphism is differential for colorectal cancer and adenomas.

The null effects of the *GSTM1* and *GSTT1* polymorphisms in the present study are in agreement with previous observations regarding colorectal adenomas[23,34]. Some of the previous studies showed an increased risk of colorectal cancer among individuals with the *GSTM1* null genotype[11,21,22], the *GSTT1* null genotype[21], or both the *GSTM1* and *GSTT1* null genotypes[21,22]. However, these findings were not replicated in other studies on colorectal cancer[14,15,35]. Previous studies found no effect of smoking on the association with *GSTM1* and *GSTT1*, either singly or in combination, in relation to colorectal adenomas[23] and cancer[15,22,33]. The *GSTM1* and *GSTT1* non-null genotypes as determined by gel electrophoresis include heterozygous genotypes (*i.e.*, one active and one inactive allele). One study differentiated the heterozygous genotype from the homozygous non-null genotype for *GSTM1* and *GSTT1* by TaqMan assay[36]. Both heterozygous and homozygous null genotypes of *GSTM1* were associated with a decreased risk of colorectal adenomas irrespective of smoking status, while adenoma risk was increased in association with both heterozygous and homozygous null genotypes of *GSTT1* among ever-smokers, but not among never-smokers[36]. However, it is unclear whether heterozygosity in either *GSTM1* or *GSTT1* affects enzyme activity[18].

Few studies have addressed the combined effect of the *CYP1A1* polymorphisms and the *GSTM1* and/or *GSTT1* null genotype in relation to colorectal adenomas and cancer. The combination of *CYP1A1*2A* and *GSTM1* null genotype was shown to be unrelated to colorectal adenomas[16]. There was no interaction between the two *CYP1A1* polymorphisms and either *GSTM1* or *GSTT1* null genotype on the risk of colorectal cancer[14]. A Japanese study showed a decreased risk of colorectal cancer for the combination of *CYP1A*2C* and *GSTT1* non-null genotype[15]. The present study indicated a decreased risk of colorectal adenomas for the combination

Table 5 Associations between combinations of genetic polymorphisms and colorectal adenomas with stratification by smoking category

Genotype 1	Genotype 2	< 20 pack-years		⩾ 20 pack-years		Interaction (*P*)
		n[1]	OR (95%CI)[2]	*n*[1]	OR (95%CI)[2]	
*CYP1A1*2A*	*GSTM1*					0.44
0[3]	Non-null	25/87	1.00 (referent)	50/95	1.78 (1.00-3.14)	
0	Null	34/120	0.94 (0.52-1.69)	65/86	2.53 (1.45-4.43)	
⩾ 1	Non-null	44/177	0.86 (0.49-1.50)	81/147	1.82 (1.07-3.10)	
⩾ 1	Null	56/169	1.19 (0.69-2.05)	100/171	1.92 (1.14-3.22)	
*CYP1A1*2A*	*GSTT1*					0.56
0[3]	Non-null	29/98	1.00 (referent)	63/112	1.94 (1.15-3.28)	
0	Null	30/109	0.95 (0.53-1.71)	52/69	2.53 (1.44-4.44)	
⩾ 1	Non-null	60/170	1.26 (0.75-2.11)	106/172	2.07 (1.27-3.39)	
⩾ 1	Null	40/176	0.81 (0.47-1.40)	75/146	1.70 (1.02-2.82)	
*CYP1A1*2A*	*GSTM1* + *GSTT1*					0.40
0[3]	0[4]	14/42	1.00 (referent)	27/60	1.34 (0.62-2.89)	
0	1	26/101	0.76 (0.36-1.62)	59/87	2.02 (1.00-4.07)	
0	2 (both null)	19/64	0.87 (0.39-1.94)	29/34	2.46 (1.11-5.46)	
⩾ 1	0	25/92	0.83 (0.39-1.78)	52/79	1.89 (0.93-3.86)	
⩾ 1	1	54/163	1.01 (0.51-2.01)	83/161	1.52 (0.78-2.98)	
⩾ 1	2 (both null)	21/91	0.73 (0.33-1.59)	46/78	1.66 (0.81-3.40)	
*CYP1A1*2A*	*NQO1* C609T					0.76
0[3]	CC	20/73	1.00 (referent)	43/60	2.55 (1.34-4.86)	
0	CT + TT	39/134	1.04 (0.56-1.94)	72/121	2.15 (1.20-3.86)	
⩾ 1	CC	32/148	0.81 (0.43-1.54)	66/131	1.69 (0.94-3.04)	
⩾ 1	CT + TT	68/198	1.29 (0.72-2.29)	115/187	2.23 (1.28-3.89)	
*CYP1A1*2C*	*GSTM1*					0.78
0[2]	Non-null	47/150	1.00 (referent)	80/144	1.74 (1.13-2.69)	
0	Null	59/179	1.04 (0.67-1.63)	95/138	2.15 (1.40-3.31)	
⩾ 1	Non-null	22/114	0.63 (0.36-1.11)	51/98	1.59 (0.98-2.57)	
⩾ 1	Null	31/110	0.94 (0.55-1.58)	70/119	1.77 (1.13-2.78)	
*CYP1A1*2C*	*GSTT1*					0.11
0[2]	Non-null	60/151	1.00 (referent)	94/170	1.39 (0.93-2.07)	
0	Null	46/178	0.66 (0.42-1.03)	81/112	1.80 (1.18-2.75)	
⩾ 1	Non-null	29/117	0.65 (0.39-1.09)	75/114	1.62 (1.06-2.48)	
⩾ 1	Null	24/107	0.59 (0.34-1.01)	46/103	1.06 (0.66-1.69)	
*CYP1A1*2C*	*GSTM1* + *GSTT1*					0.22
0[2]	0[4]	29/71	1.00 (referent)	45/85	1.26 (0.71-2.23)	
0	1	49/159	0.73 (0.42-1.26)	84/144	1.41 (0.84-2.37)	
0	2 (both null)	28/99	0.69 (0.38-1.28)	46/53	2.03 (1.12-3.69)	
⩾ 1	0	10/63	0.39 (0.17-0.87)	34/54	1.46 (0.79-2.72)	
⩾ 1	1	31/105	0.75 (0.41-1.36)	58/104	1.31 (0.76-2.26)	
⩾ 1	2 (both null)	12/56	0.53 (0.25-1.15)	29/59	1.09 (0.58-2.05)	
*CYP1A1*2C*	*NQO1* C609T					0.34
0[2]	CC	34/127	1.00 (referent)	68/93	2.64 (1.60-4.36)	
0	CT + TT	72/202	1.33 (0.83-2.12)	107/189	2.10 (1.33-3.31)	
⩾ 1	CC	18/94	0.75 (0.39-1.41)	41/98	1.41 (0.83-2.41)	
⩾ 1	CT + TT	35/130	1.03 (0.60-1.77)	80/119	2.47 (1.53-4.00)	

[1]Number of cases/controls; [2]Adjusted for age, hospital, rank in the Self-Defense Forces, body mass index, cigarette smoking, alcohol use, leisure-time physical activity and parental history of colorectal cancer; [3]Number of variant alleles; [4]Number of null genotypes.

of *CYP1A1*2C* and *GSTT1* null genotype, although the interaction was not significant. Inconsistent findings regarding the combination of *CYP1A1*2C* and *GSTT1* are probably ascribed to random fluctuation, although further studies are needed.

The risk of adenomas was lowest for the combination of *CYP1A1*2C* and the *GSTM1* and *GSTT1* non-null genotypes among never-smokers or light smokers, and an increased risk associated with heavy smoking was most evident among men without *CYP1A1*2C* who had the *GSTM1* and *GSTT1* null genotypes. Caution is required in interpreting the findings because of the small number of each combination of the three polymorphisms when stratified by smoking. Nonetheless, further investigation is warranted because the association of the three-polymorphism combination with colorectal cancer or adenomas has not been investigated previously.

The present study did not corroborate an increased risk of colorectal adenomas associated with the combination of *CYP1A1*2C* and *NQO1 609T* alleles reported among non-Hispanic whites[26], but showed a decreased risk among men harboring the *CYP1A1*2C* allele and the *NQO1* 609CC genotype. In that study[26], however, there were only a few subjects with both the *CYP1A1*2C* and *NQO1 609T* alleles (12 cases and 26 controls), accounting for only 4.0% of the cases and 1.8% of the controls,

because these variant alleles are much less frequent in Caucasians than in Asians[10,31]. In a study of Caucasians[26], the OR for the combination of *CYP1A1*2C* allele and *NQO1* 609CC genotype was 0.6 (95%CI: 0.3-1.2), as compared with the same referent combination as used in the present study. This finding is thus compatible with the present observation. It should be noted that the borderline significant interaction between *CYP1A1*2C* and *NQO1* C609T resulted from the eight statistical tests (Table 3). The probability of detecting at least one statistically significant result is 0.34, even when none of the eight interactions are present. The combined effect of these two polymorphisms requires careful interpretation, but requires further studies for mechanistic plausibility. Exposure to activated carcinogens may be lowered in individuals with both the *CYP1A1*2C* allele and the *NQO1* 609CC genotype for faster activation and detoxification.

The advantages of our study were that colonoscopy was done non-selectively in a defined population and that the absence of polyp lesions was confirmed in the control subjects by complete colonoscopy. Ethnic homogeneity was another advantage. There were several limitations. Statistical adjustment was not made for dietary factors because such data were not available. Only men were included, and the findings may not be applicable to women. Smoking is much less prevalent in women than in men in Japan[15]. The study subjects were not representative of Japanese men in the general population, but selection was unlikely to have occurred with regard to the genetic polymorphisms under study. The allele and genotype frequencies in the present study population were almost the same as those observed among Japanese individuals elsewhere. Among community controls (n = 778) in a Japanese case-control study of colorectal cancer[15], the frequencies of the *CYP1A1*2A*, *CYP1A1*2C* and *NQO1* 609T alleles were 37%, 23% and 38%, respectively, and the *GSTM1* and *GSTT1* null genotypes accounted for 54% and 44%, respectively. In a random sample of approximately 300 Japanese adults[37], the frequencies of the *CYP1A1*2C* and *NQO1* 609T alleles were 21% and 38%, respectively. Finally, the study size was not sufficiently large to detect a moderately increased risk for the variant homozygote. With two-sided a = 0.05, the power of detecting a 1.5-fold increase in the risk for the variant homozygote in the additive model was 0.66 for *CYP1A1*2A*, 0.39 for *CYP1A1*2C* and 0.65 for *NQO1* C609T.

In conclusion, the combination of *CYP1A1*2C* and *NQO1* 609CC genotype was associated with a decreased risk of colorectal adenomas, regardless of smoking status, in Japanese men. Future studies are needed to clarify the biological mechanisms involved.

COMMENTS

Background

Cigarette smoking has consistently been related to an increased risk of colorectal adenomas, and possibly of colorectal cancer, but the biological mechanisms remain unknown.

Research frontiers

Tobacco smoke contains various types of carcinogens, which are activated by phase Ⅰ enzymes and/or detoxified by phase Ⅱ enzymes. It is a matter of interest whether or not functional genetic polymorphisms of the metabolic enzymes are related to colorectal adenomas and cancer.

Innovations and breakthroughs

Few studies have examined the association of genetic polymorphisms of phase Ⅰ and phase Ⅱ enzymes in combination with colorectal adenomas or cancer. Adenoma risk differed by the combination of genetic polymorphisms of CYP1A1 (phase Ⅰ enzyme) and NAD(P)H-quinone oxidoreductase 1 (NQO1) (phase Ⅱ enzyme), and the association was not modified by smoking.

Applications

The findings confer clues to understanding the biological mechanisms of the association between smoking and colorectal adenomas and cancer.

Terminology

CYP1A1 is responsible for bioactivation of tobacco carcinogens. *CYP1A1*2A* and *CYP1A1*2C* polymorphisms are putatively functional, and have been related to increased risk of tobacco-related cancers; Glutathione *S*-transferases are involved in detoxification of chemical carcinogens, and individuals with the *GSTM1* and/or *GSTT1* null genotype may be susceptible to increased risk of cancer; NQO1 also acts as a phase Ⅱ enzyme, and the 609C>T polymorphism results in loss of NQO1 activity.

Peer review

This was a good study. The study subjects were all male officials in the Self-Defense Forces. Besides smoking, they may have risk factors, such as alcohol drinking and dietary habits, similar to those in the general population.

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BRIEF ARTICLE

Clinical and pathological differences between serum immunoglobulin G4-positive and -negative type 1 autoimmune pancreatitis

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Abstract

AIM: To identify clinical and pathological differences between serum immunoglobulin G4 (IgG4)-positive (SIP) and IgG4-negative (SIN) type 1 autoimmune pancreatitis (AIP) in South Korea.

METHODS: AIP was diagnosed by the international consensus diagnostic criteria. The medical records and pathology were retrospectively reviewed and IgG4-positive cells were counted in a high power field (HPF). Type I AIP was defined as a high serum level of IgG4 or histological finding. SIN type 1 AIP was defined as a histological evidence of type 1 AIP and a normal serum IgG4 level. The clinical and pathological findings were compared between the two groups. The analysis was performed using Student's *t* test, Fischer's exact test and Mann-Whitney's *U* test. A *P* value of < 0.05 was considered statistically significant. As repeated comparison was made, *P* values of less than 5% ($P < 0.05$) were considered significant.

RESULTS: Twenty five patients with definite type 1 AIP (19 histologically and six serologically diagnosed cases) were enrolled. The mean tissue IgG4 concentrations were significantly higher in SIP than SIN group (40 cells per HPF *vs* 18 cells per HPF, $P = 0.02$). Among eight SIN patients, the tissue IgG4 concentrations were less than 15 cells per HPF in most of cases, except one. The sensitivity of serum IgG4 was 68% (17 SIP and eight SIN AIP). Other organ involvement was more frequently associated with SIP than SIN AIP (59% *vs* 26%, $P = 0.016$). However, the relapse rate and diffuse swelling of the pancreas were not associated with serum IgG4 level. The concentrations of IgG4-positive cells per HPF were higher in SIP than SIN AIP (40 *vs* 18, $P = 0.02$).

CONCLUSION: The sensitivity of serum IgG4 was 68% in type 1 AIP. High serum IgG4 level was associated with other organ involvement and tissue IgG4 concentration but did not affect the relapse rate in type 1 AIP.

Key words: Autoimmunity; Chronic pancreatitis; Immunoglobulin G4-related systemic disease; Lymphoplasmacytic sclerosing pancreatitis; Immunoglobulin G4

Core tip: Type 1 autoimmune pancreatitis (AIP) is one

of the immunoglobulin G4 (IgG4)-related diseases and serum IgG4 is known as a useful diagnostic marker. However, the sensitivity of serum IgG4 is variable. The sensitivity of serum IgG4 was not sufficient (68%) in definite type 1 AIP. The demographic findings were not different between SIP and SIN type 1 AIP, but other organ involvement was significantly more common in SIP than in SIN type 1 AIP. High serum IgG4 level was associated with other organ involvement and tissue IgG4 concentration, but did not affect the relapse rate in type 1 AIP.

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INTRODUCTION

Autoimmune pancreatitis (AIP) is a type of chronic pancreatitis with irregular narrowing of the pancreatic duct and systemic fibroinflammatory disease. AIP is characterized by a remarkable response to steroid therapy. According to a multicenter nationwide study in Korea, the prevalence of AIP was 2.0% among 814 patients with chronic pancreatitis[1]. An early report from Japan that proposed the term lymphoplasmacytic sclerosing pancreatitis (LPSP) described some specific morphological features of AIP, such as diffuse lymphoplasmacytic infiltration with marked interstitial fibrosis and obliterative phlebitis[2].

The Japan Pancreas Society proposed diagnostic criteria for the first time in 2002, and the characteristic features of AIP were defined as the elevation of serum immunoglobulin G4 (IgG4) and LPSP on pathology[3]. However, emerging evidence suggests the presence of two AIP types that have different clinical profiles and outcomes. In 2003, a Mayo clinic group found two distinct histological patterns, which were designated LPSP and idiopathic duct-centric chronic pancreatitis (IDCP)[4]. IDCP was characterized by inflammatory infiltrates that were denser in the lobules than in interlobular fibrotic areas.

Recently, the expert panel in the international consensus study has agreed that there are two distinct histopathological types of AIP[5]. Type 1 AIP has dense periductal lymphoplasmacytic infiltrate with storiform fibrosis and obliterative phlebitis, whereas type 2 is distinguished from type 1 by granulocyte epithelial lesions, less prominent lymphoplasmacytic infiltrate, and less prominent storiform fibrosis. Recently, international consensus diagnostic criteria (ICDC) for AIP were developed based on the agreement of an international panel of experts and ICDC include both types 1 and 2 AIP[6].

According to the ICDC, the radiological imaging and the response to steroids are common features of both types 1 and 2 AIP. However, typical serological abnormalities, such as serum IgG4 elevation and other organ involvement, can be seen only in type 1. Thus, for a definitive diagnosis of type 2 AIP, histological confirmation is always necessary. Type 2 AIP is associated with inflammatory bowel disease and affects younger patients without a gender predilection[7]. Both types of AIP respond to steroid very well, but type 2 AIP has a lower relapse rate than type 1 AIP[7].

Although elevation of serum IgG4 is the one of the characteristic features in type 1 AIP, the sensitivity of serum IgG4 is variable. The initial Japanese study reported that the sensitivities of IgG4 were 90.9%[8]. However, other studies reported the sensitivity of IgG4 as approximately 70.0%[9-12]. The problem of the previous studies was that there was no clear classification of AIP type because the study was performed before the concept of type 2 AIP was established. If the study population had included more type 2 AIP, the sensitivity of IgG4 would have been low. However, a recent multicenter study also showed that the sensitivity was only 63.0% among histologically proven type 1 AIP[13]. Type 1 AIP is considered as the pancreatic manifestation of IgG4-related systemic disease in which tissue infiltration of IgG4-positive plasma cells is a characteristic feature[14-16]. However, the reason for the variable level of serum IgG4, the relation between serum level and tissue concentration of IgG4, and clinical significance of serum IgG4 level in type 1 AIP is unknown and remains an interesting issue.

The aim of this study was to find clinical and pathological differences between serum IgG4-positive (SIP) and serum IGg4-negative (SIN) type 1 AIP in Korea.

MATERIALS AND METHODS

Patients

From January 2005 to May 2011, all patients with AIP were retrospectively reviewed at the Seoul National University Hospital. The diagnosis of AIP was based on the ICDC[6] and patients without available serum IgG4 level were excluded. Patients with definite AIP were enrolled. The institutional review board of Seoul National University Hospital approved the study.

Definition of AIP type

The histology was obtained before steroid therapy in all cases. If the histology was available, type 1 AIP was defined as LPSP and type 2 as IDCP. The serum IgG4 level was obtained before steroid therapy and tissue acquisition. If tissue was not obtained, type 1 AIP was also defined if the serum IgG4 level was higher than upper limit of normal value (134 mg/dL). If the patients had no or unclear pathological findings and serum IgG4 level was normal, the patients were classified as indeterminate type and excluded in this study.

Radiological analysis

Pancreatic imaging was categorized as diffuse or segmen-

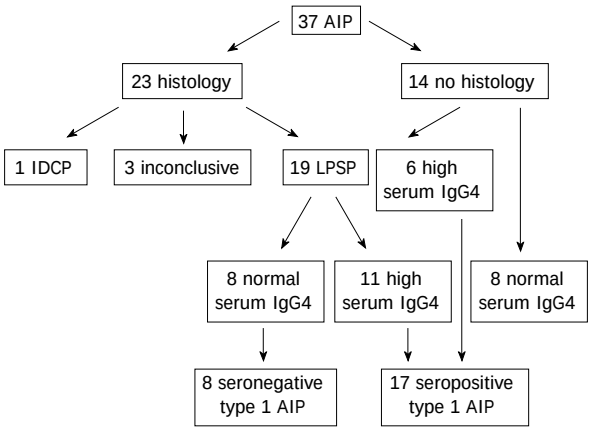

Figure 1 Enrolled patients and classification of autoimmune pancreatitis. Among 37 patients with autoimmune pancreatitis (AIP), one case was type 2 AIP and 19 patients were type 1 AIP by histology. The pathological diagnosis was inconclusive in three cases among 23 tissue samples. Among 14 patients without histology, eight patients were excluded because of normal serum immunoglobulin G4 (IgG4) levels. Ultimately, 25 patients with definite type 1 AIP (19 histologically and six serologically diagnosed cases) were enrolled in this study. LPSP: Lymphoplasmacytic sclerosing pancreatitis; IDCP: Idiopathic duct-centric chronic pancreatitis.

tal swelling by computed tomography (CT) scan. The presence of extrapancreatic lesions included sclerosing cholangitis, sclerosing sialoadenitis, lymphadenopathy, retroperitoneal fibrosis, and ulcerative colitis. Sclerosing cholangitis was defined as the presence of benign stricture of the bile duct on cholangiography. The stricture of only lower bile duct was not included in sclerosing cholangitis. The presence of sialoadenitis, lymphadenopathy and retroperitoneal fibrosis was determined based on CT findings.

Steroid therapy and relapse

Steroid therapy was done at 0.6 mg/kg per day of prednisolone for one month and gradually tapered to a maintenance dose over three months. Steroid maintenance therapy (5 mg/d) was administered for 6 mo to prevent relapse. Relapse was defined as a recurrence of symptoms with the development of pancreatic or extrapancreatic abnormal findings on imaging studies.

Histological examination

Surgically resected or core biopsied specimens were reviewed by a specialist pathologist without any clinical information. Fine needle aspiration specimens were not considered as available histology and not reviewed. All specimens were stained with anti-IgG4 antibody for immunohistochemical examination. The number of IgG4-positive plasma cells was counted in a high power field (HPF). In surgical specimens, LPSP was defined with at least three of the four characteristic features which are (1) dense infiltration of plasma cells and lymphocytes, particularly periductal; (2) peculiar storiform fibrosis; (3) venulitis with lymphocytes and plasma cells often leading to obliteration of the affected veins; and (4) abundant (> 10 cells per HPF) IgG4-positive plasma cells. In biopsy specimens, AIP was considered with lymphoplasmacytic infiltration with fibrosis and abundant (> 10 cells per HPF) IgG4-positive plasma cells.

Statistical analysis

Statistical analysis was done with statistical software (SPSS version 19.0 for Windows, SPSS Inc, Chicago, IL, United States; MedCalc version 11.5.0.0, MedCalc Software, Mariakerke, Belgium). The data were compared between two groups. The analysis was performed using Student's *t* test, Fischer's exact test and Mann-Whitney's *U* test. A *P* value of < 0.05 was considered statistically significant. As repeated comparisons were made, *P* values of less than 5% ($P < 0.05$) were considered significant.

RESULTS

Enrolled patients and classification of AIP

Thirty seven patients with AIP were enrolled and histology was available for 23 patients (Figure 1). Among 23 patients with histology, 19 patients showed typical finding of type 1 AIP and were confirmed as type 1 AIP. Only one patient was histologically confirmed as a type 2 AIP and had a history of ulcerative colitis. The pathological diagnosis was inconclusive in three cases among eight core biopsies. One type 2 and three SIN AIP patients with inconclusive pathology were excluded from this study. Among 19 patients with type 1 AIP, 11 patients had high serum IgG4 level and eight patients had normal levels. Among 14 patients without histology, six patients had elevated serum IgG4 levels (146, 213, 250, 279, 300 and 4000 mg/dL) and were included in type 1 AIP. Another eight patients with normal serum IgG4 levels were classified as indeterminate AIP and excluded from this study. Ultimately, 17 patients with SIP type 1 AIP and eight patients with SIN type 1 AIP were enrolled in this study. The median age was 61 years (range, 33-84 years) and males were predominant (72%). The sensitivity of serum IgG4 was 68.0%.

Comparison of SIP and SIN type 1 AIP

The mean age of the two groups was similar (62 years *vs* 60 years in SIP and SIN type 1 AIP) and there was no difference in sex between two groups (Table 1). The diffuse type of AIP seemed to be more common in the SIP than in the SIN group (47% *vs* 31%) but the difference was not significant ($P = 0.39$). The median serum IgG4 level was 312 mg/dL (normal range, 145-4000 mg/dL) in the SIP group and was 33 mg/dL (normal range, 6-75 mg/dL) in the SIN group and the difference was significant ($P = 0.03$). The patients of the SIP group were more likely to have other organ involvement than those of the SIN group (59% *vs* 26%, $P = 0.016$). Among the SIP group, sclerosing cholangitis was the most common (four cases) and sialoadenitis was also common (three cases) as other organ involvement. Retroperitoneal fibrosis, mediastinal lymphadenitis and lacrimal gland also represented other organ involvements. Among the SIN group, one patient

Table 1 Comparison of clinical characteristics of serum immunoglobulin G4-positive and negative type 1 autoimmune pancreatitis patients *n* (%)

Variables	SIP	SIN	*P* value
Patients	17	8	
Mean age, yr	62 (33-84)	60 (42-72)	0.359
Sex (M/F)	13:4	5:3	0.172
Diffuse type	8 (47)	3 (31)	0.390
Median serum IgG4 (mg/dL)	312 (145-4000)	33 (6-75)	0.030
Other organ involvement	10 (59)	1 (26)	0.016
Histologic examination			
Resection	5 (26)	6 (75)	0.018
Biopsy	6 (32)	2 (25)	
Not done	6 (32)		
Mean follow up, mo	30	16	0.075
Relapse	6 (35)	2 (25)	0.850

SIP: Serum immunoglobulin G4 (IgG4)-positive; SIN: Serum IgG4-negative; F: Female; M: Male.

had retroperitoneal fibrosis. Only one patient with sclerosing cholangitis was pathologically confirmed as an other organ involvement and other patients were diagnosed with only image and steroid responsiveness. The surgical resection rate was higher in the SIN than in the SIP group (75% *vs* 26%, $P = 0.018$). The mean follow up duration was not different between the two groups (30 mo *vs* 16 mo in SIP and SIN groups, $P = 0.075$). All patients, except those who received surgical resection, received steroid treatment and the response rate was 100% in both SIP and SIN groups. The relapse rate was not different between the two groups (36% *vs* 25% in SIP and SIN group, $P = 0.80$). The mean interval from steroid treatment and relapse was not different between the two groups (14 mo *vs* 11 mo in SIP and SIN groups, $P = 0.82$).

Correlation between serum and tissue IgG4 concentrations

Among the 25 patients with type 1 AIP, 19 patients had tissue specimens, which included 11 SIP and 8 SIN groups. The mean tissue IgG4 concentrations were significantly higher in the SIP than the SIN group (40 cells per HPF *vs* 18 cells per HPF, $P = 0.02$). Among eight SIN patients, the tissue IgG4 concentrations were less than 15 cells per HPF in most of cases, except one (Figure 2). Among 11 SIP patients, the tissue IgG4 concentrations were more than 25 cells per HPF, except for one case (15 cells per HPF). However, there was no linear correlation between serum and tissue IgG4 concentration among the 11 SIP patients.

Clinical features of eight patients with SIN type 1 AIP

The clinical features of eight patients with SIN type 1 AIP are summarized in Table 2. Three cases were typical diffuse type AIP. However, surgical resection was done in two cases because serum IgG4 was normal and the possibility of malignancy could not be excluded in the early period (2005). For four cases with segmental type, surgical resections were performed because the possibility of malignancy could not be excluded by imaging at that time. Only one patient had retroperitoneal fibrosis and experienced disease relapse. Six patients who received surgical resection could be confirmed as type 1 AIP with LPSP (level 1 criterion) and level 1/2 parenchymal imaging. One patient (case 3) had level 1 parenchymal imaging and level 2 histology. The other patient (case 4) could be diagnosed as type 1 AIP with level 1 ductal imaging, level 2 histology and response to steroids.

Table 2 Clinical features of eight patients with serum immunoglobulin G4-negative type 1 autoimmune pancreatitis

Patients	Age/ sex	Tissue	Image	OOI	Serum IgG4 (mg/dL)	Tissue IgG4 in HPF	Relapse
Case 1	72/F	Resection	Diffuse	No	75	5	No
Case 2	42/M	Resection	Diffuse	No	26	5	Yes
Case 3	71/F	Biopsy	Diffuse	RF	33	15	Yes
Case 4	61/M	Biopsy	Tail	No	39	11	No
Case 5	61/M	Resection	Body	No	43	80	No
Case 6	51/M	Resection	Tail	No	21	5	No
Case 7	66/M	Resection	Head	No	6	12	No
Case 8	53/F	Resection	Body	No	11	12	No

OOI: Other organ involvement; RF: Retroperitoneal fibrosis; HPF: High power field; F: Female; M: Male; IgG4: Immunoglobulin G4.

One patient had a relatively high tissue IgG4 concentration (80 cells per HPF) despite a low serum IgG4 level (43 mg/dL). He was 61-year-old male and a mass was detected incidentally at the body of the pancreas. Magnetic resonance image (MRI) findings also showed a slightly exophytic mass of iso-attenuation at the body of the pancreas with distal parenchymal atrophy and an abrupt cutting of the pancreatic duct was noticed with upstream ductal dilatation (Figure 3). Image findings were compatible with pancreatic cancer and a distal pancreatectomy was performed. Gross pathological finding showed a 1.3 cm × 1.2 cm × 3 cm sized white solid mass with uncertain margins. Microscopy showed dense periductal lymphoplasmacytic infiltration, storiform fibrosis and obliterative phlebitis (Figure 4A). The IgG4 immunohistochemistry also showed dense infiltration (80 cells per HPF) (Figure 4B). After the operation, he did not develop any symptoms or signs of recurrence for 3 years of follow-up.

DISCUSSION

IgG4-related disease was recognized as a systemic disease since 2003[17] and AIP was proposed as one of the IgG4-related sclerosing diseases in 2006[18]. Since then, two histopathological subtypes, LPSP and IDCP, have been recognized[19]. Type 1 AIP is now considered as the pancreatic manifestation of an IgG4-related systemic fibroinflammatory diseases involving the salivary gland, bile duct, and retroperitoneum. Thus, serum IgG4 is a useful marker for the diagnosis of type 1 AIP and most diagnostic criteria of AIP include serum IgG4 elevation as one of the criteria[6,9,20]. However, the sensitivity of serum IgG4 is variable and different among countries. If the

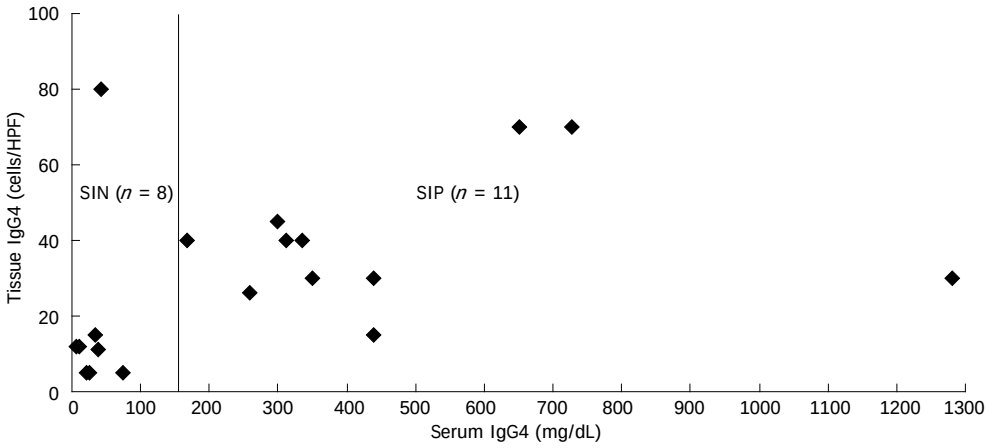

Figure 2 Correlation between serum and tissue immunoglobulin G4 concentrations. Among eight serum immunoglobulin G4 (IgG4)-negative (SIN) patients, the tissue IgG4 concentrations were less than 15 cells per high power filed (HPF) in most of cases, except one. Among 11 serum IgG4-positive (SIP) patients, the tissue IgG4 concentrations were more than 25 cells per HPF, except one case (15 cells per HPF). There was no linear correlation between serum and tissue IgG4 concentration among the 11 SIP patients.

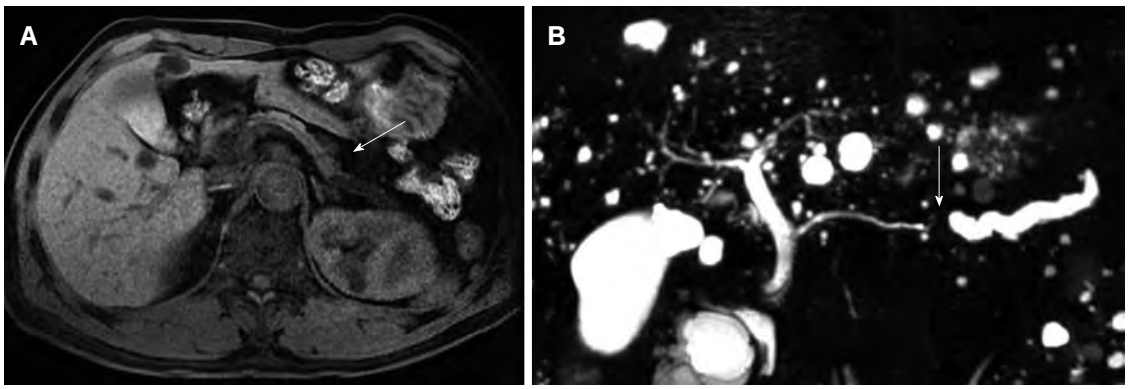

Figure 3 Magnetic resonance image of 61-year-old male patient with normal serum immunoglobulin G4. A: Magnetic resonance image shows slightly exophytic mass of iso-attenuation at the body of pancreas; B: Distal parenchymal atrophy and abrupt cutting of pancreatic duct with upstream ductal dilatation.

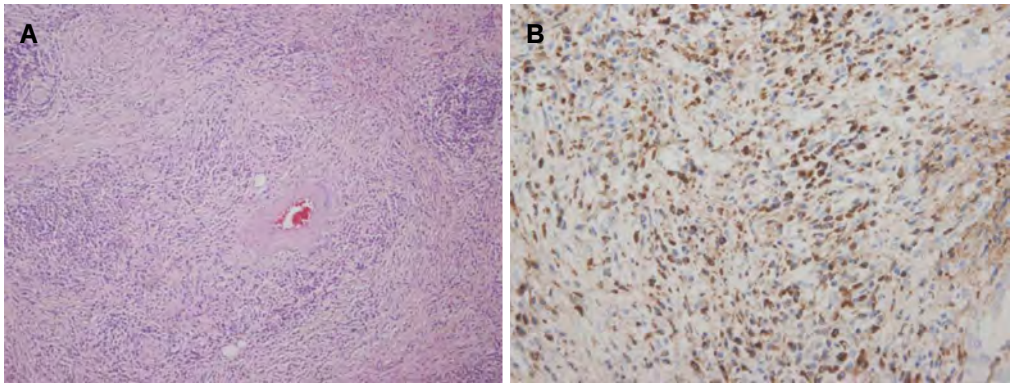

Figure 4 Histology and immunoglobulin G4 immunohistochemical staining. A: Hematoxylin and eosin staining shows typical finding of lymphoplasmacytic sclerosing pancreatitis (× 200); B: Immunoglobulin G4 (IgG4) staining shows dense infiltration of IgG4 positive cells (× 400).

study population includes more type 2 AIP, the sensitivity of IgG4 may be low because serum IgG4 is not usually elevated in type 2 AIP. A recent international multicenter study, which enrolled 713 patients with AIP from eight countries, reported that sensitivity of serum IgG4 was only 63% among 204 patients with histologically proven type 1 AIP[13]. The relatively low sensitivity of serum IgG4 can make the diagnosis of AIP in the clinical setting confusing. In our study, four patients with segmental type AIP underwent unnecessary surgical resection.

We questioned why the serum IgG4 test was not sufficiently sensitive if type 1 AIP is a type of IgG4-related

systemic disease. Therefore, we conducted our study to analyze the clinical and pathological differences between SIP and SIN type 1 AIP. Unfortunately, there have been few studies concerning the normal serum IgG4 AIP[21,22]. One study included 58 AIP patients including 13 normal serum IgG4 AIP[21] but histology was available in only 14 cases (six cases among 13 SIN AIP). Another study included 27 patients with AIP, including seven SIN AIP[22]. Histology was not available in any cases because endoscopic ultrasonography guided fine needle aspiration was performed in 26 cases using 22 gauge needle, not to diagnose AIP, but to exclude pancreatic malignancy. Thus, it could not be determined that all of the enrolled patients were really type 1 AIP in both studies. To exclude possible type 2 AIP, our study enrolled 19 patients with histologically proven type 1 AIP and six patients who were clinically diagnosed as type 1 AIP with elevated serum IgG4 levels. Of course, there is the possibility of type 2 AIP despite the elevated serum IgG4 level among six patients, because serum IgG4 elevation was detected in 23% among 47 patients with histologically proven type 2 AIP according to a recent study[13]. However, the possibility might be very low, because the serum IgG4 level was relatively high (213, 250, 279, 300, 4000 mg/dL), except in one case (146 mg/dL) and type 2 AIP was reported to be relatively rare in Asian countries, including South Korea[13]. In addition, five patients had other organ involvement, which is rarely be seen in type 2 AIP[7].

The surgical resection rate was higher in the SIN than the SIP group. One reason could be a difficult diagnosis of AIP. If the lesion is in the body/tail and serum IgG4 is normal, the clinicians would not suspect the possibility of AIP and would not hesitate to perform a surgical resection. Another reason might be selection bias of this study, because we excluded eight patients with normal serum IgG4 and no histology. The eight patients received steroid treatment and their steroid responsiveness was 100%. One patient experienced a relapse.

In this study, the clinical profiles of type 1 AIP were similar to another recent multicenter study including 327 Asian patients[12]. The higher mean age (over 60 year), male predominance, common other organ involvement, especially sclerosing cholangitis and frequent relapse, are common features of Asian patients of AIP that are similar to our study. Interestingly, the important clinical difference between SIP and SIN type 1 AIP was the frequency of other organ involvement. Other organ involvement was significantly more common in SIP than SIN type 1 AIP (59% *vs* 26%). Only one patient among the SIN group had retroperitoneal fibrosis. This result implies that other organ involvement can affect the serum IgG4 level. Mikulicz's disease refers to idiopathic symmetrical swelling of the lacrimal, submandibular gland and is an IgG4-related systemic disease. A recent study reported that the serum IgG4 level is very high (894 mg/dL) in Mikulicz's disease and significantly higher in patients with extrasalivary gland involvement[23]. More frequent other organ involvement in our SIP type 1 AIP is similar to the results of previous studies[21,22,24].

Another reason for variable serum IgG4 levels may be the number of IgG4-positive plasma cells in the tissue. As expected, the mean tissue IgG4 concentration was significantly low in SIN compared with SIP type 1 AIP. All patients in the SIP group had high IgG4 concentrations (over 25 cells per HPF), except one case (15 cells per HPF). However, the patients in the SIN group had very low IgG4 concentrations (below 15 cells per HPF), except one case (80 cells per HPF). The data might lead us to conclude that the IgG4 concentration of pancreatic tissue can influence the sensitivity of serum IgG4 in type 1 AIP. However, the serum level of IgG4 had no correlation with tissue IgG4 concentration in SIP type 1 AIP. Thus the serum level may be influenced by not only tissue concentration, but also other factors, such as the size of the involved pancreas and other organ involvement. We think that this is the first study to investigate the correlation between serum and tissue IgG4 concentration in type 1 AIP.

Other possible clinical roles of serum IgG4, other than as a diagnostic marker, are uncertain and are a interesting issue in type 1 AIP. The clinical use of serum IgG4 may be relevant in three settings: monitoring of therapy, monitoring for disease relapse and prediction of relapse. The large multicenter study in Japan reported that IgG4 levels failed to normalize in 115/182 (63%) of the patients treated with steroids[25]. The study suggested that serial IgG4 levels are helpful in identifying early relapse. However, only 30% of patients with persistent IgG4 elevation relapsed, whereas relapse was also seen in 10% of patients with normal IgG4 levels. The results regarding the value of initial serum IgG4 levels in predicting relapse vary among studies, some reporting higher relapse rate in patients with elevated serum IgG4 levels[22,26], whereas others failed to observe any association[7,27-29]. To clarify the role of serum IgG4 in predicting relapse, type 2 AIP should be excluded in the normal serum IgG4 group, because type 2 AIP is known for rare relapse[7]. The positive study might include some patients with type 2 AIP. In our study, the relapse rate was similar between the two groups of type 1 AIP. Thus, our data supports the view that initial serum IgG4 levels cannot predict relapse in type 1 AIP.

In conclusion, the sensitivity of serum IgG4 was not sufficient (68%) for definite diagnosis of type 1 AIP. The demographic findings were similar between SIP and SIN type 1 AIP, but other organ involvement was significantly more common in SIP than SIN type 1 AIP. High serum IgG4 level was associated with other organ involvement and tissue IgG4 concentration, but did not affect the relapse rate in type 1 AIP.

COMMENTS

Background

Type 1 autoimmune pancreatitis (AIP) is one of the immunoglobulin G4 (IgG4)-related diseases and serum IgG4 is known as a useful diagnostic marker.

However, the sensitivity of serum IgG4 is variable. AIP is a type of chronic pancreatitis with irregular narrowing of the pancreatic duct and systemic fibro-inflammatory disease and is characterized by a remarkable response to steroid therapy.

Research frontiers

IgG4-related disease was recognized as a systemic disease in 2003 and AIP was proposed as one of the IgG4-related sclerosing diseases in 2006. Two histopathological subtypes, lymphoplasmacytic sclerosing pancreatitis and idiopathic duct-centric chronic pancreatitis, have been recognized, and type 1 AIP is now considered as the pancreatic manifestation of an IgG4-related systemic fibroinflammatory diseases involving the salivary gland, bile duct, and retroperitoneum. Thus, serum IgG4 is a useful marker for the diagnosis of type 1 AIP and most diagnostic criteria of AIP include serum IgG4 elevation as one of the criteria. However, the sensitivity of serum IgG4 is variable and different among countries.

Innovations and breakthroughs

The sensitivity of serum IgG4 was not sufficient (68%) for defining type 1 AIP. The demographic findings were similar between serum IgG4-positive (SIP) and serum IgG4-negative (SIN) type 1 AIP, but other organ involvement was significantly more common in SIP than SIN type 1 AIP. High serum IgG4 level was associated with other organ involvement and tissue IgG4 concentration, but did not affect the relapse rate in type 1 AIP.

Peer review

The authors compared the clinical and pathological differences between serum IgG4-positive and IgG4-negative type 1 autoimmune pancreatitis and demonstrated that the sensitivity of serum IgG4 was 68% in type 1 AIP. The high serum IgG4 level was associated with other organ involvement and tissue IgG4 concentration, but did not affect the relapse rate in type 1 AIP.

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BRIEF ARTICLE

Massive presacral bleeding during rectal surgery: From anatomy to clinical practice

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Author contributions: Lou Z, Zhang W, Meng RG and Fu CG performed the majority of patient treatment; Lou Z and Zhang W collected the clinical data and provided financial support for this work; Lou Z and Zhang W designed the study and wrote the manuscript.

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Abstract

AIM: To investigate control of two different types of massive presacral bleeding according to the anatomy of the presacral venous system.

METHODS: A retrospective review was performed in 1628 patients with middle or low rectal carcinoma who were treated surgically in the Department of Colorectal Surgery, Changhai Hospital, Shanghai, China from January 2008 to December 2012. In four of these patients, the presacral venous plexus (n = 2) or basivertebral veins (n = 2) were injured with massive presacral bleeding during mobilization of the rectum. The first two patients with low rectal carcinoma were operated upon by a junior associate professor and the source of bleeding was the presacral venous plexus. The other two patients with recurrent rectal carcinoma were both women and the source of bleeding was the basivertebral veins.

RESULTS: Two different techniques were used to control the bleeding. In the first two patients with massive bleeding from the presacral venous plexus, we used suture ligation around the venous plexus in the area with intact presacral fascia that communicated with the site of bleeding (surrounding suture ligation). In the second two patients with massive bleeding from the basivertebral veins, the pelvis was packed with gauze, which resulted in recurrent bleeding as soon as it was removed. Following this, we used electrocautery applied through one epiploic appendix pressed with a long Kelly clamp over the bleeding sacral neural foramen where was felt like a pit Electrocautery adjusted to the highest setting was then applied to the clamp to "weld" closed the bleeding point. Postoperatively, the blood loss was minimal and the drain tube was removed on days 4-7.

CONCLUSION: Surrounding suture ligation and epiploic appendices welding are effective techniques for controlling massive presacral bleeding from presacral venous plexus and sacral neural foramen, respectively.

Key words: Massive presacral bleeding; Rectal surgery; Suture ligation; Welding

Core tip: Massive presacral bleeding is an uncommon but potentially life-threatening complication of rectal surgery. It is difficult to control the bleeding and several alternative techniques for hemostasis have been proposed. We described the use of two simple and effective techniques for controlling two different types of massive presacral bleeding, classified according to the anatomy of the presacral venous system.

Lou Z, Zhang W, Meng RG, Fu CG. Massive presacral bleeding during rectal surgery: From anatomy to clinical practice. *World J Gastroenterol* 2013; 19(25): 4039-4044 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i25/4039.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i25.4039

INTRODUCTION

Massive presacral bleeding is a potentially life-threatening complication of rectal surgery and remains one of the most challenging intraoperative emergencies to colorectal surgeons[1,2]. The incidence and the mortality have been reported as high as 9.4% and 4.3%, respectively[3,4]. Total mesorectal excision was introduced in 1982 and is considered the gold standard with an acceptable intraoperative risk for rectal carcinoma[5]. However, massive presacral bleeding remains inevitable, especially in recurrent rectal carcinoma or in operations performed by junior colorectal surgeons. Several hemostatic techniques for controlling this intraoperative emergency have been proposed, such as the use of thumbtacks, bone wax, balloon tamponade, and endoscopic stapling[6-8]. However, some techniques fail to arrest the bleeding[9], resulting in shock and even death.

Based on the anatomy of presacral venous system, massive presacral bleeding can be divided into two different types. In our opinion, the key feature in controlling massive presacral bleeding is correct judgment of the bleeding type. Here, we report our experience with massive presacral bleeding and describe the use of two simple and effective techniques (surrounding suture ligation and epiploic appendices welding) for controlling two different types of massive presacral bleeding according to the anatomy of the presacral venous system. To the best of our knowledge, there are no reports of this hemostatic strategy in the literature.

MATERIALS AND METHODS

This was a retrospective review of 1628 patients with middle or low rectal carcinoma who were treated surgically in the Department of Colorectal Surgery, Changhai Hospital, Shanghai, China from January 2008 to December 2012 (Table 1). All the patients who sustained massive presacral bleeding during mobilization of the rectum were recorded.

In four of these patients, the presacral venous plexus (n = 2) or basivertebral veins (n = 2) were injured. The first two patients (a 63-year-old woman and a 58-year-old man) with low rectal carcinoma were operated upon by a junior associate professor and the source of bleeding was the presacral venous plexus. The other two patients with recurrent rectal carcinoma were both women (aged 69 and 72 years, respectively). The rectal stumps were found to be densely adherent to the surrounding structures and the source of bleeding was the basivertebral veins. Two different techniques were used to control the bleeding.

Table 1 Adjuvant therapy for low rectal dissection in 1628 patients n (%)

Adjuvant therapy	Patients	Patients with bleeding
Rectal carcinoma	1606	2 (0.12)
Without neoadjuvant therapy	1463	2 (0.14)
With neoadjuvant radiotherapy	89	0 (0.00)
With neoadjuvant chemotherapy	29	0 (0.00)
With neoadjuvant radiochemotherapy	25	0 (0.00)
Recurrent rectal carcinoma	22	2 (9.00)
With preoperative radiotherapy	6	0 (0.00)
With preoperative chemotherapy	1	0 (0.00)
With preoperative radiochemotherapy	3	0 (0.00)
Without preoperative radiotherapy	12	2 (16.7)

RESULTS

In the first patient with massive bleeding from the presacral venous plexus, suture ligation was used initially to control the bleeding, which exacerbated the bleeding. As an alternative, the pelvis was packed with gauze, which resulted in recurrent bleeding as soon as the packing was removed. Following this, we tried to perform suture ligation around the venous plexus in the area with intact presacral fascia that communicated with the bleeding site (surrounding suture ligation). The bleeding stopped after 11 attempts at suture ligation. The patient underwent a super-low anterior resection with protective ileostomy. The estimated blood loss was 2000 mL. In the second patient with massive bleeding from the presacral venous plexus, we used the same technique to control bleeding with eight attempts at surrounding suture ligation. The estimated blood loss was 800 mL.

In the following two patients with massive bleeding from the basivertebral veins, the pelvis was packed with gauze, which resulted in recurrent bleeding as soon as it was removed. In the first of these patients, we tried to control bleeding by surrounding suture ligation initially. However, this technique was unsuccessful. Following this, we used electrocautery applied through one epiploic appendix pressed with a long Kelly clamp over the bleeding sacral neural foramen where was felt like a pit. First, fingertip pressure was applied directly to the sacral neural foramen to control the bleeding. Then, one epiploic appendix, 1-2 cm in diameter, was excised and mounted on a long Kelly clamp. The finger was rapidly withdrawn and the epiploic appendices pressed directly over the sacral neural foramen. Electrocautery adjusted to the highest setting was applied to the clamp to "weld" closed the bleeding point. The bleeding stopped after 3 min. In this patient, blood loss was 6000 mL. In the last patient with massive bleeding from the basivertebral veins, we used the same technique to control bleeding within 10 min. The estimated blood loss was 600 mL. Intraoperative data are shown in Table 2.

Postoperatively, the blood loss was minimal and the drain tube was removed on days 4-7. Patients were discharged on day 8 and they all returned for ileostomy reversal 3 mo later.

DISCUSSION

Massive presacral bleeding is considered to be an intraoperative emergency during rectal surgery. The anatomy of the presacral venous system makes it vulnerable to serious bleeding that can often be difficult to control[10]. The

Table 2 Patients with massive presacral bleeding

Patient	Sex	Age (yr)	TNM stage	Surgical procedure	Blood loss (mL)	Procedures used to control bleeding	Postoperative complication
1	Female	63	T4N0M0	AR + ileostomy	2000	Surrounding suture ligation	None
2	Male	58	T4N1M0	AR + ileostomy	800	Surrounding suture ligation	None
3	Female	69	Recurrent	AR + ileostomy	6000	Epiploic appendices welding	None
4	Female	72	Recurrent	APR	600	Epiploic appendices welding	None

TNM: Tumor-node-metastasis; AR: Anterior resection; APR: Abdominoperineal resection.

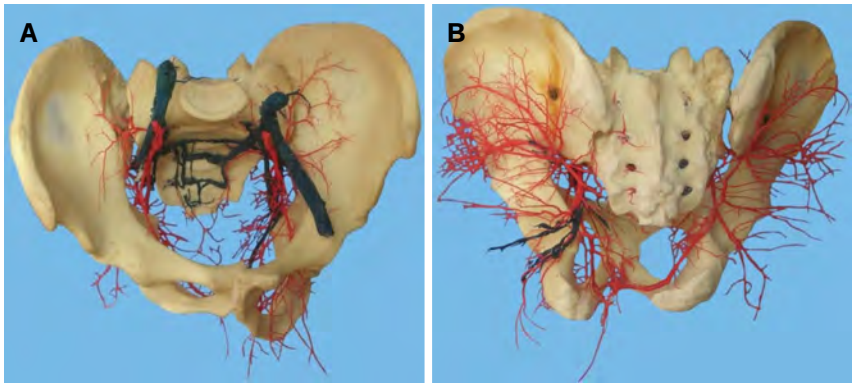

Figure 1 Presacral vascular cast. A: Front view; B: Dorsal view.

presacral venous plexus runs into the pelvic fascia that covers the anterior aspect of the sacrum. It is formed by the two lateral sacral veins, the middle sacral vein, and the in-between communicating veins. These veins are avalvular and communicate *via* the basivertebral veins with the internal vertebral venous system (Figure 1)[11]. Massive presacral bleeding can be divided into two different types according to the anatomy. The first type of bleeding arises from the presacral venous plexus. It may be massive, but can be stopped by suture ligation. The other type is massive, high-pressure bleeding that can be controlled only by pressing the sacrum with the finger or gauze. This type of bleeding originates from the sacral neural foramen where the basivertebral vein is injured[12]. When the patient is in the lithotomy position, the hydrostatic pressure is increased 2-3 times above the pressure in the inferior vena cava[13]. This avalvular system communicates with the vertebral veins, which explains why it is difficult to stop the bleeding.

Intraoperative massive bleeding may be more common during difficult operations in patients with large and fixed tumors, neoadjuvant radiotherapy, and recurrent rectal carcinoma[14]. The rate of massive presacral bleeding was higher in patients with recurrent rectal carcinoma than in those with rectal carcinoma (9.0% *vs* 0.12%, $P = 0.001$). The higher incidence of this emergency in patients with recurrent rectal carcinoma might be related to the more difficult dissection that results from fibrosis and anatomical disruption in this area. The expectation that resection of locally advanced tumors carries a higher risk of presacral vessel lesions was not confirmed in our study because we studied a small number of cases in a single institution.

Incorrect pelvic contraction or inappropriate manipulation is the most common cause of injury to the presacral venous plexus in patients without the above common contributing factors. In this series, two operations with massive presacral bleeding were performed by a junior associate professor. Initial suture ligation was performed incorrectly, which caused more massive bleeding and blood loss was estimated at 2000 mL. Massive bleeding was eventually controlled using or surrounding suture ligation technique. Blood loss was likely related to the extent and site of intraoperative vessel injury, the specific management of the bleeding, and the expertise of the surgeon[1].

The main clinical characteristics of massive presacral bleeding include: (1) bleeding that occurs suddenly during mobilization of the rectum, which can quickly lead to hemorrhagic shock and even result in death; (2) gushing of blood from the pelvic floor, which makes the bleeding site undetectable; (3) ligation of the internal iliac vessel is futile; and (4) bleeding does not stop, even in hemorrhagic shock. According to previous reports, blood loss in presacral bleeding ranges between 300 and 7800 mL[12]. Most of these patients need blood transfusion. In our study, blood loss ranged between 600 and 6000 mL (mean, 2350 mL), and three patients needed blood transfusions. In patients with colorectal carcinoma undergoing surgery, blood transfusion is associated with adverse clinical outcomes, including increased mortality[15]. Therefore, it is important to use a simple and effective procedure to control massive presacral bleeding in rectal surgery.

In our experience, whenever massive presacral bleeding occurs, the first step is direct pressure with the finger at the bleeding point. At the same time, surgeons should inform an anesthetist to prepare sufficient blood. When the bleeding point cannot be exposed clearly, gauze should be pressed directly over the presacral area and the pressure maintained for 15-20 min. The blood surround-

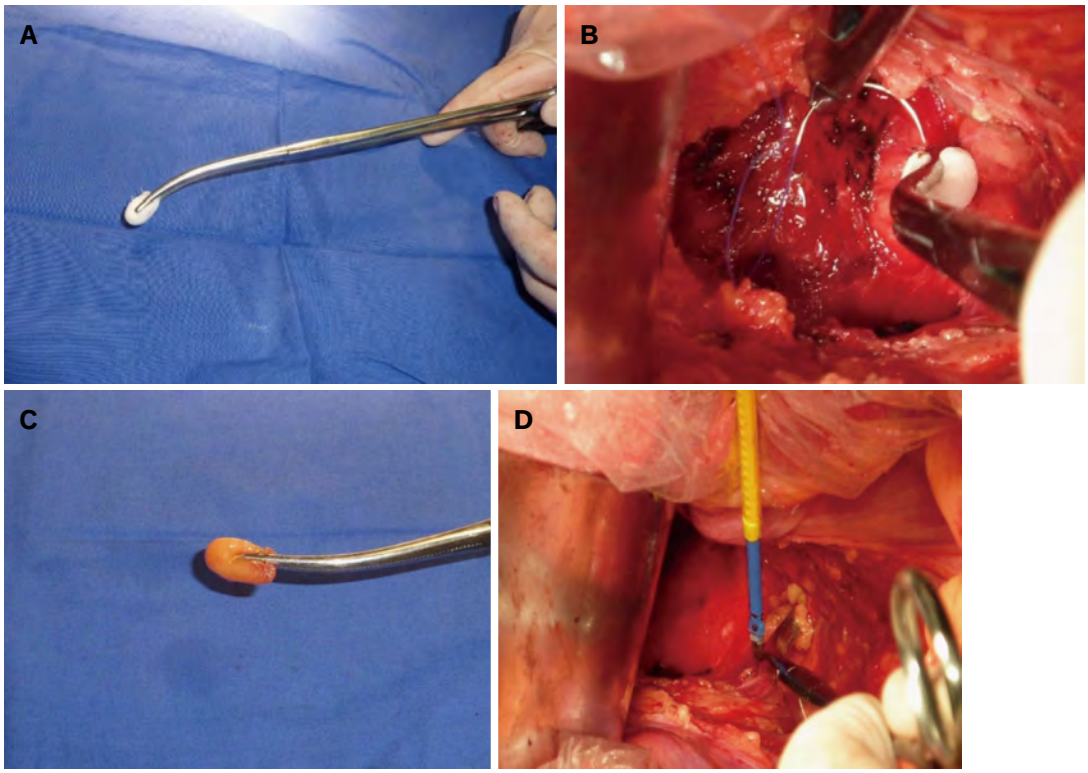

Figure 2 Bleeding point originated from the presacral venous plexus and a sacral neural foramen where the basivertebral veins were injured. A: Continuous pressure over the bleeding site using a gauze nut at the tip of a long Kelly clamp; B: Venous branches surrounding the gauze nut could be identified, and were suture ligated one by one with 3-0 suture thread; C: Continuous pressure over the bleeding site using the epiploic appendices at the tip of a long Kelly clamp; D: Electrocautery applied through the epiploic appendices pressed with a long Kelly clamp over the bleeding vessel.

ing the gauze should be removed by suction. If possible, the specimen should be removed to achieve better exposure. Next, the surgeon should remove the packing gauze, slowly exposing the bleeding point. The bleeding type should been distinguished as soon as possible and an appropriate hemostatic technique can be deployed.

In the first type of massive presacral bleeding, the bleeding point originates from the presacral venous plexus. In our experience, appropriate suture ligation remains an effective method to control this type of bleeding. It should be performed by an experienced surgeon, maintaining continuous pressure over the bleeding site using a gauze nut at the tip of a long Kelly clamp. Surgeons should continue to mobilize the rectum to achieve better exposure if possible. In cases in which the venous branches surrounding the gauze nut can be identified, they are suture ligated one by one with suture thread (VCP772D; Ethicon). Importantly, the suture-ligated tissues should include the presacral fascia, presacral veins, and deep connective tissues. Suture ligation should be performed where the presacral fascia is intact. Jiang *et al*[16] have reported that circular suture ligation of the venous plexus in the area with intact presacral fascia that surrounds the bleeding site is an effective and simple technique to control presacral venous bleeding. In the present study, this type of bleeding was successfully controlled in two patients by the surrounding suture ligation technique (Figure 2A and B).

However, there are several limitations to the surrounding suture ligation technique. First, it can be difficult for bleeding occurring at the bottom of a narrow pelvis, which is typical in patients with obesity[16]. Second, previous rectal surgery can lead to fibrosis of the presacral area, which increases the difficulty in identification of presacral vein distribution and suture ligation. Assessment of the vein locations according to the typical pattern of vein distribution could be wrong, leading to failure of bleeding control. We performed surgery for recurrent rectal carcinoma in two cases. Surrounding suture ligation technique was ineffective because vessel distribution was difficult to identify. Lastly, for bleeding coming from a retracted vein inside the sacrum, other techniques may be used to control the massive bleeding.

In the second type of massive presacral bleeding, the bleeding point originates from a sacral neural foramen where the basivertebral veins are injured.

Harrison *et al*[17] have reported a technique of muscle fragment welding to control presacral bleeding during rectal mobilization. We performed this technique in two cases whose bleeding points originated from a sacral neural foramen during surgery for recurrent rectal carcinoma. This type of bleeding was effectively controlled using electrocautery applied through the epiploic appendices pressed with a long Kelly clamp over the bleeding vessel (Figure 2C and D). Compared with the technique of muscle fragment welding, it is easier to excise one epiploic appendix than a muscle fragment. Because of the round shape of the epiploic appendices, it is easier to fill

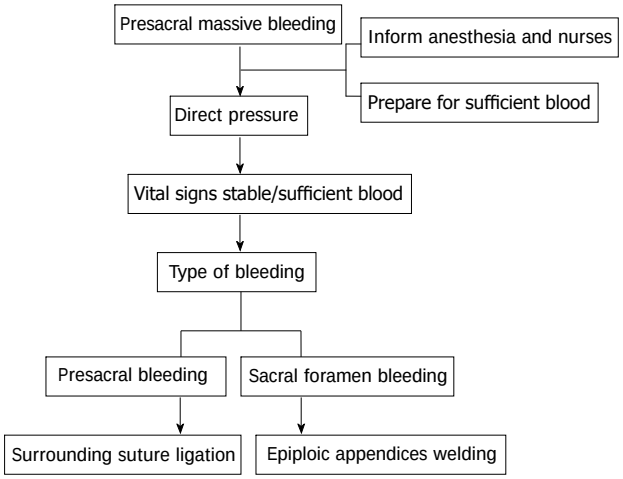

Figure 3 Process in the management of massive presacral bleeding.

the sacral neural foramen. The cauterized epiploic appendices usually adhere to the presacral tissue as a charred coagulum. Hemostasis was immediate and permanent, and no major complications were noted. This technique is intended to deliver heat energy through the forceps to the epiploic appendices. The epiploic appendices act primarily as a fluid-containing electrode that allows conduction of energy and heat to the basivertebral veins. The temperature increases gradually and coagulation is achieved. As in a previous study, necrosis and subsequent abscess development were not seen in our patients, and this may be related to the hypervascular nature of the presacral area and revascularization of the small segment of the epiploic appendices[18].

Alternative methods have been described in the literature. Pelvic packing effectively controls massive presacral bleeding. Intra-abdominal packing should be familiar to colorectal surgeons because when other attempts to provide hemostasis fail, it can be the last resort to control life-threatening bleeding[19]. Packing gauze must be carefully removed at a planned second laparotomy when the patient has stabilized hemodynamically. However, there is a risk of infection or secondary complication from foreign bodies.

Nowadays hemostatic agents are readily available. Some authors have reported that they are effective in stopping bleeding from presacral veins[20-30]. However, in our experience, they are ineffective in stopping massive presacral bleeding. Hemostatic agents may be considered in cases of little bleeding when other techniques have failed.

In conclusion, surrounding suture ligation and epiploic appendices welding are safe, readily available, and highly effective techniques for controlling massive presacral bleeding from the presacral venous plexus and sacral neural foramen, respectively (Figure 3).

COMMENTS

Background

Massive presacral bleeding is a potentially life-threatening complication of rectal surgery and remains one of the most challenging intraoperative emergencies. The incidence and mortality have been reported to be as high as 9.4% and 4.3%, respectively. Total mesorectal excision was introduced in 1982 and is considered a gold standard with an acceptable intraoperative risk during surgery for rectal carcinoma. However, massive presacral bleeding remains inevitable, especially in surgery for recurrent rectal carcinoma or during operations performed by junior colorectal surgeons.

Research frontiers

Several hemostatic techniques for controlling this intraoperative emergency have been proposed, such as the use of thumbtacks, bone wax, balloon tamponade, and endoscopic stapling. However, some techniques fail to arrest the bleeding, resulting in shock and even death.

Innovations and breakthroughs

Based on the anatomy of the presacral venous system, massive presacral bleeding can be divided into two different types. The key factor in controlling massive presacral bleeding is correct assessment of the bleeding type. This article reports two simple and effective techniques for controlling two different types of massive presacral bleeding, classified according to the anatomy of the presacral venous system. According to the authors, there are no reports of this hemostatic strategy in the literature.

Applications

Surrounding suture ligation and epiploic appendices welding are safe, readily available, and highly effective techniques for controlling massive presacral bleeding from the presacral venous plexus and sacral neural foramen, respectively.

Terminology

Presacral bleeding is considered to be an intraoperative emergency in rectal surgery. The anatomy of the presacral venous system makes it vulnerable to serious bleeding that can often be difficult to control.

Peer review

The literature review indicates a large body of work on presacral bleeding already. Often a greater number of techniques described equates to a lack of a gold standard of care, which can be problematic in whatever field. This article outlines two useful and effective techniques to deal with this severe, although not frequent, complication of rectal surgery. The results are interesting and suggest that surrounding suture ligation and epiploic appendices welding for controlling two different types of massive presacral bleeding are simple and effective techniques.

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BRIEF ARTICLE

Recurrent abdominal liposarcoma: Analysis of 19 cases and prognostic factors

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Abstract

AIM: To evaluate the clinical outcome of re-operation for recurrent abdominal liposarcoma following multidisciplinary team cooperation.

METHODS: Nineteen consecutive patients who had recurrent abdominal liposarcoma underwent re-operation by the retroperitoneal sarcoma team at our institution from May 2009 to January 2012. Patient demographic and clinical data were reviewed retrospectively. Multidisciplinary team discussions were held prior to treatment, and re-operation was deemed the best treatment. The categories of the extent of resection were as follows: gross total resection (GTR), palliative resection and partial resection. Surgical techniques were divided into discrete lesion resection and combined contiguous multivisceral resection (CMR). Tumor size was determined as the largest diameter of the specimen. Patients were followed up at approximately 3-monthly intervals. For survival analysis, a univariate analysis was performed using the Kaplan-Meier method, and a multivariate analysis was performed using the Cox proportional hazards model.

RESULTS: Nineteen patients with recurrent abdominal liposarcoma (RAL) underwent 32 re-operations at our institute. A total of 51 operations were reviewed with a total follow-up time ranging from 4 to 120 (47.4 ± 34.2) mo. The GTR rate in the CMR group was higher than that in the non-CMR group (P = 0.034). CMR was positively correlated with intra-operative bleeding (correlation coefficient = 0.514, P = 0.010). Six cases with severe postoperative complications were recorded. Patients with tumor sizes greater than 20 cm carried a significant risk of profuse intra-operative bleeding (P = 0.009). The ratio of a highly malignant subtype (de-differentiated or pleomorphic) in recurrent cases was higher compared to primary cases (P = 0.027). Both single-factor survival using the Kaplan-Meier model and multivariate analysis using the Cox proportional hazards model showed that overall survival was correlated with resection extent and pathological subtype (P < 0.001 and P = 0.02), however, relapse-free interval (RFI) was only correlated with resection extent (P = 0.002).

CONCLUSION: Close follow-up should be conducted in patients with RAL. Early re-operation for relapse is preferred and gross resection most likely prolongs the RFI.

Key words: Overall survival; Recurrent abdominal liposarcoma; Relapse-free interval

Core tip: Recurrent abdominal liposarcoma (RAL) is an intractable disease encountered by both general surgeons and surgical oncologists. RAL commonly affects multiple organs, and re-operation for RAL is often difficult and is associated with significant risk, even when debulking is imminent. The high likelihood of postoperative complications and a lower survival outcome are

detractors for repeat operations. A multidisciplinary team approach, realistic risk stratification, and careful management may help increase the success rate of gross total resection, lower these complication rates, improve survival, and increase the quality of life of these patients. Overall survival, relapse-free interval and other clinical follow-up data are also presented in detail in this study.

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INTRODUCTION

Liposarcoma is the most common retroperitoneal sarcoma[1,2]. It accounts for more than 20% of all sarcomas in adults and up to 41% of all retroperitoneal sarcomas[3,4]. Liposarcomas also originate from the mesentery, gastrointestinal wall, and even from solitary organs, which has been reported sporadically[4-11]. Complete surgical resection is the only effective treatment method for retroperitoneal liposarcomas[3,12,13].

However, liposarcomas are associated with a high local recurrence rate[14-16]. Re-operation is the only effective treatment for recurrent abdominal liposarcoma (RAL)[17]. For those who are not amenable to complete radical resection, debulking resection should be performed to relieve symptoms, reduce complications, and increase the life span[18]. However, there is no consensus concerning the utility of repeat debulking resections. RAL commonly affects multiple organs, and re-operation for RAL is often difficult and is associated with significant risk, even when debulking is imminent. The high likelihood of postoperative complications and a lower survival outcome are detractors for repeat operations.

A multidisciplinary team approach, realistic risk stratification, and careful management may help lower these complication rates, improve survival, and increase the quality of life of these patients. We have treated 19 RAL patients over the past 3 years using a multidisciplinary team approach. The clinical and follow-up data of these patients were retrospectively analyzed and summarized.

MATERIALS AND METHODS

Patient enrollment and operation selection

Between May 2009 and Jan 2012, 19 consecutive patients with RAL were treated by the retroperitoneal sarcoma team at our institution. Patients were identified by reviewing a database that accrued data prospectively. Histology was reviewed and classified according to the World Health Organization classification[19,20]. The multidisciplinary team were involved in case discussions which were held prior to treatment, and repeat resection was deemed the best treatment. The multidisciplinary team members included general surgeons, a pathologist, radiologist, oncologist, radiologist, urologist and gynecologist. Multivisceral resection was recommended only in cases of expected gross tumor resection. The operative plan was explained to the patient in detail, and informed consent was obtained before surgery.

Extent of resection

The categories of the extent of resection were as follows: gross total resection (GTR), whether the margin was histologically free or not; palliative resection; and partial resection. Palliative resections were performed when the gross disease could not be completely removed and less than a 1 cm rim of tumor remained. Partial resections were defined as visually more than a 1 cm rim of remaining tumor. Surgical techniques were divided into discrete lesion resection (DLR) and combined contiguous multivisceral resection (CMR). Tumor size was determined as the largest diameter of the specimen.

Clinical data

Patients' demographic and clinical data were reviewed retrospectively and included age, gender, disease onset date, combined resected organ, pathology subtype, tumor size, intra-operative bleeding, post-operative complications, disease relapse date and survival time in order to analyze prognostic factors.

Follow up

Patients were followed-up at approximately 3-mo intervals. The relapse-free interval (RFI) was defined as the time between initial surgery and confirmation of clinical recurrence.

Statistical analysis

The median and standard error were used to present continuous variables. Fisher's test or a crosstab analysis was performed to compare variables between groups. For survival analysis, a univariate analysis was performed using the Kaplan-Meier method, and a multivariate analysis was performed using the Cox proportional hazards model. $P < 0.05$ was considered statistically significant.

RESULTS

Patient clinical characteristics

Nineteen patients with RAL underwent 32 re-operations at our institute. The patient demographic, surgical, and pathological data are summarized in Table 1. A total of 51 operations were reviewed. The recurrences were tracked from Mar 2002 to Aug 2011, with a total follow-up time ranging from 4 to 120 (47.4 ± 34.2) mo.

Surgical treatment

The surgical methods and resection extent are summarized in Table 2. Five of the nineteen patients underwent the primary operation at our institute. The resected or-

Table 1 Patient demographics and clinical data *n* (%)

Variables	Mean/median
Age (yr)	
mean ± SD	55 ± 10.8
Median (range)	58 (34-84)
Gender	
Male	12 (63.2)
Female	7 (36.8)
No. of operations	
Two	11 (57.9)
Three	4 (21.1)
Four	3 (15.9)
Five	1 (5.3)
Follow-up time (mo)	
mean ± SD	48.9 ± 34.8
Range	4-120
Primary tumor location	
Retroperitoneum	13 (68.4)
Mesentery	3 (15.8)
Omentum	1 (5.3)
Small intestine	1 (5.3)

Table 2 Surgical methods and resection extent of primary and recurrent liposarcomas *n* (%)

Variables	DLR	CMR	Total
Primary tumor			
GTR	11 (57.89)	4 (21.05)	15 (78.94)
Palliative resection	3 (15.79)	0 (0.00)	3 (15.79)
Partial resection	1 (5.26)	0 (0.00)	1 (5.26)
Total	15 (78.95)	4 (21.05)	19 (100.00)
Recurrent tumor			
GTR	5 (15.63)	15 (46.88)	20 (62.50)
Palliative resection	2 (6.25)	6 (18.75)	8 (25.00)
Partial resection	3 (9.38)	1 (3.13)	4 (12.50)
Total	10 (31.25)	22 (68.75)	32 (100.00)

DLR: Discrete lesion resection; CMR: Contiguous multivisceral resection; GTR: Gross total resection.

gans included the small intestine (n = 14), colon (n = 11), kidney (n = 8), spleen (n = 7), pancreas (n = 5), stomach, appendix, ovary (n = 3 each), and liver, bladder, testicle, and abdominal wall (n = 1 each). The GTR rate in the CMR group was higher than that in the non-CMR group (P = 0.034). Only one CMR case underwent partial resection. This patient had a spontaneous enterobrosis and therefore required an emergency operation. He lived for 3 mo after this salvage treatment. The median intra-operative blood loss was 500 mL. Thirteen cases had bleeding ranging from 500-4000 (1300 ± 1100) mL; bleeding in 12 of these 13 cases occurred during CMR. CMR was positively correlated with intra-operative bleeding (correlation coefficient = 0.514, P = 0.010). Six cases with severe postoperative complications were recorded. Two cases experienced anastomotic leakage, and the other four experienced either pleural effusion, subdiaphragmatic effusion, abdominal abscess, or an abdominal wall wound infection.

Pathology data

The primary tumor size was recorded in nine patients, including one patient with multiple lesions; the other eight tumors ranged in size from 13-38 (22.6 ± 9.9) cm. A total of 24 relapse cases were observed who had measurable specimens with tumor sizes ranging from 4-46 (27.2 ± 14.5) cm, and 8 cases had multiple lesions. The median size was 20 cm for all specimens. The resection extent, surgical approach, and operative blood loss were compared according to tumor size. The relapse cases were subgrouped by median tumor size when comparing the clinical data with the number of cases. Patients with tumor sizes greater than 20 cm carried a significant risk of profuse intra-operative bleeding (P = 0.009), as detailed in Table 3.

Table 3 Comparison of clinical data according to recurrent tumor size

Tumor size	< 20 cm	> 20 cm	Total
GTR	4	10	14
Palliative resection	3	5	8
Partial resection	2	0	2
DLR	4	2	6
CMR	6	12	18
Bleeding (< 500 mL)	4	6	10
Profuse bleeding (≥ 500 mL)[1]	1	13	14

[1]P = 0.009 between different recurrent tumor size group. DLR: Discrete lesion resection; CMR: Contiguous multivisceral resection; GTR: Gross total resection.

The pathological subtypes were significantly different between recurrent and primary tumors. The subtype frequently changed with each recurrence within the same patient. In this series, well-differentiated and myxoid liposarcomas were more commonly found within the primary tumor; however, dedifferentiated liposarcomas were more common in recurrent tumors. The ratio of a highly malignant subtype (dedifferentiated or pleomorphic) in the recurrent cases was higher compared to the primary cases (5/9 *vs* 23/9, P = 0.027).

Follow-up and survival analysis

Survival was tracked during the follow-up period. Six patients died of their disease after an overall survival (OS) of 8-90 (33.7 ± 29.7) mo. Single-factor survival was analyzed according to surgical method, resection extent, tumor location, tumor size, and pathological subtype of the primary disease. Patients with a GTR of the primary tumor had a longer survival than those with a palliative or partial resection (P = 0.001, Figure 1A). Patients who underwent a CMR at first operation had a slightly longer survival (P = 0.081, Figure 1B). Patients with a primary retroperitoneal liposarcoma had a worse survival than liposarcoma at any other site (mesentery, omentum and small intestine, P = 0.054, Figure 1C). Patients with a less malignant subtype of primary liposarcoma (well differentiated and myxoid cell type) tended to live longer than those with a more highly malignant subtype (dedifferentiated and pleomorphic cell type, P = 0.002, Figure 1D). Multivariate analysis using the Cox proportional hazards model showed that OS correlated with resection extent and pathological subtype (P < 0.001 and P = 0.02).

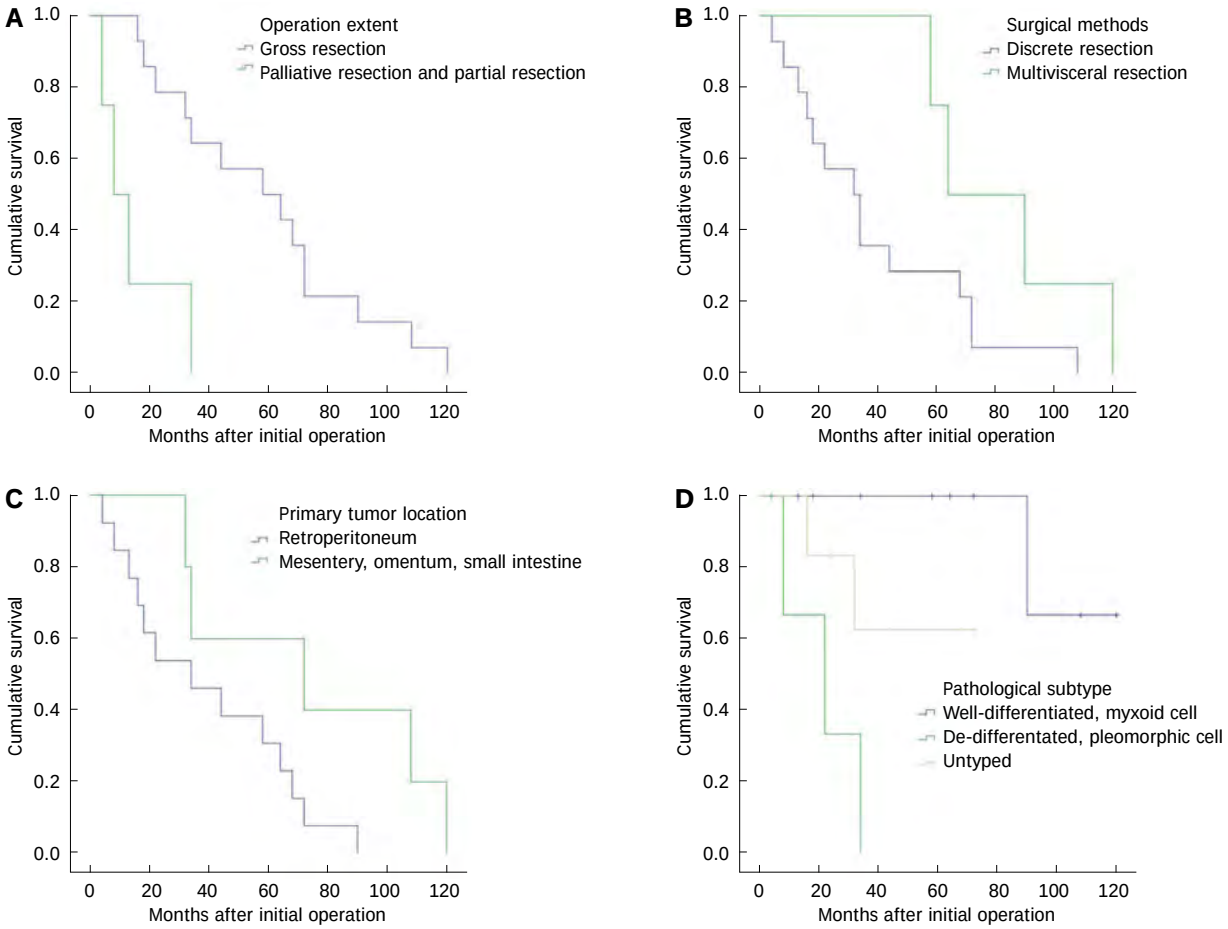

Figure 1 Relationship between overall survival and operation extent (A), surgical methods (B), tumor origin (C), and pathological subtype (D) in patients who underwent resection of a primary abdominal liposarcoma.

The RFI of the primary surgical treatment ranged from 2-84 (22.0 ± 21.2) mo. The RFI differed between GTR patients (6-84/27.0 ± 21.2 mo) and patients who underwent partial or palliative resections (2-4/3.3 ± 1.0 mo, $P = 0.001$). Eighteen recurrences were observed after a gross or palliative resection for recurrent tumor, and the RFI was 1-28 (8.3 ± 7.4) mo. Of these, 11 were post-GTR (RFI = 4-28/12.5 ± 7.4 mo) and seven were post-palliative resection (RFI = 3-6/4 ± 1.3 mo). Eight post-GTR cases had a follow-up of 3-30 (10.3 ± 10.1) mo with no relapse. Patients who underwent GTR had a longer RFI than those who underwent palliative resection ($P = 0.01$).

The RFI was compared according to the revision operation time, surgical method, resection extent, primary tumor location, tumor size, simultaneous tumor number, and pathological subtype. The RFI was shorter in patients who underwent more than 2 operations ($P = 0.035$, Figure 2A). No significant differences in RFI were found between CMR and DLR ($P = 0.599$, Figure 2B). However, there was a significant difference between GTR cases and non-GTR cases ($P < 0.001$, Figure 2C). Patients with well-differentiated liposarcomas had a longer RFI compared to those with other liposarcoma subtypes ($P = 0.007$, Figure 2D). When grouped by median tumor size (20 cm) or simultaneous tumor number (solitary or multiple), no significant difference was observed ($P = 0.54$, Figure 2E and $P = 0.33$, Figure 2F). A multivariate analysis using a Cox proportional hazards model showed that the RFI only correlated with resection extent ($P = 0.002$).

DISCUSSION

Liposarcoma is the most common mesenchymal tumor in the abdomen. To date, surgical resection is the only effective treatment for liposarcoma. Unfortunately, these tumors are almost always very large at the time of diagnosis due to their slow growth and often vague symptoms[11], which make GTR difficult. These tumors are known for their frequent local recurrence and expansive growth with contiguous organ infiltration, which are the main causes of death from this disease. There is no strong evidence that chemotherapy or radiotherapy is curative[21,22]. Re-operation is still the mainstay of treatment, but is associated with significant risk. Using a multidisciplinary team approach, the surgical management of RAL has been improved at our institute. Very few studies have focused on the re-operative treatment of RAL.

In this series, we reviewed 19 patients with RAL who

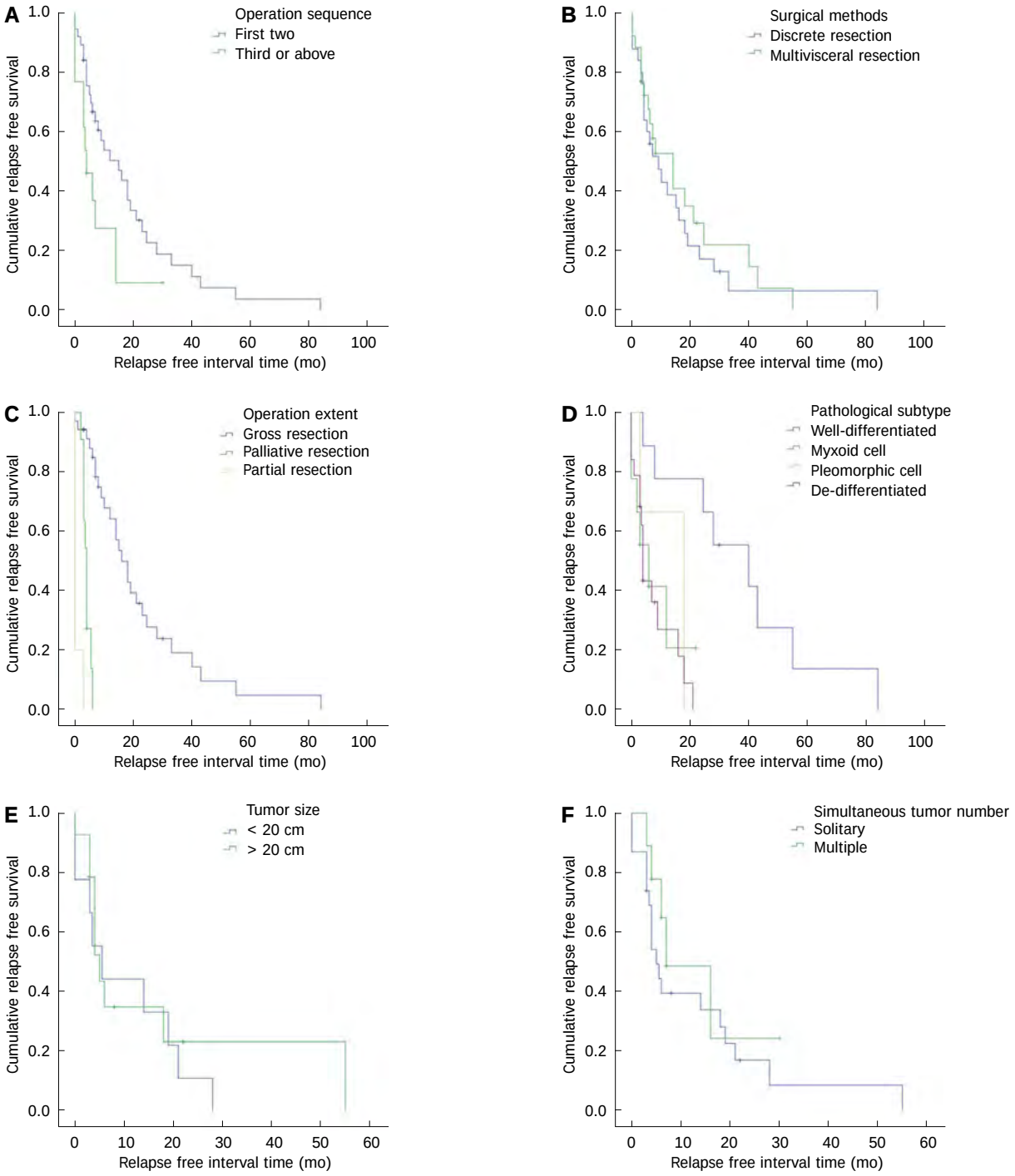

Figure 2 Relationship between the relapse-free interval and operation sequence (A), surgical method (B), operation extent (C), pathological subtype (D), tumor size (E), and simultaneous tumor number (F) in patients who underwent resection of an abdominal liposarcoma.

underwent 32 re-operations. All 19 patients had a successful re-operation with no intra-operative mortalities. However, the surgical treatment of RAL was associated with intra-operative bleeding and postoperative complications. These were most notable in cases where CMR was anticipated. The most common postoperative complications were anastomotic leak and effusion/infection.

There is no consensus in the literature regarding the guiding principles of surgical treatment for RAL. A large series of 177 primary retroperitoneal liposarcoma patients demonstrated that the pathological subtype on gross resection was the most significant prognostic factor[16]. In our multi-disciplinary team, the benefits and risks of re-operation were evaluated, and plans were formulated for all the RALs we encountered. GTR is the preferred approach for patients with RAL when CMR is necessary. If there was no possibility of gross resection, palliative resection was performed without multivisceral resection. Partial resections for RAL should only be performed in patients with intolerable symptoms (*e.g.*, extreme increas-

ing intra-abdominal pressure, grave complications, and in some emergency conditions). CMR should be avoided in patients who have undergone partial resection because this does not result in cure and incurs greater morbidity. One partial resection included an enterectomy due to spontaneous perforation caused by the RAL.

In this study, 75% (15/20) of patients who underwent GTR involved CMR. There is no similar study from our institute or similar data in the literature. It is unknown whether CMRs increase the GTR rate for RAL. However, the GTR rate was higher in CMR cases than in non-CMR cases for RALs. In operations for the primary tumor, there were more non-combined resections in GTR patients (57.9% *vs* 21.1%). The tumor size was 4-46 (median 20) cm, which is similar to that in another retrospective study of 21 cases of primary retroperitoneal liposarcoma[25]. The most frequently combined resected organ was the small intestine, which is in contrast to another study reporting the kidney[24]. In our study, the small intestine was associated with a risk of anastomotic leak. Tumor size was also correlated with intra-operative profuse bleeding (> 20 cm, $P = 0.009$). Additionally, a pathologic subtype change was observed in the RALs compared to the primary tumors or previous relapsed tumors. A pathologic subtype change predicted deterioration in repeat relapse cases[25]. Dedifferentiated liposarcomas were more commonly found as recurrent tumors[14,26-28].

There have been no studies that have focused on recurrent abdominal liposarcomas or retroperitoneal liposarcomas. Most reported studies are single cases or include less than 3 cases in a report. However, several studies have described primary and recurrent retroperitoneal liposarcoma, with more than 10 cases reported since 1991[23,25,29-32], but no primary mesentery or omental liposarcomas have been described. In our study, patients with primary retroperitoneal liposarcoma had a poorer survival, however, this was not statistically significant ($P = 0.054$). It is generally recognized that complete or gross total resection at the initial operation is very important, resulting in a more favorable prognosis[33]. In our study, patients who underwent gross resection of the primary tumor had a longer survival than those who underwent a palliative or partial resection. CMR for retroperitoneal sarcoma was recommended for the initial operation in a study of 77 patients due to an infiltrative tumor pattern[34]. Dedifferentiated tumors tend to present more often as a recurrence[35,36], frequently require multi-organ resection, and carry a shorter disease-free interval when compared to well-differentiated subtypes[25]; a similar result was observed for well-differentiated tumors in this study. OS was correlated with the resection extent and pathological subtype ($P < 0.001$ and $P = 0.02$). CMRs may increase the chance of complete resection.

Macroscopic complete resection for recurrent retroperitoneal liposarcoma has been recommended[37]. It is believed that palliative resection is worthwhile for treating the troublesome symptoms of recurrence in patients who have little chance of gross resection[32]. Repeat operations were performed in our study, and the RFI was shorter in patients who underwent more than two operations. GTR was a significant prognostic factor for the RFI ($P < 0.001$). Tumor subtype in a well-differentiated liposarcoma resulted in a significantly longer RFI compared to other types, according to the Kaplan-Meier analysis. The surgical extent was the only significant prognostic factor, as demonstrated by the Cox regression model. This showed that GTR was the major factor affecting the relapse time regardless of whether the tumor was a primary or recurrent tumor. Our results show that surgical management is the key factor in the successful treatment of abdominal liposarcoma. Multidisciplinary team cooperation has the advantage of a well-designed surgical management plan. Whether tumor size affects OS in addition to the relapse-free interval is controversial. Some authors have reported that large tumor size is negatively associated with prognosis[23,29] as large tumors require more difficult operations. However, other reports have shown no obvious difference in OS or relapse-free interval according to tumor size[25,37]. Tumor size did not affect the RFI in our study. However, it was one of the factors associated with the GTR rate, which indirectly affected OS. Multidisciplinary team approaches and multivisceral resections used in the surgical management of these cases reduced the risk of tumor residue when operating on larger abdominal liposarcomas.

Most abdominal liposarcomas are asymptomatic in the early stages. As the tumor grows patients may experience abdominal distention or other symptoms related to the tumor compressing contiguous organs, vessels, or even the ureter. Some tumors were large when the patients presented to the hospital, and it was difficult to completely resect these tumors at the time of surgery. The abdominal liposarcomas were often recurrent, particularly those with a highly malignant subtype. It is important that such patients have appropriate follow-up. However, to date, follow-up has not been standardized. The relapse time after the initial operation has been reported to vary due to the surgical extent and pathologic subtype. Postoperative adjuvant chemotherapy or radiotherapy was also not recommended as there is little evidence of benefit[38,39]. Proactive re-operation for RAL is strongly recommended. In such cases, close follow-up is necessary to identify relapse early.

RAL is a difficult disease to treat. The surgical treatment of RALs can be particularly challenging for surgical oncologists. GTR is the most important positive prognostic factor for these patients, and proactive surgical treatment is recommended. A multidisciplinary team approach most likely increases the chance of GTR, and CMR is frequently required to achieve gross tumor clearance. Palliative or partial resections are indicated in patients with recurrent disease and insufferable symptoms.

COMMENTS

Background

Abdominal liposarcomas are associated with a high local recurrence rate. Re-operation is the only effective treatment for recurrent abdominal liposarcoma

(RAL). For those who are not amenable to complete radical resection, debulking resection may relieve symptoms, reduce complications, and increase the life span. However, RAL commonly affects multiple organs, and re-operation for RAL is often difficult and is associated with significant risk, even when debulking is imminent, There is no consensus concerning the utility of repeat debulking resections. The high likelihood of post-operative complications and a lower survival outcome are detractors for repeat surgery.

Research frontiers

Re-operation is widely accepted as the treatment for recurrent abdominal liposarcoma. However, repeat re-operation for RAL is associated with high risk and a high complication rate. There are no recommended general criteria regarding when or how the re-operation should be performed. A multidisciplinary team approach, realistic risk stratification, and careful management may help lower the complication rate and improve survival.

Innovations and breakthroughs

Recurrent abdominal liposarcoma is an intractable disease encountered by general surgeons or surgical oncologists. It is generally believed that chemotherapy or radiotherapy provide minor help for patients with abdominal liposarcoma. Macroscopic complete resection or gross total resection is still the only treatment that correlates with overall survival or disease-free survival. However, recurrent lesions involve several adjacent organs in most cases. Multiple contiguous organ resections should be carried out under such conditions, however, this is associated with significant risks of failing to resect the lesion completely, multiple complications and even intra- or post-operative death. With the advantage of a multidisciplinary approach, the surgical oncologist can prepare for the treatment of this difficult disease, enhance the successful rate of gross resection and lower the morbidity and mortality related to the operation. This preliminary study summarized the outcome of multidisciplinary team cooperation in the treatment of abdominal liposarcoma which can be subsequently improved.

Applications

The study results suggest that repeat re-operation for recurrent abdominal liposarcoma with multidisciplinary team cooperation may help lower the complication rates, improve survival, and increase the quality of life of these patients.

Terminology

Recurrent abdominal liposarcoma: Recurrent abdominal liposarcoma is a disease where the liposarcoma relapses mainly in the peritoneal cavity, whether the liposarcoma originated from the retroperitoneal area or another region. Gross total resection: is the same as macroscopic complete resection, and means that the tumor is totally resected whether the pathological margin is negative or positive.

Peer review

This is a good retrospective study in which authors analyze the clinical outcome of repeated re-operation on recurrent abdominal liposarcoma. The results are interesting and suggest that repeated re-operation on recurrent abdominal liposarcoma under multidisciplinary team cooperation gain satisfactory clinical outcome.

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BRIEF ARTICLE

Probiotics improve survival of septic rats by suppressing conditioned pathogens in ascites

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Abstract

AIM: To investigate the benefits of probiotics treatment in septic rats.

METHODS: The septic rats were induced by cecal ligation and puncture. The animals of control, septic model and probiotics treated groups were treated with vehicle and mixed probiotics, respectively. The mixture of probiotics included *Bifidobacterium longum*, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. We observed the survival of septic rats using different amounts of mixed probiotics. We also detected the bacterial population in ascites and blood of experimental sepsis using cultivation and real-time polymerase chain reaction. The severity of mucosal inflammation in colonic tissues was determined.

RESULTS: Probiotics treatment improved survival of the rats significantly and this effect was dose dependent. The survival rate was 30% for vehicle-treated septic model group. However, 1 and 1/4 doses of probiotics treatment increased survival rate significantly compared with septic model group (80% and 55% *vs* 30%, $P < 0.05$). The total viable counts of bacteria in ascites decreased significantly in probiotics treated group compared with septic model group (5.20 ± 0.57 *vs* 9.81 ± 0.67, $P < 0.05$). The total positive rate of hemoculture decreased significantly in probiotics treated group compared with septic model group (33.3% *vs* 100.0%, $P < 0.05$). The population of *Escherichia coli* and *Staphylococcus aureus* in ascites of probiotics treated group were decreased significantly compared with that of septic model group (3.93 ± 0.73 *vs* 8.80 ± 0.83, $P < 0.05$; 2.80 ± 1.04 *vs* 5.39 ± 1.21, $P < 0.05$). With probiotics treatment, there was a decrease in the scores of inflammatory cell infiltration into the intestinal mucosa in septic animals (1.50 ± 0.25 *vs* 2.88 ± 0.14, $P < 0.01$).

CONCLUSION: *Escherichia coli* and *Staphylococcus aureus* may be primary pathogens in septic rats. Probiotics improve survival of septic rats by suppressing these conditioned pathogens.

Key words: Sepsis; Probiotics; Pathogens; *Escherichia coli*; *Staphylococcus aureus*

Core tip: We observed the survival of septic rats treated with different amounts of mixed probiotics. The data indicated that conditioned pathogens such as *Escherichia coli* and *Staphylococcus aureus* may be primary pathogens of septic rats in our study. Probiotics improve the survival of septic rats by suppressing the conditioned pathogens.

Liu DQ, Gao QY, Liu HB, Li DH, Wu SW. Probiotics improve survival of septic rats by suppressing conditioned pathogens in ascites. *World J Gastroenterol* 2013; 19(25): 4053-4059 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i25/4053.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i25.4053

INTRODUCTION

Sepsis is the systemic inflammatory response to infection and one of the most common causes of death in critically-illed patients[1]. Each year, more than 750000 clinical cases of death occur due to sepsis, and the mortalities from severe sepsis were 20%-30% in the period of 1979-2000 in the United States[2,3]. Microbial infection initiates and promotes systemic inflammatory responses by increasing cytokines release and neutrophils recruitment in target organs and inducing systemic inflammatory response syndrome and multiple organ dysfunction syndrome[4]. It has been demonstrated that intestinal microbes play an important role in sepsis[5]. Cecum is a pouch of large intestines connecting the terminal ileum to the ascending colon and home to a large number of anaerobic and aerobic microbes[6]. Cecal ligation and puncture (CLP) of rats produce cecal ischemia and polymicrobial infection[7]. The bacteria of colonic contents will spill into the abdomen, and produce severe peritonitis and bacteremia[8]. So the CLP has been used as a classic animal model of sepsis[9-11].

There is a complex microbial population in intestinal tract, some of which are probiotics. When administered in adequate amounts, probiotics confer a health benefit to the host[12]. The products of probiotics include mucin, organic acids, branched chain fatty acids, H_2, CO_2, ammonia, amines and vitamins. These products regulate host health through different pathways such as regulating energy, gene expression and cell differentiation, producing anti-inflammatory agents and keeping gut homeostasis[13,14]. The probiotics include *Bifidobacteria, Lactobacilli, Enterococci, Streptococci, Propionibacteria, Bacillus,* and yeasts. A variety of species of probiotics have been shown to benefit human gastrointestinal health[15-17]. However, the mechanisms of probiotics in improving survival in sepsis are unclear. In this study, we sought to address this question in a septic model of Wistar rats.

MATERIALS AND METHODS

Animal experiments

Male Wistar rats (8-10 wk old, Animal Center of Academy of Malitary Medical Sciences, China) were housed on a 12:12 h light-dark cycle under pathogen-free conditions with free access to food and water. We performed CLP, a clinically relevant animal model for human sepsis[18,19]. The animals were anesthetized by 10% chloral hydrate (3 mL/kg *via* intraperitoneal injection). After a midline incision was made in the abdomen, we isolated the cecum gently and placed a ligature 2.0 cm from the cecal tip using 2-0 silk suture. Ligated cecal stump was punctured by a 12-gauge needle. Colonic contents were extruded into abdominal cavity. We put back the cecum into its normal position and closed the abdomen by suturing muscle and skin, respectively. For control animals, the cecum was isolated without ligating and puncturing. The probiotic mixture consisted of three different viable strains. One dose of the probiotic mixture contained 1×10^7 CFU *Bifidobacterium longum* (ATCC 15697), 1×10^6 CFU *Lactobacillus bulgaricus* (ATCC 11842) and 1×10^6 CFU *Streptococcus thermophilus* (ATCC 19987). Before administration, the probiotic mixture was reconstituted in sterile water for 10 min at 37 ℃. We gave probiotics to animals of treated groups through intragastric administration. The animals of control and septic model groups were treated with vehicle (sterile water). The first administration of probiotics or vehicle was started 6 h after surgery. Thereafter, it was administered once a day for 3 d.

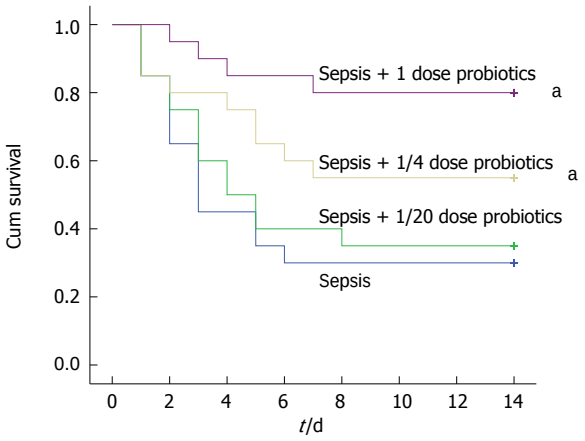

Figure 1 Survival in experimental sepsis. Survival was analysed in Wistar rats subjected to cecal ligation and puncture. Probiotics (1, 1/4 or 1/20 doses) or vehicle treatment started 6 h later and thereafter administered once a day for 3 d. All animals were observed for two weeks to compare their survival rates (n = 20; $^aP < 0.05$ *vs* septic model group).

Samples collection

All surviving animals were anaesthetised by 10% chloral hydrate (3 mL/kg, *via* intraperitoneal injection) after a 72 h period of CLP. Samples of blood and ascites were harvested for both anaerobic and aerobic microbial analysis immediately. Another portion of ascites was stored at -80 ℃ for DNA extraction. Then rats were killed by cervical dislocation, and colonic tissues were collected in neutral buffered formalin for histological analysis.

Microbial analysis of blood and ascites

Serial 10-fold dilutions were made in 0.9% sterile saline. We spread 20 μL of 10^0-10^{-7} dilutions on the nonselective blood-agar (Jinzhang Co, Ltd., Tianjin, China) surface. For anaerobic incubation, the anaerobic blood-agar dishes (Jinzhang Co, Ltd., Tianjin, China) were placed in anaerobic bags (bioMérieux, France) immediately. The time of aerobic incubation was shorter (24 h) than anaerobic (48 h) at 37 ℃. The colonies were determined in appropriate dilution, and total viable counts of original samples were calculated. Different colonies were separated and isolated for 2-3 times. We identified bacterial species using colony morphology and Gram's stain. Microstation microbe analysis system (Biolog, Winooski, VT, United States) was used for advanced identification.

Table 1 Comparison of bacterial spectrum and total viable count in ascites between septic model group and probiotics treated group

Group	Bacterial spectrums in ascites	Total viable counts (Log_{10} cells/mL ascites)
Septic model group	*Escherichia coli, Enterococcus faecalis, Staphylococcus aureus, Enterococcus avium, Streptococcus viridans, Streptococcus agalactiae, Micrococcus luteus, Enterococcus gallinarum, Enterococcus durans, Enterococcus malodoratus, Streptococcus ferus, Morganella morganii ss morganii, Acinetobacter radioresistens, Streptococcus criceti, Lactobacillus reuteri, Veillonella criceti\ratti, Desulfovibrio fructosivorans, Clostridium oroticum, Lactobacillus bifermentans*	9.81 ± 0.67
Probiotics treated group	*Escherichia coli, Enterococcus faecalis, Staphylococcus aureus, Enterococcus malodoratus, Morganella morganii ss morganii, Enterococcus durans, Streptococcus viridans, Prevotella dentioola, Desulfovibrio fructosivorcms, Bacterorides ovatus, Prevotella nigrescens*	5.20 ± 0.57[a]

Probiotics (1 dose) or vehicle treatment started 6 h later and thereafter administered once a day for 3 d. Samples of ascites were harvested for both anaerobic and aerobic culture. The bacterial spectrum of ascites was lower in probiotics treated group than in septic model group. The total viable counts of bacteria in ascites decreased significantly in probiotics treated group compared with septic model group (n = 18; [a]P < 0.05 *vs* septic model group).

Quantitative real-time polymerase chain reaction

Bacterial genomic DNA was extracted from ascites of rats using the QIAamp DNA mini kit (Qiagen, Hilden, United States) according to the manufacturer's protocol. We obtained 16S rRNA sequences of bacteria from the Ribosomal Database: (http://rdp.cme.msu.edu/), and designed primers for the specific bacterial strain using Primer 5.0 software package. The genomic DNA was used as template for the amplification of specimen and control standard bacterial strain through real-time polymerase chain reaction (PCR). PCR cycles were as follows: initial denaturation at 94 ℃ for 4 min, followed by 40 cycles of 94 ℃ for 30 s, 55 ℃ for 30 s, 72 ℃ for 40 s. PCR primers were: *Escherichia coli* forward: 5'-CATGCCGCGTGTATGAAGAA-3' and reverse: 5'-CGGGTAACGTCAATGAGCAAA-3'; *Enterococcus faecalis* forward: 5'-CAGCAGTAGGGAATCTTCGGCAATG-3' and reverse: 5'-AGCCTCAGCGTCAGTTACAGACCAG 3'; *Staphylococcus aureus* forward: 5'-CGTCAGCTCGTGTCGTGAGATGTTG-3' and reverse: 5'-GCGGTTTCGCTACCCTTTGTATTGT-3'. The real-time PCR was performed using FastStart SYBR Green Master (Roche, Basel, Switzerland) and IQ5 PCR system (BIO-RAD, Hercules, CA, United States).

Table 2 Comparison of bacterial spectrum and total positive rate of hemoculture between septic model group and probiotics treated group

Group	Bacterial spectrums of hemoculture	Total positive rate of hemoculture
Septic model group	*Escherichia coli, Staphylococcus aureus, Curtobacterium pusillum, CDC group II-E subgroup A*	100%
Probiotics treated group	*Escherichia coli, Staphylococcus aureus*	33.3%[a]

Probiotics (1 dose) or vehicle treatment started 6 h later and thereafter administered once a day for 3 d. Samples of blood were harvested for both anaerobic and aerobic culture. The bacterial spectrum of hemoculture was lower in probiotics treated group than in septic model group. The total positive rate of hemoculture decreased significantly in probiotics treated group compared with septic model group (n = 18; [a]P < 0.05 *vs* septic model group).

Histological examination of intestinal inflammation

The colonic tissues of at least four rats in each group were fixed in neutral buffered formalin, and processed for histological analysis. The sections of colonic tissues were stained by haematoxylin-eosin. Colonic sections were assessed for the severity of mucosal inflammation based on the following: infiltration of neutrophils and mononuclear cells into the intestinal mucosa (0, scant to normal; 1, minimal to mild; 2, mild to moderate; 3, moderate to severe; 4, severe inflammation)[20,21], and four fields of each sample were assessed. Moreover, epithelial thickness was measured under microscope (Leica, Frankfurt, Germany).

Statistical analysis

Data were expressed as the mean ± SD. All statistical analyses were performed using SPSS 17.0 software package. Survival analysis was shown in Kaplan-Meier survival curves. Survival comparisons between two subgroups were performed by the log-rank test. Differences between two groups were analysed using unpaired t test for continuous variables and the χ^2 test for nominal variables. A P value of less than 0.05 was considered statistically significant.

RESULTS

Probiotics improve the survival of rats with experimental sepsis

One hundred male Wistar rats were divided into five groups (control group, septic model group and three sepsis plus treatment groups) for survival analysis. We gave probiotic mixture (1, 1/4 or 1/20 doses) to animals in three treated groups by intragastric administration (once a day for 3 d). The animals of control and septic model

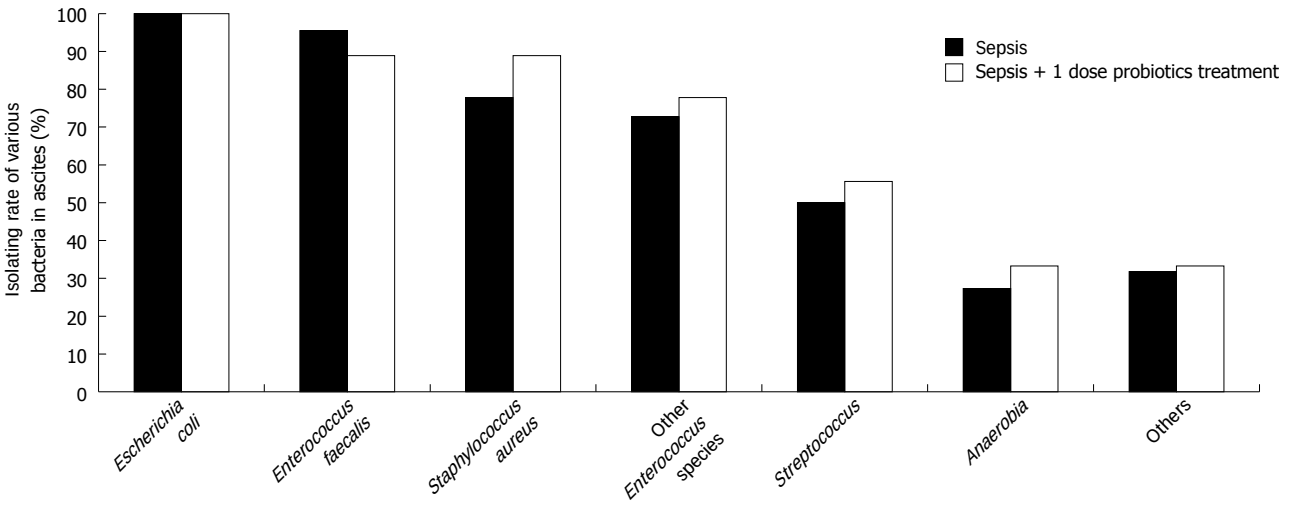

Figure 2 Comparison of isolating rate of various bacteria in ascites between sepsis and probiotics treated group. Probiotics (1 dose) or vehicle treatment started 6 h later and thereafter administered once a day for 3 d. Samples of ascites were harvested for both anaerobic and aerobic culture. There was no statistical significance in isolating rates between two groups for all bacterial species ($P > 0.05$). In addition, "other *Entertrococcus* species" include *Enterococcus avium, Enterococcus gallinarum, Enterococcus durans* and *Enterococcus malodoratus*. "Anaerobia" include *Lactobacillus reuteri, Veillonella cricetiratti, Desulfovibrio fructosivorans, Clostridium oroticum, Lactobacillus bifermentans, Prevotella dentioola, Bacterorides ovatus* and *Prevotella nigrescens*. "Others" include *Micrococcus luteus, Morganella morganii ss morganii* and *Acinetobacter radioresistens*.

groups were treated with vehicle only. We observed all animals for two weeks. The animals in control group survived normally. The majority of rats who had CLP showed clear signs of sepsis such as piloerection, lethargy, malaise and forming ascites. Probiotics attenuated the clinical manifestations of sepsis. Probiotics treatment also improved survival significantly and this effect was dose dependent. The survival rate was the lowest (30%, 6/20 rats) in the vehicle-treated septic model group. There was no protective effect using 1/20 dose probiotics (survival rate was 35%, 7/20 rats). However, 1 and 1/4 doses of probiotics treatment increased survival rate significantly (80%, 16/20 rats and 55%, 11/20 rats) compared with vehicle treated septic model group ($P < 0.05$) (Figure 1).

Probiotics inhibit bacteria in blood and ascites of rat experimental sepsis

The consequence of survival analysis indicated that 1 dose probiotics treatment was more effective than other doses. Therefore, we divided 80 male Wistar rats into three groups (control group, 8 rats; septic model group, 50 rats; and sepsis plus 1 dose probiotics treated group, 22 rats). Probiotics or vehicle were given to the animals through intragastric administration (once a day for 3 d), respectively. All animals (8 rats) in the control group survived normally. Forty-four percent of animals (22 rats) were alive in septic model group, and 81.8% of animals (18 rats) were alive in probiotics treated group. We harvested samples after a 72 h period of CLP. The microbial composition of blood and ascites were analysed. No bacteria were determined in blood and ascites of control group. The bacterial spectrum of ascites (Table 1) and blood (Table 2) was lower in probiotics treated group than in septic model group. There was no statistical significance in isolating rates between two groups for all bacterial species ($P > 0.05$, Figure 2). However, the total viable counts of bacteria in ascites decreased significantly in probiotics treated group compared with septic model group ($P < 0.05$, Table 1). Similarly, the total positive rate of hemoculture decreased significantly in probiotics treated group compared with septic model group ($P < 0.05$, Table 2).

The consequence of bacterial cultivation indicated that *Escherichia coli, Enterococcus faecalis* and *Staphylococcus aureus* were predominant microbial population in ascites of sepsis. For this reason, we detected the population of these bacteria in ascites using quantitative real-time PCR. The data indicated that all population of these bacteria decreased significantly in probiotics treated group compared with septic model group ($P < 0.05$, Figure 3).

Probiotics improve colonic mucosal inflammation of experimental sepsis

With probiotics treatment, there was a decrease in the infiltration of neutrophils and mononuclear cells into the intestinal mucosa in septic animals ($P < 0.05$, Figure 4). No apparent differences of epithelial cell hyperplasia were found between the rats in probiotics treated group and septic model group (data not shown).

DISCUSSION

Despite the development of antibiotics and other intensive care treatment, sepsis has a high mortality. CLP of rats is one of animal models of human sepsis. Because colonic contents are extruded into abdominal cavity, various microbes proliferate in ascites immediately. Therefore, the bacteria from feces cause polymicrobial infection, bacteremia and lethal peritonitis[22,23]. In this study, we treated the experimental septic rats with mixture of

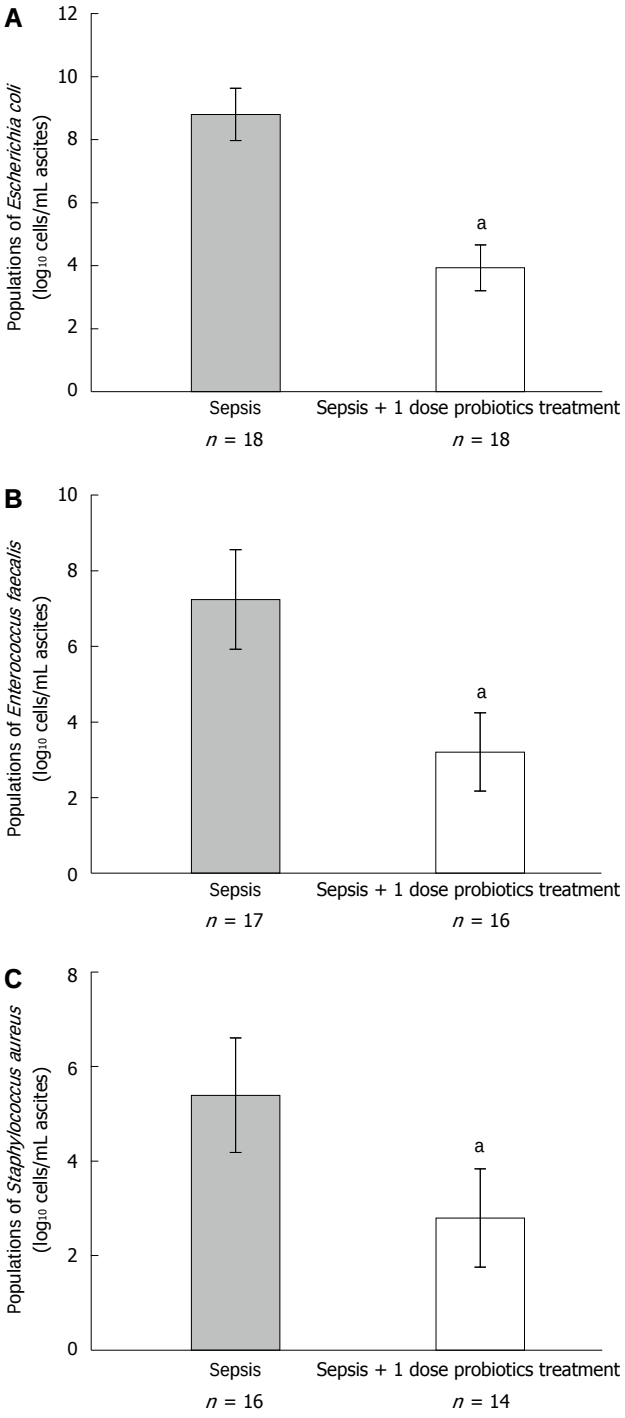

Figure 3 Comparison of predominant bacterial populations in ascites between sepsis and probiotics treated groups. Probiotics (1 dose) or vehicle treatment started 6 h later and thereafter administered once a day for 3 d. Bacterial genomic DNA was extracted and analysed by quantitative real-time PCR as described previously. The population of *Escherichia coli* (A), *Enterococcus faecalis* (B) and *Staphylococcus aureus* (C) were compared between two groups ($^{a}P < 0.05$ *vs* septic model group).

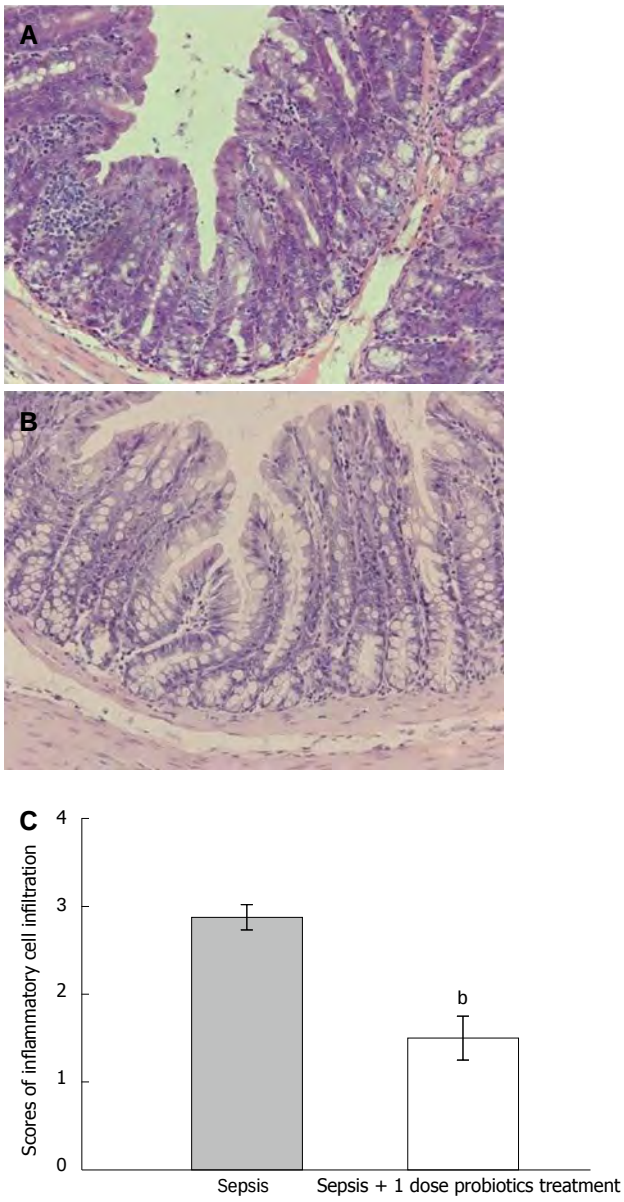

Figure 4 Colonic mucosal inflammation of experimental sepsis. Probiotics (1 dose) or vehicle treatment started 6 h later and thereafter administered once a day for 3 d. We harvested colonic tissues after a 72 h period of cecal ligation and puncture. The sections of colonic tissues were stained by haematoxylin-eosin (× 400). Four fields of each sample were assessed. More neutrophils and mononuclear cells infiltrated into the intestinal mucosa in septic model group (A) than probiotics treatment group (B). We also compared scores of inflammatory cell infiltration between two groups (C) ($^{b}P < 0.01$ *vs* septic model group).

three live probiotics. We also analysed the survival of probiotics treated septic animals. It was demonstrated that probiotics improved the survival of rats with experimental sepsis and this effect was dose dependent. No protective effect was observed using the lowest concentration of probiotics (1/20 dose). However, 1 and 1/4 doses of probiotics treatment increased survival significantly compared with septic model group. Therefore, we treated septic animals in subsequent experiments using 1 dose of probiotics all the time.

Ascites culture data indicates that more pathogens grew in septic model group (10^{9}-10^{10} cells/mL) than in probiotic treated group (10^{4}-10^{5} cells/mL). Both aerobes and anaerobes were detected in ascitic samples, although the majority of microbes were aerobes. Cecum contained anaerobes, facultative aerobes and aerobes. Furthermore, the amounts of anaerobes were greater than those of aerobes[24,25]. However, in our study, aerobes had been

isolated frequently from septic ascitic samples such as *Escherichia coli* (isolating rate was 100%), *Enterococcus faecalis* (95.5%) and *Staphylococcus aureus* (77.8%). The total isolating rate of anaerobes was less than 30%. The main reason for this phenomenon was "oxygen". When the operation of CLP was performed in experimental sepsis, the anaerobes were exposed to oxygen directly. Furthermore, some oxygen was stored in abdominal cavity of animal after operation. For these reasons, the majority of anaerobes were killed by oxygen. Thereafter, the aerobes which were minority in original colonic contents proliferated immediately. When we gave probiotics to septic rats, the bacterial spectrum of ascites and blood was lower than in the septic model group. Meanwhile, probiotics decreased total viable counts of pathogens in septic ascites significantly. In addition, the data of hemoculture showed that *Escherichia coli* and *Staphylococcus aureus* usually were detected in septic model group. Probiotics decreased the positive rate of hemoculture in septic rats.

It seemed that *Escherichia coli* and *Staphylococcus aureus* are the primary pathogens of CLP rats in septic model in our study. On one hand, we detected the population of these bacteria in ascites by quantitative real-time PCR. All population of these bacteria decreased significantly in probiotics treated group compared with septic model group. On the other hand, inflammatory response of intestinal mucosa was lessened in probiotics treated group compared with septic model group. All these data indicated that the mixture of probiotics improved the survival in a murine model of polymicrobial sepsis by suppressing the conditioned pathogens. However, the reasons for this suppression are not clear. There are two potential reasons: first, the decreased bacterial number may result from the inhibition of bacterial proliferation; second, a less bacteria infiltration or promoted bacterial killing[26-30].

Based on what had been mentioned above, we draw a conclusion that conditioned pathogens (*Escherichia coli* and *Staphylococcus aureus*) may be primary pathogens of CLP rats in septic model in our study. Probiotics (*Bifidobacterium longum*, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*) contribute to improving the survival in an animal sepsis model by suppressing the conditioned pathogens.

COMMENTS

Background

Sepsis is the systemic inflammatory response to infection. Microbial infection initiates and promotes systemic inflammatory responses. A variety of species of probiotics have been shown to benefit human gastrointestinal health. However, the mechanisms of probiotics in improving sepsis are unclear. In this study, the authors sought to address this question in the septic model of Wistar rats.

Research frontiers

Recently, more and more gastrointestinal diseases have been treated using probiotics. The probiotics and their products keep gastrointestinal tract homeostasis and regulate immune responses. For example, probiotics can regulate IgE production level and maturation of T cell in the gut.

Innovations and breakthroughs

To study the benefits of probiotics for sepsis, the authors observed the survival of cecal ligation and puncture (CLP) rats using different amounts mixed probiotics. The mixture of probiotics included *Bifidobacterium longum*, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. They also detected bacterial populations in ascites and blood of CLP rats using cultivation and real-time polymerase chain reaction. The data suggested that *Escherichia coli* and *Staphylococcus aureu* may be primary pathogens in the septic model. Probiotics improve survival in the septic model by suppressing the conditioned pathogens.

Applications

The results of this study suggest that probiotics improve survival in the septic model by suppressing the conditioned pathogens. This study helps to know whether probiotics can improve the clinical course of sepsis.

Terminology

CLP of rats produces cecal ischemia and polymicrobial infection. The bacteria of fecal contents will spill into the abdomen, and produce severe peritonitis and bacteremia. So CLP has been used as a classic animal model of sepsis. There are complex microbial populations in intestinal tract. Some of them are probiotics. When administered in adequate amounts, probiotics confer a health benefit to the host. The products of probiotics include mucin, organic acids, branched chain fatty acids, H_2, CO_2, ammonia, amines and vitamins. These products regulate host health through different pathways such as regulating energy, gene expression and cell differentiation, producing anti-inflammatory agents and keeping gut homeostasis.

Peer review

This study investigated the potential benefit of probiotic supplement in preventing septic death by studying the survival rates in rats treated with different doses of a probiotic mixture and then further investigated the effects of probiotics administration on the bacteria proliferation in blood and ascites in a cecal ligation and puncture sepsis model. This study shows the importance of knowing whether probiotics can improve the survival of experimental sepsis.

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BRIEF ARTICLE

Impact of postoperative complications on long-term survival after radical resection for gastric cancer

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Abstract

AIM: To investigate the potential impact of complications in gastric cancer patients who survive the initial postoperative period.

METHODS: Between January 1, 2005 and December 31, 2006, 432 patients who received curative gastrectomy with D2 lymph node dissection for gastric cancer at our department were studied. Associations between clinicopathological factors [age, sex, American Society of Anesthesiologists grade, body mass index, tumor-node-metastases (TNM) stage and tumor grade], including postoperative complications (defined as any deviation from an uneventful postoperative course within 30 d of the operation and survival rates) and treatment-specific factors (blood transfusion, neoadjuvant therapy and duration of surgery). Patients were divided into 2 groups: with (n = 54) or without (n = 378) complications. Survival curves were compared between the groups, and univariate and multivariate models were conducted to identify independent prognostic factors.

RESULTS: Among the 432 patients evaluated, 61 complications occurred affecting 54 patients (12.50%). Complications included anastomotic leakages, gastric motility disorders, anastomotic block, wound infections, intra-abdominal abscesses, infectious diarrhea, bleeding, bowel obstructions, arrhythmias, angina pectoris, pneumonia, atelectasis, thrombosis, unexplained fever, delirium, ocular fungal infection and multiple organ failure. American Society of Anesthesiologists grade, body mass index, combined organ resection and median duration of operation were associated with higher postoperative complications. The 1-, 3- and 5-year survival rates were 83.3%, 53.2% and 37.5%, respectively. In the univariate analysis, the size of lesions, TNM stage, blood transfusion, lymphovascular invasion, perineural invasion, neoadjuvant chemotherapy, and postoperative complications were significant predictors of overall survival. In the multivariate analysis, only TNM stage and the presence of complications remained significant predictors of reduced survival.

CONCLUSION: The occurrence of in-hospital postoperative complications was an independent predictor of worse 5-year overall survival rate after radical resection of gastric cancer.

Key words: Gastric cancer; Perioperative complication; Surgical resection; Complications

Core tip: The concept of perioperative complications as a risk factor for survival is well known in gastric cancer, however, the potential impact of complications for patients who survive the initial postoperative period has not been determined. We showed that the occurrence of in-hospital postoperative complications is an independent predictor of worse 5-year overall survival after radical resection of gastric cancer. In 432 patients evaluated, 61 complications occurred affecting 54 patients (12.50%). American Society of Anesthesiologists grade, body mass index, combined organ resection and median duration of operation were associated with higher post-

operative complications. The 1-, 3- and 5-year survival rates were 83.3%, 53.2% and 37.5%, respectively.

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INTRODUCTION

Globally, gastric cancer ranks fourth and fifth in males and females, respectively, in terms of incidence, and ranks third and fifth in males and females, respectively, in terms of mortality[1]. China is classified as a high incidence area for gastric cancer. Stomach cancer has the third highest incidence and is the second leading cause of death among all cancers[2]. Surgery is the cornerstone in the treatment of gastric cancer. Although postoperative complications after surgical resection of gastric cancer are common, the potential long-term impact of these complications for patients who survive the initial postoperative period is not well understood. Western countries have published complication rates ranging from 35% to 46%, and mortality rates from 4% to 16% after D2 lymph node dissection[3-5]. Major complications include anastomotic leakage, intra-abdominal bleeding, intra-abdominal abscess, intestinal obstruction and wound infection. Previous investigations focused on the immediate effect of postoperative complications and their impact on acute perioperative course and length of hospital stay. The concept of perioperative complications as a risk factor for survival is well known in other cancer surgery, such as esophageal, colorectal cancer liver metastases, hilar cholangiocarcinoma and colorectal cancer[6-9]. To date, few studies have determined the potential impact of early surgical complications on long-term survival for patients with gastric cancer. The aim of this study was to assess the impact of prognostic factors, in particular perioperative complications, on the long-term survival of patients undergoing radical resection for gastric cancer.

MATERIALS AND METHODS

Patients

Patients' medical records and clinicopathological data during the period from January 1, 2005 to December 31, 2006 were studied retrospectively at the Department of Gastrointestinal Surgery, First Clinic Medical School of Yangzhou University, Yangzhou, China. Patients' inclusion criteria were: (1) All patients in the study had histologically confirmed gastric adenocarcinoma and received curative gastrectomy with D2 lymph node dissection; (2) Information regarding postoperative complications and mortality was available for each patient studied. As a result, 432 patients were eligible for analysis. All these patients were followed up for a minimum of 60 mo after gastric resection. These 432 patients comprised 263 men (60.88%) and 169 women (39.12%). Their median age was 64 years (range, 28-83 years). The follow-up of patients after surgery was 5 years. Numbers of subtotal and total gastric resection were 186 and 246, respectively, and gastrointestinal reconstruction comprised 82 Billroth Ⅰ, 68 Billroth Ⅱ and 282 Roux-en-Y anastomoses. This research was in compliance with the Helsinki Declaration and was approved by the ethics committee of the First Clinic Medical School of Yangzhou University. The main characteristics of 432 people included study are summarized in Table 1.

Assessment of complications

Complications were defined as any deviation from an uneventful postoperative course within 30 d of the operation. A recently published standardized complication classification system (Clavien-Dindo classification) was used to grade postoperative complications[10]. Briefly, grade I complications include any deviation from the normal postoperative course not needing specific treatment, as well as wound infections treated topically at the bedside. Grade Ⅱ complications can be treated solely by drugs, blood transfusion, physiotherapy and nutritional support. Grade Ⅲ complications require interventional or surgical treatment, without (Ⅲa) or with (Ⅲb) general anesthesia. Grade Ⅳ complications are life-threatening complications requiring intensive-care unit management (Ⅳa, single organ dysfunction; Ⅳb, multiple organ dysfunction). Grade Ⅴ represents death of the patient. In the present study, if a patient had more than 1 complication, the grade used for analysis was defined by the highest-ranked complication.

Follow-up

Complete follow-up was available for all study patients. Follow-up was calculated from the date of surgery. Follow-up data were obtained by phone, letter, and the outpatient clinical database. The end of the follow-up period was 5 years after surgery.

Statistical analysis

The impact of clinicopathological and therapy-related variables with a potential influence on a postoperative complication were investigated. For this purpose, patients with postoperative complications were compared with those who recovered normally. The two groups were compared by univariate analysis with respect to clinicopathological factors [age, sex, American Society of Anesthesiologists (ASA) grade, body mass index (BMI), tumor-node-metastases (TNM) stage and tumor grade] and treatment-specific (blood transfusion, neoadjuvant therapy and duration of surgery) variables. For the outcome analysis, the patients were divided into two groups: those with complications and those with no postoperative complications. To investigate the impact of such complications on postoperative

Table 1 Intergroup comparison of epidemiological and treatment-related variables in 432 patients

Variable	Postoperative complications (n = 54)	No postoperative complications (n = 378)	P value
Age (yr), median (range)	60 (28-80)	59 (23-76)	0.516[1]
Sex			0.236[2]
Male	37	226	
Female	17	152	
ASA grade			0.000[2]
Ⅰ	24	341	
Ⅱ	18	33	
Ⅲ	11	4	
Ⅳ	1	0	
Body mass index (kg/m^2)			0.040[2]
< 28	42	332	
⩾ 28	12	46	
TNM stage			0.358[2]
Ⅰ	5	49	
Ⅱ	9	94	
Ⅲ	28	174	
Ⅳ	12	61	
Tumor size (cm), mean (range)	4.9 (2.0-12.0)	4.7 (0.5-12.0)	0.338[1]
Combined organ resection			0.000[2]
No	35	346	
Yes	19	32	
Neoadjuvant therapy			0.126[2]
No	50	366	
Yes	4	12	
Median (range) duration of operation (min)	220 (175-310)	195 (160-300)	0.000[1]

[1]t test; [2]Pearson χ^2. ASA: American Society of Anesthesiologists; TNM: Tumor-node-metastases.

outcome, the two groups were compared using Kaplan-Meier survival curves. Postoperative complications are listed in Table 2. Assessment of the oncological relevance of the complication was based on an analysis of the two groups of patients. The 5-year survival rates were first subjected to univariate analysis, followed by multivariate analysis. Three patients who died within 30 d after surgery were excluded from the survival analysis.

RESULTS

Complications

Sixty-one complications occurred, affecting 54 patients (12.50%). Complications were graded into seven categories according to their severity. The details are given in Table 2. Ten patients suffered from gastric resection-related complications. Thirteen patients experienced infectious complications. Eight patients had bleeding complications and six had bowel obstructions. Five patients developed cardiac complications. Eleven patients had pulmonary complications. Two had thrombosis and six patients had other complications. Three patients died during hospitalization within 5 to 26 d after the initial gastrectomy, representing an in-hospital mortality of 0.69%. Two patients died as a result of postoperative sepsis and multiple organ failure. One died from acute respiratory distress syndrome.

Table 2 Post-operative complication types, frequencies and severities

Variables	Number
Gastric resection-related complications	
Anastomotic leakages	5
Gastric motility disorders	4
Anastomotic block	1
Infectious complications	
Wound infection	7
Intra-abdominal abscess	5
Infectious diarrhea	1
Bleeding complications	
Anastomotic bleeding	2
Intra-abdominal bleeding	5
Subcutaneous hematoma surrounding drainage tubes	1
Bowel obstructions	6
Pulmonary complications	
Pneumonia	10
Atelectasis	1
Cardiac complications	
Arrhythmia	4
Angina pectoris	1
Thrombosis	
Deep venous thrombosis	1
Portal venous thrombosis	1
Other complications	
Delirium	2
Unexpected fever	2
Ocular fungal infection	1
Multiple-organ failure	1
Incidence and severity (Clavien-Dindo grade)	
Ⅰ	8
Ⅱ	35
Ⅲa	11
Ⅲb	2
Ⅳa	1
Ⅳb	1
Ⅴ	3

Risk factors for post-operative complication

The variables age, sex, ASA grade, BMI, TNM stage, mean tumor size, combined organ resection, neoadjuvant therapy and median duration of operation were investigated by Pearson χ^2 test or by t test. Only ASA grade, BMI, combined organ resection and median duration of operation had an independent impact on the occurrence of complications ($P < 0.05$).

Survival

The 1-, 3- and 5-year survival rates were 83.3%, 53.2% and 37.5%, respectively. In the univariate analysis, survival was not influenced by gender, age or BMI. In contrast, the size of the lesions, TNM stage, blood transfusion, lymphovascular invasion, perineural invasion, neoadjuvant chemotherapy and postoperative complications were significant predictors of overall survival (Table 3). In the multivariate analysis, perineural invasion, the size of the lesions, blood transfusion, lymphovascular invasion and neoadjuvant chemotherapy were no longer predictive factors for reduced survival. However, the TNM stage and the presence of complications remained significant predictors of reduced survival (Table 4).

Table 3 Univariate survival analysis of gastric cancer patients according to various clinicopathological variables and complications

Variable	Patients	5-yr survival	Log rank χ^2 test	*P* value
Gender			2.847	0.092
Male	262	34.00%		
Female	167	43.70%		
Age (yr)			1.157	0.282
≤ 60	230	40.00%		
> 60	199	35.20%		
BMI (kg/m^2)			0.018	0.893
≤ 26	278	38.50%		
> 26	151	36.40%		
Size of lesions (cm)			8.130	0.004
< 5	269	42.00%		
≥ 5	160	30.60%		
TNM stage			60.453	0.000
Ⅰ	54	72.20%		
Ⅱ	101	54.90%		
Ⅲ	202	28.70%		
Ⅳ	72	12.70%		
Blood transfusion			4.982	0.026
Yes	74	27.00%		
No	355	40.00%		
Lymphovascular invasion			4.673	0.031
Yes	69	21.70%		
No	360	40.80%		
Perineural invasion			5.237	0.022
Yes	36	25.00%		
No	393	38.90%		
Neoadjuvant chemotherapy			7.124	0.008
No	411	38.60%		
Yes	18	16.70%		
Complications			25.946	0.000
Yes	51	21.80%		
No	378	39.90%		

BMI: Body mass index; TNM: Tumor-node-metastases.

Table 4 Predictors of survival: Multivariate analysis

Risk factor	HR (95%CI)	*P* value
Size of lesions	1.2 (0.9-1.5)	0.156
TNM stage	1.6 (1.4-1.9)	0.000
Blood transfusion	0.9 (0.5-1.4)	0.752
Lymphovascular invasion	1.0 (0.6-1.6)	0.841
Perineural invasion	0.7 (0.4-1.0)	0.107
Neoadjuvant chemotherapy	1.5 (0.8-2.5)	0.134
Post-operative complications	2.5 (1.8-3.6)	0.000

Cox regression analysis of patient survival. TNM: Tumor-node-metastases.

DISCUSSION

In the surgical approach for early and selective advanced gastric cancer, gastrectomy with D2 lymphadenectomy is justified[11,12]. Local tumor control and long-term oncological survival are dependent on the quality of the surgical treatment and the surgeon's case-load[13]. The occurrence of postoperative complications is higher in inexperienced hands, and there is a considerable difference in early surgical outcomes among centers[14,15]. Overall survival rate is higher at specialized centers. Therefore, it may be stated that gastric cancer surgery is safe at specialized centers. The postoperative complications at our institution were in the acceptable range: most patients had a smooth recovery and postoperative mortality was not high.

In this study, ASA grade, BMI, combined organ resection and duration of operation were greater in the postoperative complications group than in the no complications group. These findings are in agreement with recent reports showing similar predictors of postoperative complications. ASA grade was reported to affect surgical complications[1], but this was not consistent with prior studies reported by Kawamura *et al*[16]. A negative effect of BMI on perioperative complications of gastrectomy has also been reported[17,18]; elevated BMI was significantly associated with increased weight of the stomach extirpated *en bloc* with omentum and perigastric lymph nodes, which was found to increase operative times. Additional organ resection in surgical therapy for gastric cancer has been associated with increased complications and perioperative mortality in pursuit of a D2 lymphadenectomy[19,20]. A large retrospective study from Japan found no survival difference when patients undergoing gastrectomy alone were compared to patients with additional organ resection, however, the complication rate was greater[21]. The duration of operation was greater in the postoperative complications group than in the no complications group. Patients with advanced TNM stage, combined organ resection and elevated BMI always require a longer operation time than others.

Although this is the first study to identify the independent impact of postoperative complications among patients undergoing surgery for gastric cancer, other investigators have explored the potential relationship between postoperative complications and long-term survival beyond the initial perioperative period in other malignancies. In an analysis of 197 colorectal cancer liver metastases patients, Schiesser *et al*[22] reported 30% perioperative complications, and the median survival time of patients with perioperative complications was 3.2 years, compared to 4.4 years in those patients without complications. Similar results were reported for hilar cholangiocarcinoma[8]. Given these findings, it is logical to fully explore the potential impact of in-hospital, postoperative complications on long-term cancer survival. Mechanisms currently under discussion include more serious immunosuppression and more obvious inflammation associated with postoperative complications. Generally, postoperative complications have been suggested to lead to an extended period of immunosuppression, which permits residual tumor cells to proliferate and survive in the host. One example supporting this hypothesis is based, in part, on the finding that in several other malignancies, perioperative blood transfusions correlated with negative immunomodulatory effects and earlier cancer recurrence[23,24]. Our findings could be explained in a similar manner. Infective complications are the most common complications, including intra-abdominal abscesses, wound infections, pneumonia, infectious diarrhea and anastomotic leakage, which can also cause abdominal infection. Several studies have demonstrated a correlation

between long-term outcomes after curative resections of solid tumors and postoperative infection and sepsis[7,25-27]. Infection and sepsis potentiate proinflammatory cytokine cascades, including tumor necrosis factor-alpha and interleukins 1, 6 and 8. These immune modulators can affect the function and regulation of natural killer cells, cytotoxic T-lymphocytes and antigen-presenting cells[28-30]. Hypothetically, micrometastases may progress rapidly during brief and prolonged periods of relative immunosuppression resulting from postoperative complications. Besides, both sepsis and blood transfusion may stimulate vascular endothelial growth factor release, which is one of the most potent stimulators of metastatic growth[31,32]. This combination of transfusion and sepsis may stimulate cancer recurrence[33].

Our results show a clear association between postoperative complications and long-term survival for patients undergoing resection for gastric cancer. The study highlights the significance not only of appropriate patient selection and surgical technique, but also serves to emphasize the potential impact that postoperative monitoring and hospital care can have on long-term outcomes. Performance of a safe operation with minimal blood loss, careful lymphadenectomy and gastrointestinal reconstruction are important for reducing post-operative complications. Avoidance of complications improves long-term survival.

In summary, our study aimed to evaluate the impact of complications on survival for patients who received radical surgery for gastric cancer. We found that the occurrence of in-hospital postoperative complications was an independent predictor of worse 5-year overall survival rate after radical resection of gastric cancer.

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COMMENTS

Background

The concept of perioperative complications as a risk factor for survival is well known in gastric cancer, however, the potential impact of complications for patients who survive the initial postoperative period has not been determined. This article studies the potential impact of complications on patients who survive the initial postoperative period.

Research frontiers

Perioperative complications are an important factor after surgery. Currently, many experts have focused on lymphatic metastasis for survival; however, perioperative complications have not been unequivocally addressed. In this study, the authors demonstrate that the occurrence of in-hospital postoperative complications was an independent predictor of worse 5-year overall survival rate after radical resection of gastric cancer.

Innovations and breakthroughs

Between January 1, 2005 and December 31, 2006, 432 patients who received curative gastrectomy with D2 lymph node dissection were studied. Associations between clinicopathological factors, including postoperative complications and survival, were studied using univariate and multivariate models.

Applications

To understand survival during the initial postoperative period of gastric cancer, this article studied the potential impact of complications for patients who survive the initial postoperative period. In the univariate analysis, size of lesions, tumor-node-metastases (TNM) stage, blood transfusion, lymphovascular invasion, perineural invasion, neoadjuvant chemotherapy and postoperative complications were significant predictors of overall survival. In the multivariate analysis, only TNM stage and the presence of complications remained significant predictors of reduced survival.

Peer review

The article provides a detailed description of the survival impact of postoperative complications. This data is valuable for the treatment of gastric malignancy.

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BRIEF ARTICLE

Clinical significance of melatonin concentrations in predicting the severity of acute pancreatitis

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Abstract

AIM: To assess the value of plasma melatonin in predicting acute pancreatitis when combined with the acute physiology and chronic health evaluation Ⅱ (APACHE Ⅱ) and bedside index for severity in acute pancreatitis (BISAP) scoring systems.

METHODS: APACHE Ⅱ and BISAP scores were calculated for 55 patients with acute physiology (AP) in the first 24 h of admission to the hospital. Additionally, morning (6:00 AM) serum melatonin concentrations were measured on the first day after admission. According to the diagnosis and treatment guidelines for acute pancreatitis in China, 42 patients suffered mild AP (MAP). The other 13 patients developed severe AP (SAP). A total of 45 healthy volunteers were used in this study as controls. The ability of melatonin and the APACHE Ⅱ and BISAP scoring systems to predict SAP was evaluated using a receiver operating characteristic (ROC) curve. The optimal melatonin cutoff concentration for SAP patients, based on the ROC curve, was used to classify the patients into either a high concentration group (34 cases) or a low concentration group (21 cases). Differences in the incidence of high scores, according to the APACHE Ⅱ and BISAP scoring systems, were compared between the two groups.

RESULTS: The MAP patients had increased melatonin levels compared to the SAP (38.34 ng/L *vs* 26.77 ng/L) (P = 0.021) and control patients (38.34 ng/L *vs* 30.73 ng/L) (P = 0.003). There was no significant difference inmelatoninconcentrations between the SAP group and the control group. The accuracy of determining SAP based on the melatonin level, the APACHE Ⅱ score and the BISAP score was 0.758, 0.872, and 0.906, respectively, according to the ROC curve. A melatonin concentration ⩽ 28.74 ng/L was associated with an increased risk of developing SAP. The incidence of high scores (⩾ 3) using the BISAP system was significantly higher in patients with low melatonin concentration (⩽ 28.74 ng/L) compared to patients with high melatonin concentration (> 28.74 ng/L) (42.9% *vs* 14.7%, P = 0.02). The incidence of high APACHE Ⅱ scores (⩾ 10) between the two groups was not significantly different.

CONCLUSION: The melatonin concentration is closely related to the severity of AP and the BISAP score. Therefore, we can evaluate the severity of disease by measuring the levels of serum melatonin.

Key words: Pancreatitis; Melatonin concentrations; Predict; Cutoff; Bedside index for severity in acute pancreatitis; Acute physiology and chronic health evaluation Ⅱ

Core tip: It is important to assess the severity and changes in a patient's condition in a timely and accurate manner. Thus, a comprehensive treatment plan for acute pancreatitis patients is critical. Melatonin plays a protective role in the early course of human acute pancreatitis, and melatonin concentration variations

are closely related to the severity of acute pancreatitis and the bedside index for severity in acute pancreatitis score. We can determine the severity of disease in the clinic more objectively, accurately and rapidly by measuring the levels of serum melatonin than by using the standard scoring systems. When the serum concentration of melatonin is below 28.74 ng/L, it is possible that acute pancreatitis patients will develop severe acute pancreatitis.

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INTRODUCTION

Most patients with acute pancreatitis have a favorable prognosis. However, the mortality rate of acute pancreatitis (AP) has been reported as 6%-23%[1]. Effectively treating the disease becomes more difficult as it develops into severe AP (SAP). Therefore, it is important to assess the disease severity in a timely and accurate manner to provide comprehensive treatment to AP patients. Accurate treatment can improve the prognosis and reduce mortality[2]. As a result, there is an urgent need for an objective, accurate, fast and simple method of monitoring changes in AP patients.

Melatonin is best known as the activator of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxydase, or glutathione reductase[3-6]. Melatonin is also well-known as a scavenger of radical oxygen and nitrogen species[7-9]. Melatonin, together with reduced glutathione, vitamins C and E, uric acid, selenium, and creatinine, belongs to the category of nonenzymatic scavengers[6,10,11]. A number of studies have shown that melatonin (MT) plays a protective role in AP. In acute pancreatitis, melatonin was demonstrated to inhibit nuclear binding of nuclear factor kappa B (NF-κB). NF-κB is a transcription factor that controls the expression of genes involved in immunity and inflammation and the production of prostaglandins, cytokines, cell adhesion molecules, nitricoxide (NO), and inhibitors of apoptosis[12,13]. Melatonin has been demonstrated to reduce gene expression and synthesis of proinflammatory cytokines such as tumor necrosis factor α (TNFα) and proinflammatory interleukins such as interleukin (IL)-1β, IL-6, IL-8, and prostaglandins[1,14,15]. In addition, melatonin was also reported to modulate the processes of apoptosis and necrosis by stimulating the production of vascular endothelial growth factor to activate angiogenesis[16-18]. Furthermore, MT plays a protective role in AP-associated organ injuries in animal models[19-21]. For example, Huai *et al*[22] found that melatonin protects rats against acute pancreatitis-associated lung injury through the upregulation of IL-22 and Th22. The upregulation of IL-22 increases innate immunity in tissues and enhances regeneration.

Data on the relationship between the levels of MT in patients with AP and the severity and prognosis of this disease have not been reported. The aims of this study were to assess the value of plasma MT in determining the severity of AP and in predicting SAP. Additionally, we analyzed changes in plasma MT levels and the use of two scoring systems in AP patients.

MATERIALS AND METHODS

Patients

This study enrolled 55 consecutive patients with AP (35 men and 20 women) admitted to department of gastroenterology of our hospital between July 2010 and March 2011 (median age 51 years, range 17-82 years). The diagnosis and classification of AP were based on the diagnosis and treatment guidelines for acute pancreatitis in China (2009)[23]. SAP was diagnosed by the presence of organ failure and (or) local complications. Organ failure included shock (systolic blood pressure < 90 mmHg), pulmonary insufficiency (arterial PO_2 < 60 mmHg at room air or the need for mechanical ventilation), or renal failure (serum creatinine level > 2 mg/dL after rehydration or hemodialysis). Examples of localcomplications included pancreatic necrosis, a pseudocyst, or a pancreatic abscess. According to the diagnosis and treatment guidelines for acute pancreatitis in China, 42 cases were defined as mild AP (MAP), and 13 cases were classified as SAP. Within the population of SAP patients, there were 11 patients (84.6%) with pseudocysts and 2 patients (15.4%) with pancreatic necrosis. There were also 2 patients (15.4%) with acute renal failure. The disease etiology was biliary in 19 cases (34.5%), hyperlipidemic in 14 cases (25.5%), and idiopathic in 14 cases (25.5%). The causes of the remaining 8 cases (14.5%) were hyperlipidemic and biliary, alcoholic and biliary, or alcoholic and pancreatic (duct obstruction). There were no patient deaths during the study period (Table 1). We also analyzed 45 healthy individuals as controls for the study. There were 27 men and 18 women in the control group. The median age of the controls was 44 years (range of 24-64 years).

Monitoring

The study protocol was reviewed and approved by the local ethics committee. The study patients and healthy volunteers were enrolled after providing written informed consent. The patient-acute physiology and chronic health evaluation Ⅱ (APACHE Ⅱ) and bedside index for severity in acute pancreatitis (BISAP) scores were calculated within the first 24 h after admission in all patients with AP. The APACHE Ⅱ score is the most commonly used scoring system for determining the severity and prognosis of AP. This scoring system contains 12 monitoring indicators, and the final score is composed of an acute

Table 1 Characteristics of 55 patients with acute pancreatitis *n* (%)

Variables	Total (*n* = 55)	Mild (*n* = 42)	Severe (*n* = 13)
Age, yr (range)	51 (17-82)	51 (17-77)	50 (30-82)
Male	35 (63.6)	27 (64.3)	8 (61.5)
Female	20 (36.4)	15 (35.7)	5 (38.5)
Etiology			
Biliary	19 (34.5)	17 (40.5)	2 (15.4)
Hyperlipidemia	14 (25.5)	8 (19)	6 (46.2)
Idiopathic	14 (25.5)	11 (26.2)	3 (23.1)
Other	8 (14.5)	6 (14.3)	2 (15.4)
APACHE Ⅱ (range)	7 (2-22)	6 (2-12)	12 (6-22)
BISAP (range)	2 (0-5)	1 (0-4)	3 (2-5)
Operations	10 (18.2)	9 (21.4)	1 (7.7)
Organ failure	2 (3.6)	0 (0.0)	2 (15.4)
Pancreatic necrosis	2 (3.6)	0 (0.0)	2 (15.4)
Pseudocyst	11 (20.0)	0 (0.0)	11 (84.6)
Mortality	0 (0.0)	0 (0.0)	0 (0.0)

APACHE Ⅱ: Acute physiology and chronic health evaluation Ⅱ; BISAP: Bedside index for severity in acute pancreatitis.

physiology score, an age index and a chronic health index[24]. The BISAP scoring standard consists of five elements: blood urea nitrogen, disturbance of consciousness, systemic inflammatory response syndrome, age and pleural effusion[25]. A 3 mL sample of fasting peripheral venous blood was obtained from all patients on the first morning (6:00 AM) after admission. A blood sample was also collected from the control participants.

Laboratory methods

The blood samples from patients with AP and healthy controls were immediately centrifuged at 2500 *g* for 5 min. The sample supernatants were then stored at -80 ℃ until further investigation. The melatonin levels in serum were measured using an enzyme-linked immunosorbent assay (Changfeng Chemical Company, Shanghai, China).

Statistical analysis

The statistical analysis was performed using the SPSS 13.0 statistical program. The measurement data are expressed as the mean ± SE. Differences in MT between the mean values of various groups of experiments were compared using one-way analysis of variance and SNK post hoc analysis. The incidences of high scores for the APACHE Ⅱ and BISAP scoring systems in the high MT concentration group and the low MT concentration group were compared with a χ^2 test. A difference with a P value of < 0.05 was considered statistically significant. An receiver operating characteristic (ROC) curve was generated to analyze the ability of melatonin and the APACHE Ⅱ and BISAP scoring systems to predict SAP.

RESULTS

There was no significant difference in the age ($P = 0.751$) or sex ratio ($P = 1.000$) between patients with mild pancreatitis and severe pancreatitis. Biliary problems were the main factor in the MAP group. Conversely, most cases of SAP were caused by hyperlipidemia (46.2%). Both the APACHE Ⅱ scores and the BISAP scores in severe pancreatitis were significantly higher than in the mild cases at admission. The APACHE Ⅱ scores in the severe and mild AP cases were 12 points *vs* 6 points ($P < 0.001$), while the BISAP scores were 3 points *vs* 1 point ($P < 0.001$).

Table 2 Comparison of the capability to predict severe acute pancreatitis

Variables	Sensitivity	Specificity	Youden index	Accuracy
MT ⩽ 28.74 ng/L	73.80%	76.90%	0.507	0.758
APACHE Ⅱ score ⩾ 9.5	76.90%	83.30%	0.602	0.872
BISAP score ⩾ 2.5	76.90%	90.50%	0.674	0.906

MT: Melatonin; APACHE Ⅱ: Acute physiology and chronic health evaluation Ⅱ; BISAP: Bedside index for severity in acute pancreatitis.

The median value of melatonin levels in the MAP group, the SAP group and the control group was 38.34, 26.77 and 30.73 ng/L, respectively. The melatonin level was significantly higher in patients with mild AP compared to patients with severe pancreatitis (38.34 ± 13.76 ng/L *vs* 26.77 ± 11.88 ng/L, $P = 0.021$). A similar trend was also observed in patients with mild disease compared to controls ($P = 0.003$). There was no significant difference in melatonin levels between MAP patients and healthy individuals (38.34 ± 13.76 ng/L *vs* 30.73 ± 2.96 ng/L, $P > 0.05$)

The Youden index of MT, the APACHE Ⅱ score and the BISAP score for predicting severe acute pancreatitis was 0.507, 0.602 and 0.674, respectively. The optimal cut-off value, sensitivity, specificity, Youden index and accuracy of the respective parameters in predicting SAP are shown in Table 2. The ROC curves of MT and the APACHE Ⅱ and BISAP scoring systems are presented in Figure 1.

The optimal cutoff concentration of 28.74 ng/L for SAP, as determined from the ROC curve, was used to classify the patients into a high concentration group (34 cases) and a low concentration group (21 cases). The incidence of a high BISAP score (⩾ 3) was significantly greater in patients with alow melatonin concentration (⩽ 28.74 ng/L) compared to patients with a high melatonin concentration (> 28.74 ng/L). The incidence of a high BISAP score was 42.9% in patients with low melatonin compared to 14.7% in patients with high melatonin ($P = 0.02$). There was no significant difference in the incidence of high scores (⩾ 10) according to the APACHE Ⅱ scoring system between patients with high and low melatonin levels ($P > 0.05$) (Table 3).

DISCUSSION

In the clinic, 10%-20% of AP patients will develop severe acute pancreatitis, characterized by longer duration of disease, organ failure, systemic inflammatory response syndrome, and pancreatic necrosis. As a result, the disease pathogenesis is serious and complex. It has been reported

Table 3 Relationship between melatonin concentration and patient scores

Group[1]		APACHE Ⅱ score[2]				BISAP score[3]			
	Total cases	High score cases	Incidence	χ^2 value	*P* value	High score cases	Incidence	χ^2 value	*P* value
Low concentrations	21	8	8/21	0.821	> 0.05	9	9/21	5.422	0.02
High concentrations	34	9	9/34			5	5/34		

[1]≤ 28.74 ng/L is defined as a low concentration, > 28.74 ng/L is defined as a high concentration; [2]APACHE Ⅱ score ≥ 10 is defined as a high score, the two groups, $P > 0.05$; [3]BISAP ≥ 3 is defined as a high score. BISAP ≥ 3, low melatonin concentration compared to high concentration, $P = 0.02$. APACHE Ⅱ: Acute physiology and chronic health evaluation Ⅱ; BISAP: Bedside index for severity in acute pancreatitis.

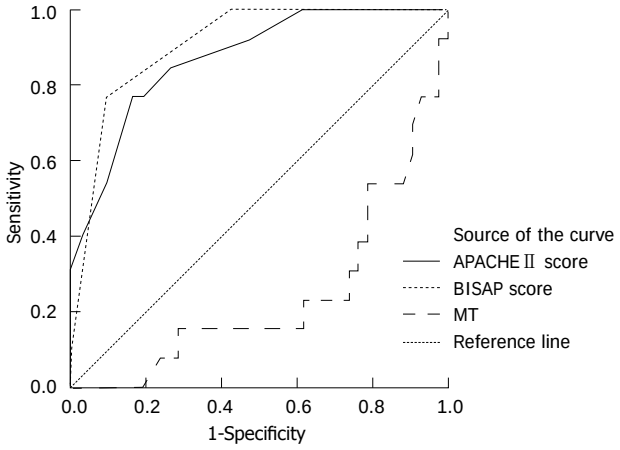

Figure 1 Receiver operating characteristic curves of melatonin, acute physiology and chronic health evaluation Ⅱ score and bedside index for severity in acute pancreatitis score to predict severe acute pancreatitis. MT: Melatonin; APACHE Ⅱ: Acute physiology and chronic health evaluation Ⅱ; BISAP: Bedside index for severity in acute pancreatitis.

that oxidative stress and lipid peroxidation caused by oxygen free radicals cause the destruction of acinar cells and abnormal expression of cytokines during AP pathogenesis[26]. Studies published in the literature concerning MT have demonstrated that it can stabilize cell membranes and protect the cells from oxidative damage. Moreover, MT can also penetrate all of the morphophysiological barriers in the human body and restore acinar cells with their lipophilic and hydrophilic characteristics[27]. Not only is melatonin itself an antioxidant; its metabolites can also reduce oxygen radicals. Additionally, melatonin can strengthen the activity of many antioxidants, such as SOD, Glutathione and CAT, and scavenges both oxygen free radicals and nitrogen free radicals[28,29]. MT has also been reported to have powerful anti-inflammatory and immunomodulatory effects by regulating the production of cytokines[30]. Furthermore, MT was found to promote the spontaneous regeneration process of pancreatic tissue through the activation of stellate cells[31]. MT is potentially capable of limiting pancreatic and associated organ damage produced during AP.

In our study, MT levels in the MAP group were significantly higher than the controls on the first day after admission. This result highlights the importance of the human endocrine system in AP development. The inflammatory response occurs in the early stage of AP prior to the activation of trypsinogen. The organism defense against inflammation occurs primarily through the action of the hypothalamus-pituitary-adrenal axis to increase the secretion of endogenous cortisol. However, the adrenal glands of patients with AP are in a state of relative insufficiency at the onset of disease[32]. Therefore, they may be protected by other mechanisms such as the recruitment of MT to reduce pancreatic damage; thus, an increase in MT will promote a mild disease course. It is well known that inflammation in acute pancreatitis is caused by the imbalance of pro-inflammatory factors and anti-inflammatory factors. This imbalance is more severe in patients with SAP. Perras *et al*[33] showed a clear negative correlation between disease severity and MT levels in patients suffering from severe inflammation. The pineal secretions from patients with profound systemic inflammatory responses were inhibited. This finding could explain why MT concentrations in the SAP group were significantly lower than those in the MAP group in this study. Our results indicate that the MT level is closely related to AP severity. Thus, a lower MT concentration is associated with more severe disease. Conversely, higher MT concentrations are associated with less severe disease. Our data are consistent with the view supported by Belyaev *et al*[32], who indicated that endogenous high levels of serum MT play a protective role in the early course of AP. Our findings have raised the hope that we may be able to control disease severity by using early detection of serum MT concentrations.

The ROC curve is used to compare the accuracy of two or more diagnostic tests. The ROC is considered the most reliable method of evaluating patient prognosis. In this study, we used the ROC curve to assess the relationship between MT and SAP by combining MT values with the APACHE Ⅱ and BISAP scoring systems. As shown in the results, the accuracy of the two scores for SAP were 0.872 and 0.906, respectively. This result indicates that both scoring systems can predict SAP accurately. However, these scoring systems are clinically cumbersome and difficult to remember for clinicians. In addition, it is time-consuming to monitor changes in condition accurately and rapidly using these systems[34,35]. The accuracy of SAP detection using MT was 0.758, and the optimal cut-off concentration was 28.74 ng/L in this study. Our data show that MT levels can predict SAP. Our results further demonstrate that the severity of disease can be determined objectively and accurately by early measurement of serum MT levels. Patients with AP may develop SAP when their MT concentration is below 28.74 ng/L.

Singh *et al*[36] reported that a BISAP score ≥ 3 was associated with an increased risk of developing organ failure. Thus, a BISAP score of 3 was used to divide the patients into the high score and low score groups. A key result of this study was the observation that MT levels were closely related to the BISAP score. The incidence of high score (≥ 3) was significantly increased in patients with low melatonin concentration (≤ 28.74 ng/L) compared to patients with high melatonin concentration (> 28.74 ng/L). Our results clearly demonstrate that a high BISAP score reflects a more severe AP condition and is associated with reduced MT concentration. Conversely, patients with higher MT concentrations had fewer incidences of a high BISAP score. Thus, our results agree with previously published data[36].

Chatzicostas *et al*[37] reported that SAP and its complications could be predicted accurately when the patient had an APACHE Ⅱ score ≥ 10. Therefore, patients were classified into high score and low score groups, with a dividing score of 10 between the groups. However, in our study there was no significant difference in the high score incidence between the low MT concentration group and the high MT concentration group. The reasons for this result include the following: (1) the APACHE Ⅱ score requires knowledge of the patient history, which may not be available if the patient is unconscious, intubated, or transferred from an outside hospital lacking detailed records, thus resulting in an incorrect number of points; and (2) the APACHE Ⅱ score includes a chronic health index, which is not directly correlated with AP. Thus, the relationship between MT levels and the APACHE Ⅱ score will require further studies.

In conclusion, the results of the present study reveal that exogenous melatonin may prevent the damage caused during acute pancreatitis due to its antioxidant, anti-inflammatory, and immunomodulatory properties. The variations of MT concentration might reflect the degree of AP severity to some extent. As a result, we can determine the severity of disease more objectively, accurately and rapidly by measuring the levels of serum melatonin. In addition, a melatonin concentration ≤ 28.74 ng/L was associated with an increased risk of developing SAP. The current clinical study was performed in a single center, and this research had some limitations. Therefore, large sample investigations will be needed to explore the value of serum melatonin in determining the severity of AP.

COMMENTS

Background

Acute pancreatitis (AP) includes both severe AP (SAP) and mild AP (MAP). SAP has a high reported mortality rate. It is important to assess the severity and changes in a patient's condition in a timely and accurate manner to provide comprehensive treatment. This approach could improve the prognosis and reduce mortality. Therefore, authors assessed the predictive value of plasma melatonin in identifying acute pancreatitis in combination with the acute physiology and chronic health evaluation Ⅱ (APACHE Ⅱ) and bedside index for severity in acute pancreatitis (BISAP) scoring systems.

Research frontiers

A number of studies have showed that melatonin (MT) plays a protective role in AP and its associated organ injuries using animal models. The research objective herein was to assess the value of plasma MT in determining the severity of AP. Additionally, authors predicted SAP and analyzed the changes of plasma MT levels and the use of two scoring systems.

Innovations and breakthroughs

The authors have evaluated the ability of melatonin and the APACHE Ⅱ and BISAP scoring systems to predict SAP by using a receiver operating characteristic (ROC) curve. The optimal cutoff concentration for SAP from the ROC curve was used to classify the patients into a high concentration group and a low concentration group. The differences in the incidence of high scores for the APACHE and BISAP scores scoring systems were compared between the two groups. In the present study, melatonin was shown to play a protective role in the early course of human acute pancreatitis, and concentration variations were closely related to the severity of AP and the BISAP score. The authors can determine the severity of AP more objectively, accurately and rapidly by measuring the levels of serum melatonin. When the melatonin concentration is at or below 28.74 ng/L, AP patients may develop SAP.

Applications

The study results suggest that exogenous melatonin may prevent the damage caused by AP due to its antioxidant, anti-inflammatory, and immunomodulatory properties. Variations of MT concentration might reflect the degree of severity of AP to some extent.

Terminology

The APACHE Ⅱ score contains 12 monitoring indicators, and the final score is composed of an acute physiology score, an age index and a chronic health index. The APACHE Ⅱ scoring system is the most commonly used scoring system for determining the severity and prognosis of AP. The BISAP scoring standard consists of five elements: blood urea nitrogen, disturbance of consciousness, systemic inflammatory response syndrome, age and pleural effusion.

Peer review

The authors focus on the clinical significance of melatonin concentrations in predicting the severity of AP. The results suggest that melatonin concentration variations are closely related to the severity of AP and the BISAP score. The serum melatonin level can be used to evaluate the severity of disease objectively, accurately and rapidly.

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META-ANALYSIS

Safety and efficacy of single-incision laparoscopic surgery for appendectomies: A meta-analysis

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Abstract

AIM: To compare single incision laparoscopic surgery for an appendectomy (SILS-A) with conventional laparoscopic appendectomy (C-LA) when implemented by experienced surgeons.

METHODS: Studies and relevant literature regarding the performance of single-incision laparoscopic surgery *vs* conventional laparoscopic surgery for appendectomy were searched for in the Cochrane Central Register of Controlled Clinical Trials, MEDLINE, EMBASE and World Health Organization international trial register. The operation time (OR time), complications, wound infection and postoperative day using SILS-A or C-LA were pooled and compared using a meta-analysis. The risk ratios and mean differences were calculated with 95%CIs to evaluate the effect of SILS-A.

RESULTS: Sixteen recent studies including 1624 patients were included in this meta-analysis. These studies demonstrated that, compared with C-LA, SILS-A has a similar OR time in adults but needs a longer OR time in children. SILS-A has similar complications, wound infection and length of the postoperative day in adults and children, and required similar doses of narcotics in children, the pooled mean different of -0.14 [95%CI: -2.73-(-2.45), $P > 0.05$], the pooled mean different of 11.47 (95%CI: 10.84-12.09, $P < 0.001$), a pooled RR of 1.15 (95%CI: 0.72-1.83, $P > 0.05$), a pooled RR of 1.9 (95%CI: 0.92-3.91, $P > 0.05$), a pooled RR of 1.01 (95%CI: 0.51-2.0, $P > 0.05$) a pooled RR of 1.86 (95%CI: 0.77-4.48, $P > 0.05$), the pooled mean different of -0.25 (95%CI: -0.50-0, $P = 0.05$) the pooled mean different of -0.01 (95%CI: -0.05-0.04, $P > 0.05$) the pooled mean different of -0.13 (95%CI: -0.49-0.23, $P > 0.05$) respectively.

CONCLUSION: SILS-A is a technically feasible and reliable approach with short-term results similar to those obtained with the C-LA procedure.

Key words: Single incision; Laparoscopy; Appendicitis; Children; Adult

Core tip: Single incision laparoscopic surgery for an appendectomy (SILS-A) is widely accepted and has become the best option for treatment of appendicitis. Compared with conventional laparoscopic appendectomy, the safety and efficacy of SILS-A is not known. This study clarified that SILS-A has a similar operation time in adults but needs more time in children, has similar complications, wound infection and length of the postoperative day in adults and children, and needs similar doses of narcotics in children.

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INTRODUCTION

Today, approximately 8% of the population will undergo appendectomy for acute appendicitis over their lifetime in Europe. An appendectomy comprises the surgical resection of the appendix and is frequently performed as an emergency process in the management of a patient suffering from acute appendicitis, a condition in which the appendix becomes inflamed and putrescent. The operation can be performed with minimally invasive surgery or as an open procedure.

Laparoscopic surgery was first used about 100 years ago, and the concept of minimally invasive surgery has significantly affected the field of traditional surgery. The first laparoscopic appendectomy was performed by the gynecologist Semm[1]. In a classic laparoscopic appendectomy, three to four incisions are required for the placement of multiple trocars. Driven by a quest toward less abdominal trauma in surgery, improved cosmesis, the potential reduction in postoperative pain, and a shorter hospital stay, specialty cameras, ports, and instruments have been developed, and minimal access surgery has undergone an accelerated process of evolution. A recent development in appendectomy has been the introduction of less invasive methods.

Single incision laparoscopic surgery applies a single multi-luminal port, or multiple mono-luminal ports, through a single skin incision. With the appearance of natural orifice translumenal endoscopic surgery, single incision laparoscopic surgery for an appendectomy (SILS-A) can be used to perform advanced[2-10], as well as preliminary procedures[11-24]. While this technique has been embraced by surgeons worldwide, the procedures and instruments used are still in the basic stages of investigation. Currently, two different methods exist for single-incision access. One involves the application of traditional, low profile laparoscopic ports that are clustered within a single skin incision, but penetration the peritoneal cavity through separate fascial incisions. The other involves the adoption of specialized ports created to provide multiple channels through a single port for one larger fascial incision. Both of methods have a good cosmetic effect. Despite its ameliorating effects, conventional laparoscopic appendectomy (C-LA) still requires three to four abdominal incisions for completion of the procedure. Each incision adds to potential morbidity risks, including bleeding, hernia, or internal organ damage[25,26]. There is little published data on the feasibility, safety, and clinical advantage of the procedure. Therefore, this study will analyze and compare the short-term surgical results of SILS-A and C-LA. The primary aim of this meta-analysis was to evaluate SILS-A *vs* C-LA; the secondary aims were to determine the difficulties, limitations or advantages of SILS-A.

MATERIALS AND METHODS

Publication search

Four bibliographic databases (Cochrane Central Register of Controlled Clinical Trials, MEDLINE, EMBASE and the World Health Organization international trial register) were searched for all relevant literature, including articles referenced in the publications. The medical subject headings (MeSH) and keywords searched for individually and in combination were as follows: "single-incision laparoscopic surgery" "multiport laparoscopic surgery" or "conventional laparoscopic" and "appendectomies". The last search was done on January 20, 2013.

Inclusion and exclusion criteria

The inclusion criteria were as follows: (1) patients should be at least 1 year of age; (2) suspected acute appendicitis on clinical and radiographic (computed tomography) grounds; (3) male or female (excluding pregnant females); (4) patients with American Society of Anesthesiology score < 3; (5) patients informed about the study, and will have read, understood and signed the patient informed; and (6) studies that provided information on at least one of the outcome measures. When a study reporting the same patient cohort was included in several publications, only the most recent or complete study was selected.

The exclusion criteria were as follows: (1) prior open laparotomy with incision through the umbilicus; (2) mental illness, dementia, or inability to provide informed consent; (3) chronic pain requiring daily medication (including opiates and NSAIDs); (4) pregnancy; (5) case reports; (6) articles that were not full text, or non-comparative studies; and (7) open operations.

Data extraction

For identified eligible studies, two reviewers using a standard form containing pre-specified outcomes would have undertaken data extraction independently. Clarification was sought where there was potential data collection but not reported. Any differences of opinion were resolved among the reviewers, and where necessary referred to a fourth party for arbitration.

Statistical analysis

Statistical analysis was performed using Review Manager (RevMan) software version 5.0.0 (The Nordic Cochrane Centre, The Cochrane Collaboration, Copenhagen, Denmark). A pooled RR and a pooled Mean Different with 95%CI were used to assess outcomes of the studies; statistical heterogeneity was tested by the χ^2 test. According to the Forest plot, heterogeneity was limited, so we used the Mantel-Haenszel fixed effect model. The significance of the pooled RR was determined by the *Z* test and statis-

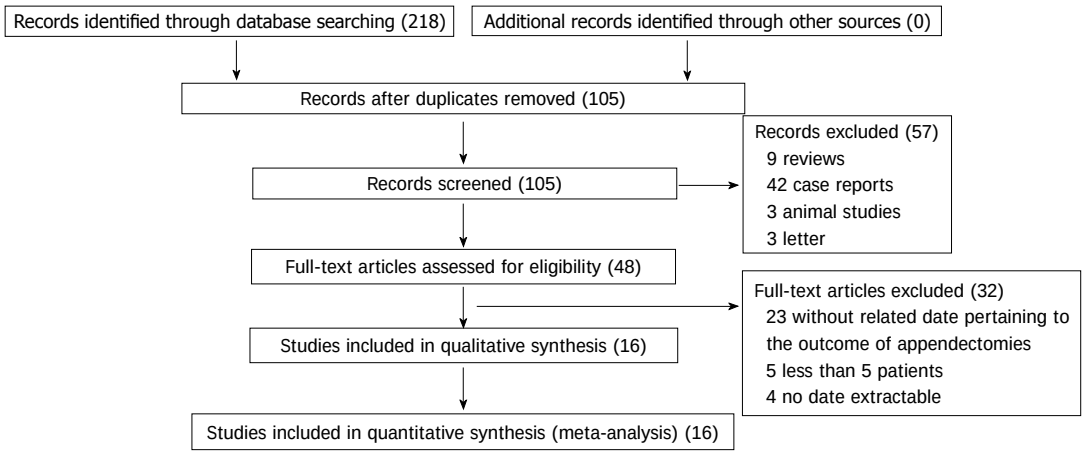


Figure 1 Flow chart for the selection of the studies.

Table 1 Main characteristics of the 10 included studies in adults

Ref.	Year	SILS-A (*n*)	C-LA (*n*)	Age (yr)		M:F	
				SILS-A	C-LA	SILS-A	C-LA
Lee *et al*[27]	2009	72	108	30.3 ± 16.4	33.6 ± 18.6	24:46	56:52
Cho *et al*[28]	2011	23	20	44.7	39.2	14:9	11:9
Teoh *et al*[29]	2011	30	60	32.97 ± 13.31	34.88 ± 11.45	19:11	38:22
Park *et al*[30]	2012	42	62	23.9 ± 11.9	29.9 ± 12.2	14:28	42:21
Kim *et al*[31]	2011	17	33	21.0	28.0	1:10	21:12
Vilallonga *et al*[32]	2012	46	41	34.2 (13.3)	37.7 (13.2)	19:27	22:19
Raakow *et al*[39]	2011	20	20	27.75 ± 8.26	31.75 ± 9.30	8:12	10:10
Amos *et al*[40]	2011	27	17	37.74 ± 18.85	33.71 ± 12.50	6:21	6:11
Chow *et al*[41]	2010	40	33	31.65 ± 15.36	29.85 ± 14.93	18:22	15:18
Kang *et al*[42]	2010	15	25	35.5 ± 13.2	37.9 ± 14.5	8:07	14:11

SILS-A: Single-incision laparoscopic surgery for appendectomy; C-LA: Conventional laparoscopic appendectomy; M: Male; F: Female.

tical significance was considered at $P < 0.05$. Publication bias was estimated using a funnel plot with an Egger's linear regression test, and funnel plot asymmetry on the natural logarithm scale of the RR was measured by a linear regression approach.

RESULTS

Study characteristics

In total, 16 studies were included in the meta-analysis[27-42]. All of these studies were published after 2009 and comprised 751 adult patients, of whom 332 were operated on using SILS-A and 419 were operated on using C-LA. The sample size of the trials ranged from 15 to 108. Eight hundred and seventy three of the patients were children, of whom 428 were operated on using SILS-A and 445 were operated on using C-LA. The sample size of the trials ranged from 8 to 180. Moreover, some studies reported single-incision laparoscopic surgery for appendicitis, but did not report information regarding C-LA and were therefore not compared in this meta-analysis. Other studies did not provide any information about SILS-A *vs* C-LA, and were excluded in present meta-analysis (Figure 1). Tables 1-4 list the main characteristics of the 16 studies included in this analysis.

Meta-analysis results

The present meta-analysis demonstrated the pooled mean different of [-0.14, 95%CI: -2.73-(-2.45), $P > 0.05$, Figure 2A], the pooled RR of 1.15 (95%CI: 0.72-1.83), $P > 0.05$, Figure 3A) a pooled RR of 1.01 (95%CI: 0.51-2.0), $P > 0.05$, Figure 4A, a pooled mean different of -0.25 (95%CI: -0.5-0.0), $P = 0.05$, Figure 5A, a pooled mean different of 11.47 (95%CI: 10.84-12.09), $P < 0.001$, Figure 2B, a pooled RR of 1.9 (95%CI: 0.92-3.91), $P > 0.05$, Figure 3B, the pooled RR of 1.86 (95%CI: 0.77-4.48), $P > 0.05$, Figure 4B, the pooled mean different of -0.01 (95%CI: -0.05-0.04), $P > 0.05$, Figure 5B, the pooled mean different of -0.13 (95%CI: -0.49-0.23), $P > 0.05$, Figure 6, respectively. It revealed that SILS-A is feasible, and appears to have results similar to C-LA in our comparisons. But, in children, SILS-A needs more operative time than C-LA.

Operation time (min): Nine studies (701 patients) provided data on operation time for adults. The pooled results indicated that SILS-A has similar results to C-LA [weighted mean differences (WMD), -0.14 (95%CI: -2.73-2.45), $P > 0.05$]. The $\chi^2$and I^2 were 21.57 ($P = 0.0006$) and 63%, respectively, indicating heterogeneity among the studies (Figure 2A). Six studies (873 patients)

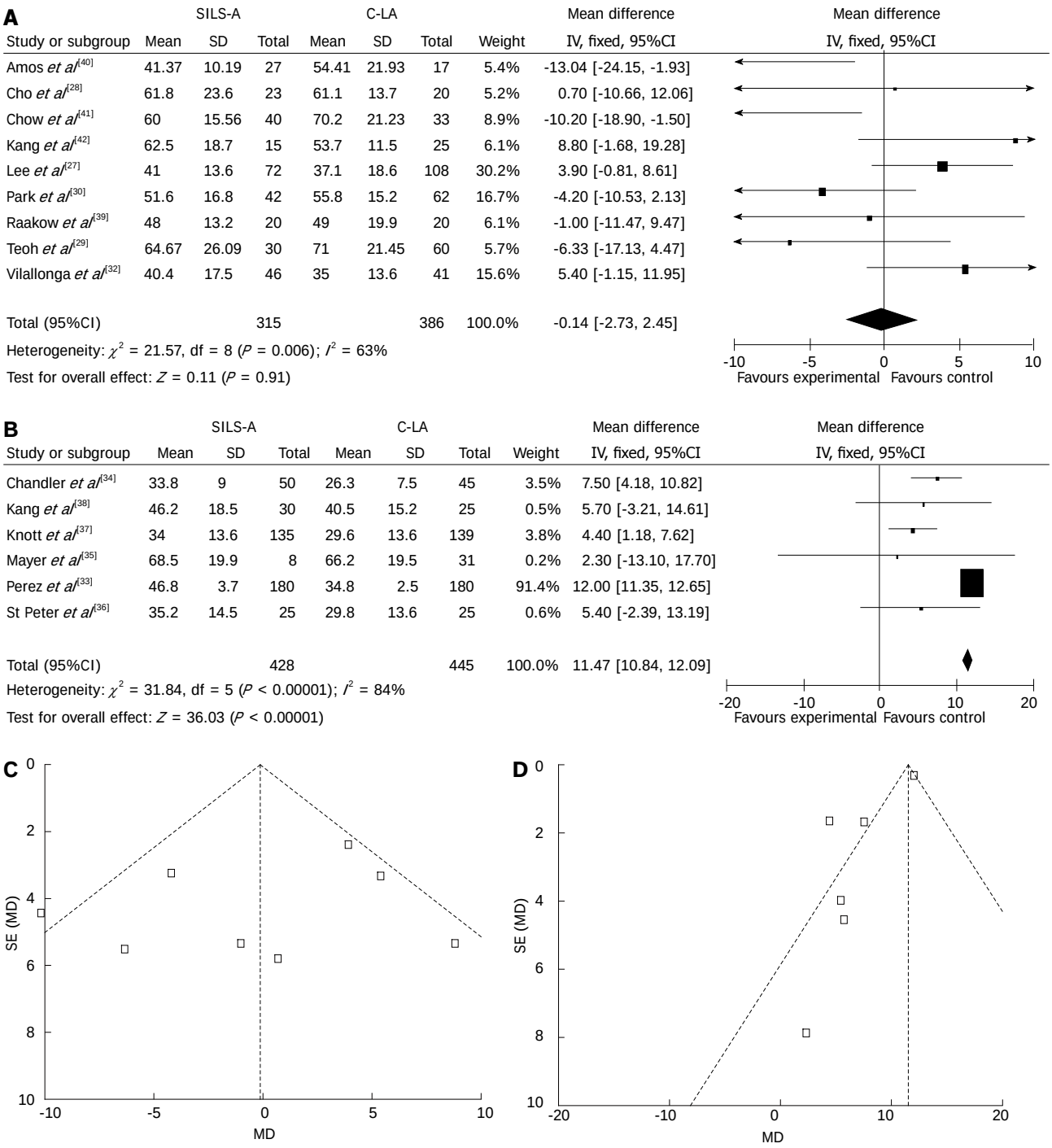


Figure 2 Forest plot of the comparison of single-incision laparoscopic surgery for appendectomies *vs* conventional laparoscopic appendectomy in terms of short-term results, outcome: operation time (min). A: Single-incision laparoscopic surgery for appendectomies (SILS-A) *vs* conventional laparoscopic appendectomy (C-LA) in terms of short-term results for adult; B: SILS-A *vs* C-LA in terms of short-term results for children; C: SILS-A *vs* C-LA in terms of short-term results for adult, mean differences (MDs); D: SILS-A *vs* C-LA in terms of short-term results for children, MDs. MDs are shown with 95%CI.

provided data on operation time for children. The pooled the indicated that SILS-A requires more time than C-LA [WMD, 11.47 (95%CI: 10.84-12.09), $P < 0.001$]. The χ^2 and I^2 were 31.84 ($P < 0.001$) and 84%, respectively, indicating heterogeneity among the studies (Figure 2B).

Complications: Ten studies (751 patients) provided data on complications in adults. Complications occurred in 27 of 332 (8.1%) patients after SILS-A and in 33 of 419 (7.8%) after C-LA. Pooling the results indicated that SILS-A had slightly, but not significantly, more complications than C-LA [WMD 1.15 (95%CI: 0.72-1.83), $P > 0.05$]. The χ^2 and I^2 were 6.16 ($P = 0.72$) and 0%, which excluded heterogeneity in the studies (Figure 3A). Six studies (873 patients) provided data on complications in children. Complications occurred in 18 of 428 (4.2%) patients after SILS-A and in 11 of 445 (2.4%) patients after C-LA. Pooling the results indicated that SILS-A and C-LA

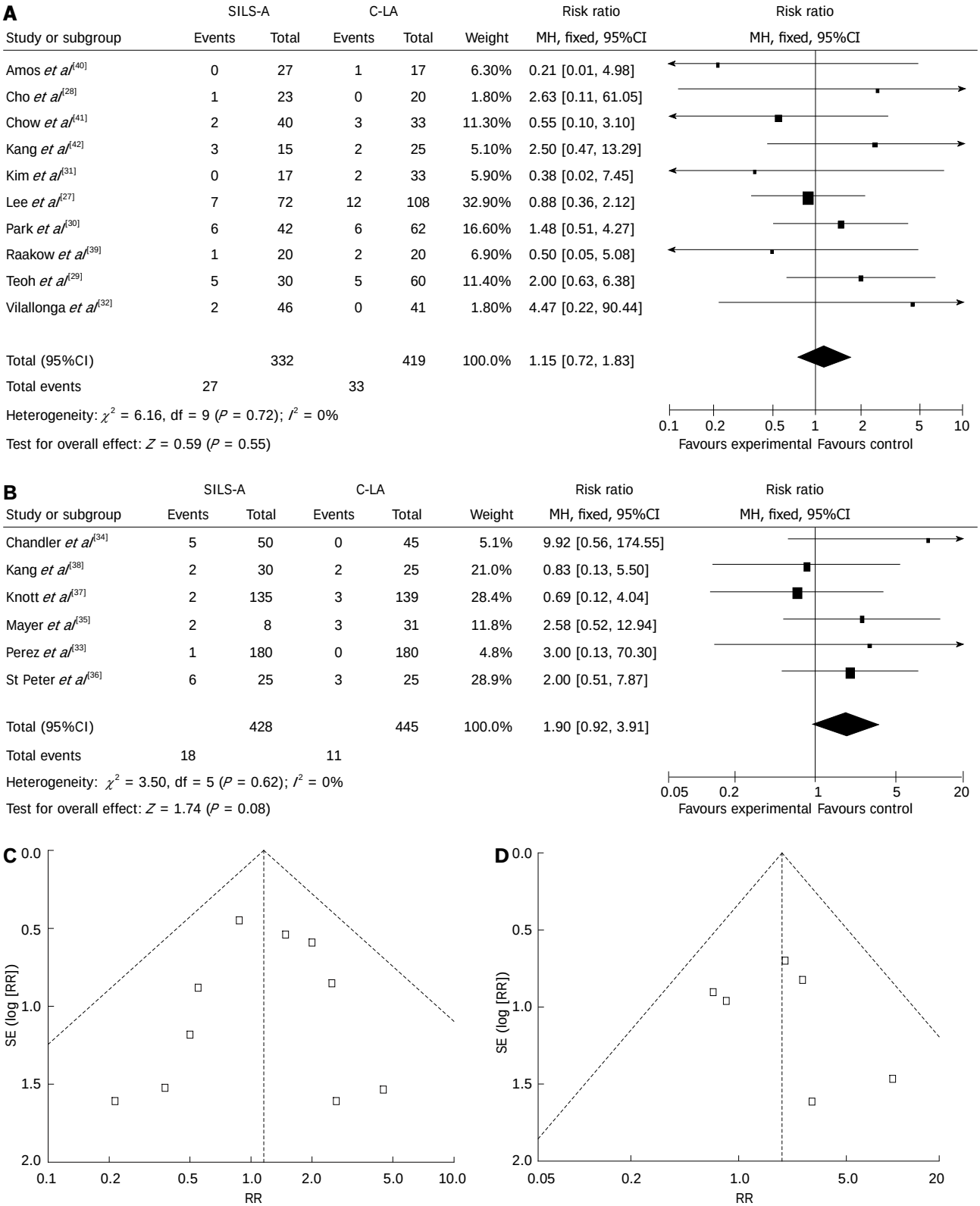


Figure 3 Forest plot of comparison: Single-incision laparoscopic surgery for appendectomies *vs* conventional laparoscopic appendectomy in terms of short-term results, outcome: Complications. A: Single-incision laparoscopic surgery for appendectomies (SILS-A) *vs* conventional laparoscopic appendectomy (C-LA) in terms of short-term results for adult; B: SILS-A *vs* C-LA in terms of short-term results for children; C: SILS-A *vs* C-LA in terms of short-term results for adult, risk ratios (RRs); D: SILS-A *vs* C-LA in terms of short-term results for children, RRs. RRs are shown with 95%CI.

have the similar levels of complications [WMD a pooled RR of 1.9 (95%CI: 0.92-3.91), $P > 0.05$]. The χ^2 and I^2 were 3.5 ($P = 0.62$) and 0%, which excluded heterogeneity in the studies (Figure 3B).

Wound infection: Seven studies (577 patients) provided

Table 2 Main characteristics of the six included studies in children

Ref.	Year	SILS-A (*n*)	C-LA (*n*)	Age		M:F	
				SILS-A	C-LA	SILS-A	C-LA
Perez *et al*[33]	2012	25	25	8.7 ± 0.6	8.9 ± 0.6	10:15	15:10
Chandler *et al*[34]	2010	50	45	11.1 ± 3.6	11.7 ± 3.8	26:24	34:11
Mayer *et al*[35]	2011	8	31	12.3 ± 2.4	12.3 ± 2.4		
St Peter *et al*[36]	2011	180	180	11.1 ± 3.5	11.1 ± 3.3	99:81	92:88
Knott *et al*[37]	2012	135	139	11.0 ± 3.5	10.9 ± 3.4	72:63	70:69
Kang *et al*[38]	2011	30	25	9.3 ± 4.0	8.7 ± 3.5	17/13	14/11

SILS-A: Single-incision laparoscopic surgery for appendectomy; C-LA: Conventional laparoscopic appendectomy; M: Male; F: Female.

Table 3 Result of the 10 included studies in adult children

Ref.	Postoperative day (d)		Complications		OR time (min)		Wound infection	
	SILS-A	C-LA	SILS-A	C-LA	SILS-A	C-LA	SILS-A	C-LA
Lee *et al*[27]	2.0 ± 1.4	2.0 ± 1.3	7	12	41.0 ± 13.6	37.1 ± 18.6	4	7
Cho *et al*[28]			1	0	61.8 ± 23.6	61.1 ± 13.7		
Teoh *et al*[29]			5	5	64.67 ± 26.09	71 ± 21.45	2	4
Park *et al*[30]	2.6 ± 1.0	2.9 ± 1.9	6	6	51.6 ± 16.8	55.8 ± 15.2	3	2
Kim *et al*[31]			0	2			0	2
Vilallonga *et al*[32]			2	0	40.4 ± 17.5	35.0 ± 13.6		
Raakow *et al*[39]	4.12 ± 0.61	4.65 ± 0.98	1	2	48.0 ± 13.2	49.0 ± 19.9	1	1
Amos *et al*[40]	3.70 ± 2.52	3.82 ± 1.24	0	1	41.37 ±10.19	54.41 ± 21.93	5	
Chow *et al*[41]	1.36 ± 0.95	2.36 ± 2.62	2	3	60.0 ± 15.56	70.2 ± 21.23	2	2
Kang *et al*[42]	6.8 ± 1.8	6.4 ± 1.6	3	2	62.5 ± 18.7	53.7 ± 11.5	1	1

OR time: Operation time; SILS-A: Single-incision laparoscopic surgery for appendectomy; C-LA: Conventional laparoscopic appendectomy.

Table 4 Result of the ix included studies in children

Ref.	Postoperative day (d)		Complications		OR time (min)		Wound infection		Doses of narcotics	
	SILS-A	C-LA	SILS-A	C-LA	SILS-A	C-LA	SILS-A	C-LA	SILS-A	C-LA
Perez *et al*[33]			1	0	46.8 ± 3.7	34.8 ± 2.5				
Chandler *et al*[34]	1.1 ± 0.4	1.2 ± 0.5	5	0	33.8 ± 9	26.3 ± 7.5	4	0	0.9 ± 0.9	1.4 ± 1.3
Mayer *et al*[35]	3.63 ± 1.2	3.68 ± 1.3	2	3	68.5 ± 19.9	66.2 ± 19.5			4.75 ± 3.3	7.33 ± 3.0
St Peter *et al*[36]	0.95 ± 0.3	0.93 ± 0.3	6	3	35.2 ± 14.5	29.8 ± 11.6	6	3	9.6 ± 4.9	8.5 ± 4.3
Knott *et al*[37]	0.92 ± 0.2	0.94 ± 0.3	2	3	34.0 ± 13.6	29.6 ± 13.6	2	3	5.7 ± 3.5	5.3 ± 3.2
Kang *et al*[38]	4.0 ± 1.5	3.8 ± 2.0	2	2	46.2 ± 18.5	40.5 ± 15.2	2	1		

OR time: Operation time; SILS-A: Single-incision laparoscopic surgery for appendectomy; C-LA: Conventional laparoscopic appendectomy.

data on wound infections in adults. Wound infections occurred in 13 of 236 (5.5%) patients after SILS-A and in 19 of 341 (5.6%) patients after C-LA. Pooling the results indicated that SILS-A and C-LA have the similar levels of wound infection [WMD 1.01 (95%CI: 0.51-2.0), $P > 0.05$]. The χ^2 and I^2 were 1.44 ($P = 0.96$) and 0%, which excludes heterogeneity in the studies (Figure 4A). Five studies (784 patients) provided data on wound infections in children. Wound infections occurred in 14 of 395 (3.5%) patients after SILS-A and in 7 of 398 (1.7%) patients after C-LA. The results indicated that SILS-A has more wound infections, but at an acceptable level. Pooling the results of wound infection [WMD 1.86 (95%CI: 0.77-4.48), $P > 0.05$]. The χ^2 and I^2 were 2.23 ($P = 0.53$) and 0%, which excluded heterogeneity in the studies (Figure 4B).

Postoperative days (d): Six studies (481 patients) provided data on postoperative days for adult. Pooling the results indicated that SILS-A has the slightly better results than C-LA [WMD -0.25 (95%CI: -0.50-0), $P > 0.05$]. The χ^2 and I^2 were 6.48 ($P = 0.26$) and 23%, respectively, indicating heterogeneity among the studies (Figure 5A). Five studies (822 patients) provided data on postoperative days for children. Pooling the results indicated that SILS-A has the same results as C-LA [WMD, -0.01 (95%CI: -0.05-0.04), $P > 0.05$]. The χ^2 and I^2 were 2.08 ($P = 0.72$) and 23%, respectively, which excluded heterogeneity in the studies (Figure 5B).

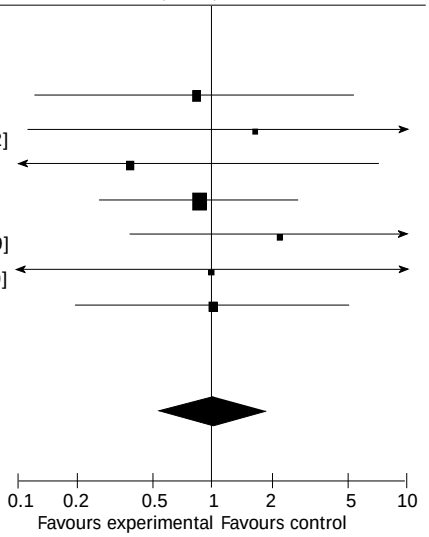
Doses of narcotics: Four studies (768 patients) provided data on doses of narcotics for children. Pooling the results indicated that SILS-A had similar results to C-LA, [WMD -0.25 (95%CI: -0.50-0), $P > 0.05$]. The χ^2 and I^2 were 14.25 ($P = 0.0003$) and 79%, respectively, indicating

A

Study or subgroup	SILSA Events	SILSA Total	C-LA Events	C-LA Total	Weight	Risk ratio MH, fixed, 95%CI
Amos *et al*[40]	0	0	0	0		Not estimable
Cho *et al*[28]	0	0	0	0		Not estimable
Chow *et al*[41]	2	40	2	33	14.1%	0.82 [0.12, 5.54]
Kang *et al*[42]	1	15	1	25	4.8%	1.67 [0.11, 24.72]
Kim *et al*[31]	0	17	2	33	11.1%	0.38 [0.02, 7.45]
Lee *et al*[27]	4	72	7	108	36.0%	0.86 [0.26, 2.82]
Park *et al*[30]	3	42	2	62	10.4%	2.21 [0.39, 12.69]
Raakow *et al*[39]	1	20	1	20	6.4%	1.00 [0.07, 14.90]
Teoh *et al*[29]	2	30	4	60	17.1%	1.00 [0.19, 5.15]
Vilallonga *et al*[32]	0	0	0	0		Not estimable
Total (95%CI)		236		341	100.0%	1.01 [0.51, 2.00]
Total events	13		19			

Heterogeneity: χ^2 = 1.44, df = 6 (P = 0.96); I^2 = 0%

Test for overall effect: Z = 0.04 (P = 0.97)

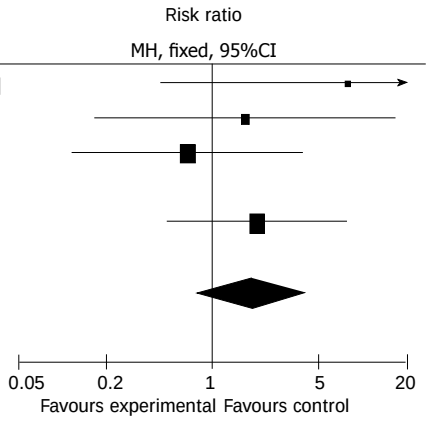


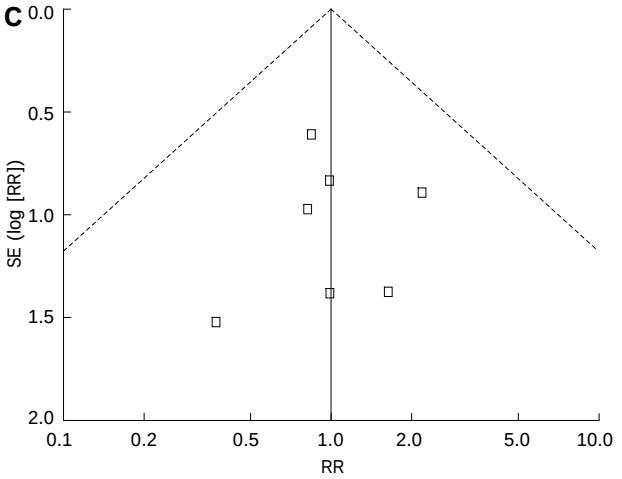
B

Study or subgroup	SILS-A Events	SILS-A Total	C-LA Events	C-LA Total	Weight	Risk ratio MH, fixed, 95%CI
Chandler *et al*[34]	4	50	0	45	6.9%	8.12 [0.45, 146.71]
Kang *et al*[38]	2	30	1	25	14.4%	1.67 [0.16, 17.32]
Knott *et al*[37]	2	135	3	139	39.0%	0.69 [0.12, 4.04]
Mayer *et al*[35]	0	0	0	0		Not estimable
St Peter *et al*[36]	6	180	3	180	39.6%	2.00 [0.51, 7.87]
Total (95%CI)		395		389	100.0%	1.86 [0.77, 4.48]
Total events	14		7			

Heterogeneity: χ^2 = 2.23, df = 3 (P = 0.53); I^2 = 0%

Test for overall effect: Z = 1.39 (P = 0.16)





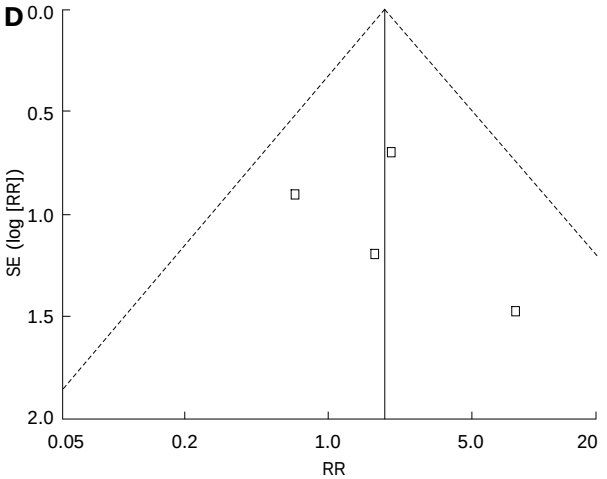


Figure 4 Forest plot of comparison: Single-incision laparoscopic surgery for appendectomies *vs* conventional laparoscopic appendectomy in terms of short-term results, outcome: wound infection. A: Single-incision laparoscopic surgery for appendectomies (SILS-A) *vs* conventional laparoscopic appendectomy (C-LA) in terms of short-term results for adult; B: SILS-A *vs* C-LA in terms of short-term results for children; C: SILS-A *vs* C-LA in terms of short-term results for adult, risk ratios (RRs); D: SILS-A *vs* C-LA in terms of short-term results for children, RRs. RRs are shown with 95%CI.

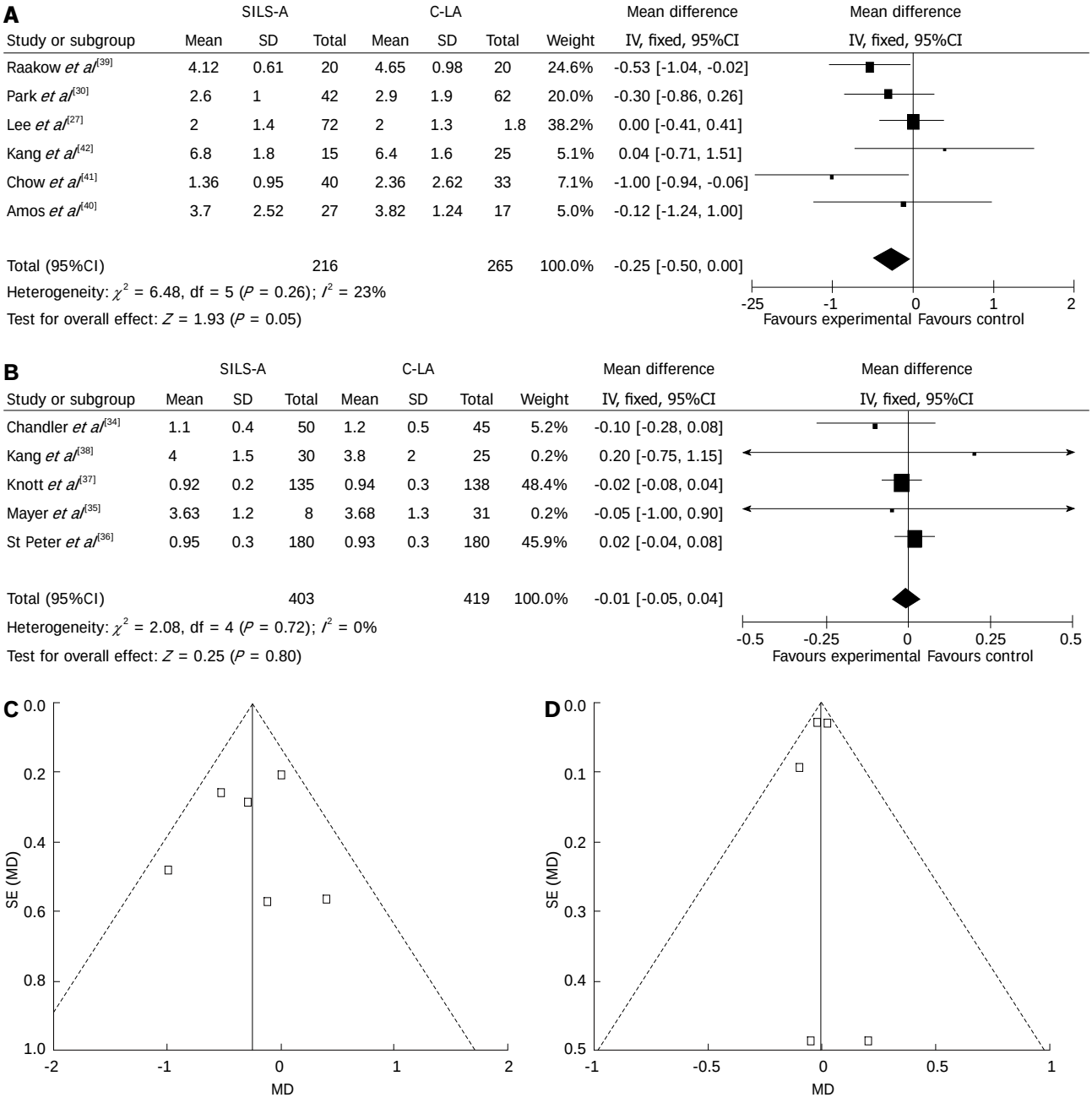


Figure 5 Forest plot of comparison: Single-incision laparoscopic surgery for appendectomies *vs* conventional laparoscopic appendectomy in terms of short-term results, outcome: postoperative day (d). A: Single-incision laparoscopic surgery for appendectomies (SILS-A) *vs* conventional laparoscopic appendectomy (C-LA) in terms of short-term results for adult; B: SILS-A *vs* C-LA in terms of short-term results for children; C: SILS-A *vs* C-LA in terms of short-term results for adult, mean differences (MDs); D: SILS-A *vs* C-LA in terms of short-term results for children, MDs. MDs are shown with 95%CI.

heterogeneity among the studies (Figure 6).

Publication bias

A funnel plot was created to access the publication bias of the literature. The shapes of the funnel plots did not reveal any evidence of obvious asymmetry (Figures 2C, 2D, 3C, 3D, 4C, 4D, 5C, 5D and 6B).

DISCUSSION

The straightforward conclusion from the 16 included studies is that compared with C-LA, SILS-A has acceptable complications, similar recovery, and the same OR times for patients.

Arguments against the use SILS-A cite the lack of evidence regarding patient benefit over open surgery or CL-A. The potential requirement for advanced instrumentation may also translate into increased costs. In addition, the lack of pneumoperitoneum leaks, triangulation, and instrument "clashing" are perceived as real disadvantages of this procedure, thereby increasing its difficulty. From our study, the umbilical incision permitted only

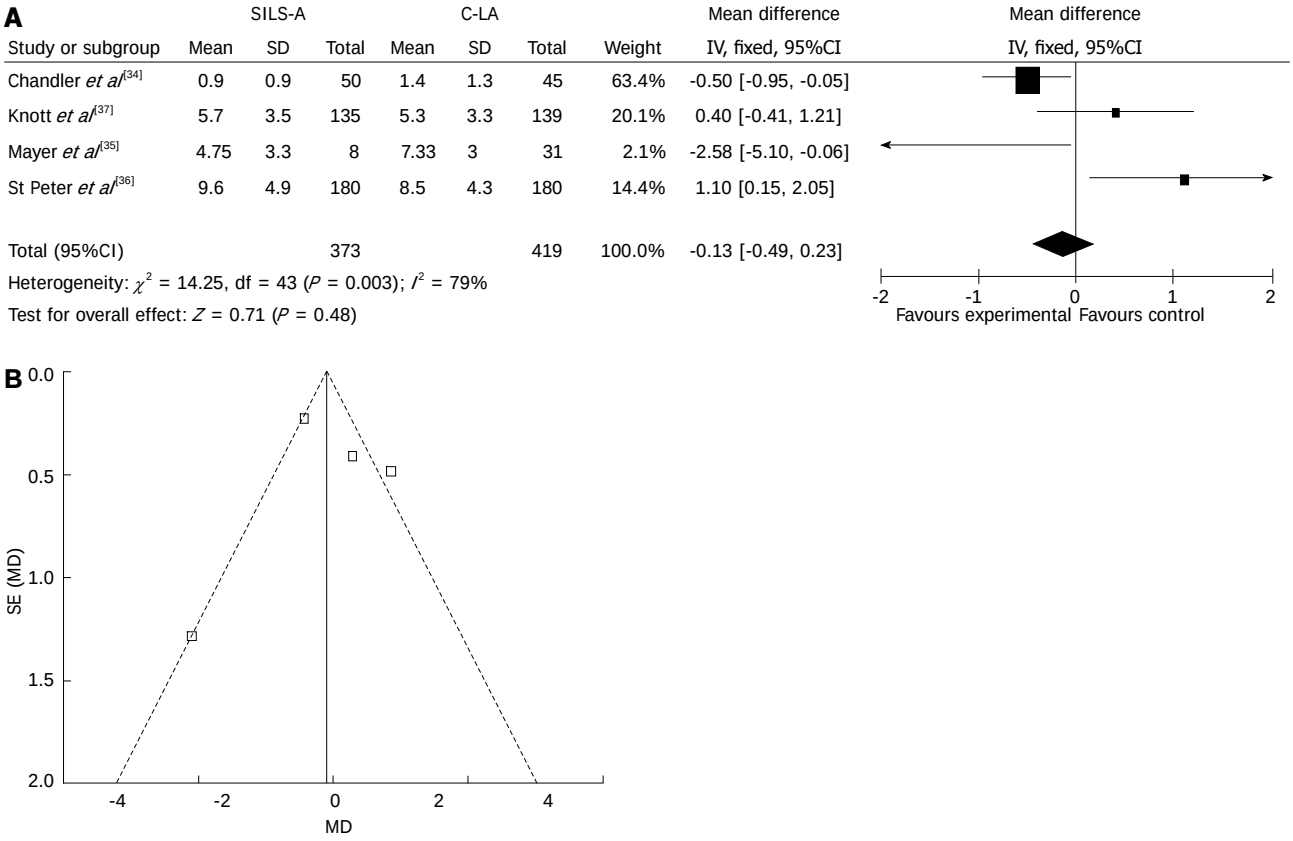


Figure 6 Forest plot of comparison: Single-incision laparoscopic surgery for appendectomies *vs* conventional laparoscopic appendectomy in terms of short-term results, outcome: doses of narcotics. A: Single-incision laparoscopic surgery for appendectomies (SILS-A) *vs* conventional laparoscopic appendectomy (C-LA) in terms of short-term results for children; B: SILS-A *vs* C-LA in terms of short-term results for children, mean differences (MDs). MDs are shown with 95%CI.

one laparoscope and one instrument into the abdominal cavity concomitantly, which ensured less trauma than the C-LA. Coaxiality was not a significant problem, except for a few of patients in whom we adopted flexible and rotating instruments. Moreover, tilting the operating table enabled us to achieve adequate exposure and dissection for the majority of patients. However, ligation of the appendix was a restricted phase of the procedure. In children, the surgery space is smaller and the lack of ancillary equipment increasing the difficulty. This is why SILS-A requires a longer time than C-LA in children at present. However, future research could be oriented toward the development of a 5-mm-diameter clip applier or sealing of the appendiceal base using energy sources, which would resolve this difficulty.

With the emergence of natural orifice translumenal endoscopic surgery, the new transumbilical approach seems to reduce the trauma of surgical access, improving postoperative pain and patient cosmesis compared to the conventional laparoscopic approach. The cosmetic outcomes of SILS-A are expected to be better if the operation is performed through the umbilicus. This is because the surgical wound is hidden within the umbilicus, leaving no visible abdominal scars. From our study, SILS-A has the same of OR times, recovery and complications as C-LA; however, SILS-A has more advantages than C-LA.

The total complication rate of 8% after SILS-A in our series was close to the 9%-14% published in the current literature for C-LA[43,44]. Extraction of the appendix through the abdominal wall is generally performed with a protected method. In our series, the risk of surgical-site infection was similar to C-LA. Although more wound infections occurred in the SILS-A group, this difference did not reach statistical significance. To answer the question of whether the wound infection rate is indeed higher for single-incision compared to C-LA, a larger number of patients are needed.

There are conflicting results regarding doses of narcotics required comparing SILS-A with C-LA, with some studies reporting higher doses of narcotics required after SILS-A[34-37] and others showing no difference. Some scholars reported that in SILS-A, early pain was more severe than in a C-LA. This might be caused by the skin incision. Although the skin incision in the umbilical area is small, the actual length of the fascia incision is much longer, and through the small incision region, all the laparoscopic equipment is used together, which stimulates the incision. From our study, there is no different between SILS-A and C-LA in children.

Postsurgical complications in patients who underwent SILS-A were treated without special side effects or complications, except for wound problems. Thus, SILS-A appears to be safe. Implementation in the identified RCT's showed a fairly low rate of complications in the SILS-A group.

However, major complications were not reduced. More large studies, with more stringent quality criteria, may improve the statistical power and provide proof of reduced morbidities. There is a common perception that although patients are released earlier after SILS-A, there are more readmissions. With a a 90 d follow up period, this is unlikely, especially in children. Although not statistically significant, it seems that SILS-A does decrease morbidities. However, the available data does not provide proof that SILS-A is superior to the conventional technique and more evidence should be provided. In addition, the quality of future trials should be higher to adequately advocate using SILS-A as the gold standard.

There are limitations to this meta-analysis. First, the sample size of some of the studies was quite low, as was the number of studies included in our meta-analysis; this may have biased the results. Second, not all of the included trials were randomized, which caused a lack of the required details. Third, we did not compare improvements in other comorbidities following SILS-A and C-LA, and these factors may be important in assessing and recommending the procedure. SILS-A is a comparatively new procedure that has become popular in recent years; therefore, there is also concern about the long-term results. The follow-up periods in most reports were 3 or 6 mo, and the studies analyzed here provided relatively short-term findings. However, we believe that, with greater awareness and the increasing popularity of SILS-A, studies comparing the two approaches in large volumes with long-term follow-up will be published.

In conclusion, this meta-analysis demonstrated that the SILS-A procedure is associated with significantly less bleeding, while providing an improved cosmetic outcome despite a modest increase the ratio of conversion. SILS-A is a technically feasible and reliable approach with short-term results similar to those obtained with C-LA. Prospective randomized studies comparing the two approaches in large patient cohorts with long-term follow-up will be needed to confirm the results reported.

COMMENTS

Background

Single incision laparoscopic surgery for an appendectomy (SILS-A) is widely accepted and has become the best option for treatment of appendicitis. Compared with conventional laparoscopic appendectomy (C-LA), the safety and efficacy of SILS-A is not known.

Research frontiers

Over the past three decades, many studies have assessed the performance of SILS-A. However, comparisons of SILS-A and C-LA for adults and children have not been published.

Innovations and breakthroughs

Based on this meta-analysis, single incision laparoscopic surgery does not increase the risk for an appendectomy. Similar associations were indicated in subgroup analyses of East Asian, Western, cohort, and high-quality studies. These findings were not presented clearly in previous systematic reviews.

Applications

Single incision laparoscopic surgery appears to be neither directly nor indirectly associated with the risk and pain of appendectomy. Further studies should seek to clarify this conclusion.

Peer review

SILS-A is rapidly becoming the focal point of attraction for specialists worldwide. This article shows the advantages of the procedure for adults and children. This analysis has great practical value for clinicians.

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CASE REPORT

Azathioprine-induced fever in autoimmune hepatitis

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Abstract

Underdiagnosis of drug-induced fever leads to extensive investigation and prolongation of hospitalization, and may lead to multiple unnecessary invasive procedures and a wrong diagnosis. Azathioprine is a widely used immunosuppressive drug. We report a case of a 53-year-old female patient diagnosed with autoimmune hepatitis treated with azathioprine, who presented to the emergency room with a 6-wk history of fever and chills without other associated symptoms. Since the patient's fever was of unknown origin, she was hospitalized. All treatment was stopped and an extensive workup to explore the source of fever and chills was performed. Results of chest X-ray, viral, urine, and blood cultures, autoimmune serology, transthoracic and transesophageal echocardiography, and abdominal ultrasound revealed no source of infection. A rechallenge test of azathioprine was performed and the fever and chills returned within a few hours. Azathioprine was established as the definite cause following rechallenge. Fever as an adverse drug reaction is often unrecognized. Azathioprine has been reported to cause drug-induced fever in patients with inflammatory bowel disease, rheumatoid arthritis, and sarcoidosis. To the best of our knowledge there have been no previous reports documenting azathioprine-induced fever in patients with autoimmune hepatitis. The occurrence of fever following the readministration of azathioprine suggests the diagnosis of drug-induced fever, particularly after the exclusion of other causes. A careful rechallenge is recommended to confirm the diagnosis.

Key words: Autoimmune hepatitis; Adverse drug reactions; Azathioprine; Drug fever

Core tip: Azathioprine is widely used in inflammatory disease such as rheumatoid arthritis, inflammatory bowel disease and post solid organ transplant such as kidney transplantation. Azathioprine is an immune modulator drug that can expose patients to various infections and clinical fever. Azathioprine needs to be remembered as a potential fever provoker in the differential diagnosis of fever origin.

Khoury T, Ollech JE, Chen S, Mizrahi M, Shalit M. Azathioprine-induced fever in autoimmune hepatitis. *World J Gastroenterol* 2013; 19(25): 4083-4086 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i25/4083.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i25.4083

INTRODUCTION

Drug reaction is an underestimated cause of fever. Drug-induced fever may be defined as a disorder characterized by the appearance of elevated body temperature 7-10 d following the administration of a specific medication, with resolution of the fever upon discontinuation of the suspected agent. Less often, the fever can appear after a long period of treatment. Drug-induced fever is estimated to account for about 10% of elevated body temperature evaluations[1,2] and fever as the sole symptom of a drug's side effect has been reported in about 3%-5%[3].

The diagnosis of drug-induced fever is usually challenging, especially in medically complicated patients with multiple treatment regimens. There are no clear guidelines for the diagnosis, which is usually made by exclusion of the responsible drug. Underdiagnosis of drug-induced fever may lead to extensive investigations, unnecessary antibiotic treatment, and prolonged hospitalization[4].

Azathioprine-induced fever is a relatively rare disorder. It has been reported in only a few patients with inflammatory bowel disease, rheumatoid arthritis, and sarcoidosis[5-7]. In several other reported cases, it was associated with accompanying manifestations such as pruritus and cutaneous rash[8,9].

We report the case of a patient with autoimmune hepatitis who developed fever and chills induced by azathioprine. Rechallenge confirmed the diagnosis. To the best of our knowledge, this is the first described case of azathioprine-induced fever in autoimmune hepatitis.

CASE REPORT

A 53-year-old female of Arab descent was diagnosed in 2009 with autoimmune hepatitis by liver biopsy, which showed moderate inflammatory activity with infiltrating plasma cells suggestive of autoimmune hepatitis. Serological tests for anti-nuclear antibody (ANA), anti-double-stranded antibodies, and anti-parietal antibodies were positive, and serum immunoglobulins IgM 329 mg/dL (normal range 65-280 mg/dL), IgA 786 mg/dL (90-450 mg/dL), IgG 3120 mg/dL (800-1700 mg/dL) were elevated (Table 1). The patient had been treated with budesonide until 6 wk before admission, when azathioprine 100 mg/d was added to the treatment regimen.

Her medical history was notable for valvular heart disease, paroxysmal atrial fibrillation, hypertension, hypothyroidism, diabetes mellitus, and microcytic anemia. Her medications included warfarin 22.5 mg once daily, metformin 850 mg 3 times daily, omeprazole 20 mg once daily, losartan 80 mg twice daily, insulin detemir 20 units once daily, insulin aspart 10 units 3 times daily, metoprolol 25 mg twice daily, spironolactone 100 mg once daily, aspirin 100 mg daily, furosemide 40 mg once daily, azathioprine 50 mg once daily, and thyroxine 50 mg once daily. She presented to the emergency room due to fever and chills of several weeks duration, without other associated symptoms. On admission, the patient was stable; blood pressure was 155/69 mmHg, pulse rate 62 beats/min, and temperature 38.4 ℃. Her physical examination was unremarkable except for small skin erosion with surrounding erythema in her anterior abdominal wall. Blood tests showed a white blood cell count of 7.700 per cubic millimeter (4-10 thousand per cubic millimeter) with 90% neutrophils (40%-70%), 5.5% lymphocytes (15%-41%) and 3.5% monocytes (1%-7%). Hemoglobin was 9.3 g (12-16 g). Results of blood chemistry, including electrolytes and kidney function, were normal except for mild elevation of liver function tests. Urinalysis showed a few leukocytes but otherwise was normal. Nasal viral cultures were negative. C-reactive protein and erythrocyte sedimentation rate were normal. Computed tomography was performed to exclude an intra-abdominal cause of fever and revealed a small area of subcutaneous inflammation adjacent to the skin erosion. Chest X-ray showed no changes suggestive of infection.

Table 1 Revised international autoimmune hepatitis group scoring system for the diagnosis of autoimmune hepatitis

Clinical feature	Score	Patient
Female gender	+2	+2
ALP:AST ratio		+2
< 1.5	+2	
1.5-3.0	0	
> 3.0	-2	
Serum globulin or IgG above normal		+2
> 2.0	+3	
1.5-2.0	+2	
1.0-1.5	+1	
< 1.0	0	
ANA, SMA, LKM1		+3
> 1:80	+3	
1:80	+2	
1:40	+1	
< 1:40	0	
Illicit drug use history		+1
Positive	-4	
Negative	+1	
Average alcohol intake daily		+2
< 25 g/d	+2	
> 60 g/d	-2	
Histologic findings		Lymphoplasmacytic infiltrate
Interface hepatitis	+3	+1
Lymphoplasmacytic infiltrate	+1	
Rosette formation	+1	
None of the above	-5	
Biliary changes	-3	
Other changes	+2	
Other autoimmune disease	+2	No other autoimmune disease
AMA positivity	-4	AMA negative
Hepatitis viral markers		+3
Positive	-3	
Negative	+3	
Aggregate score without treatment		Overall 16 points
Definite AIH	> 15	
Probable AIH	10-15	

ALP: Alkaline phosphatase; AST: Aspertate aminotransferase; AIH: Autoimmune hepatitis; ANA: Anti-nuclear antibody; SMA: Soluble ribonucleic acid; LKM: Liver-kidney microsomal; IgG: Immunoglobulin G.

The patient was admitted to the Department of Internal Medicine with an initial diagnosis of fever, most likely secondary to the localized skin infection in the abdominal wall. Azathioprine was withheld and the patient was treated with antibiotics. Within several hours the fever subsided and the patient remained afebrile throughout 5 d of hospitalization.

Following recovery and resolution of the cellulitis, the patient was discharged and azathioprine was re-administered. Within a few hours, fever and chills recurred and the patient was re-admitted to the hospital. An extensive work-up for fever of unknown origin was done including blood and urine cultures and nasal viral cultures, which were all negative. Serological tests for human immuno-

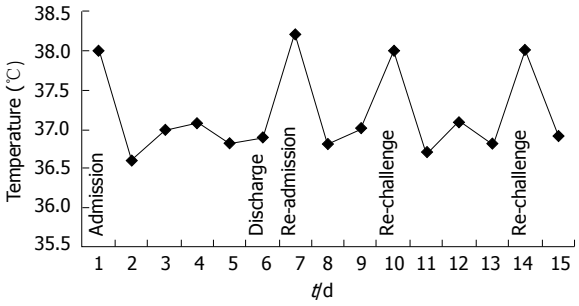

Figure 1 Patient fever scale during hospitalization and rechallenge test.

deficiency virus, hepatitis viruses, brucella, Q fever and toxoplasma were negative. Autoimmune serology showed ANA 2 of 4, anti-parietal cell antibodies 4 of 4; anti-smooth muscle, anti-mitochondrial and immunoglobulins were negative. In addition, ultrasonography of the abdomen revealed no evidence of ascites or infection. Transthoracic and transesophageal echocardiography did not show vegetation or abscess. Purified protein derivative test was negative. Again azathioprine was withheld and the fever resolved within a few hours.

With the patient's informed consent, a rechallenge with one tablet of 50 mg azathioprine was performed twice under observation. Chills appeared within 3 h and there was a gradual increase in temperature up to 38 ℃ within 7 h. Fever and chills resolved completely within 6 h after discontinuation of azathioprine.

DISCUSSION

Drug-induced fever is an underdiagnosed condition, particularly in patients treated with a large number of medications. It is described as a febrile response that coincides temporally with the administration of a drug and disappears after discontinuation of the offending agent. It is usually suspected when no other cause for fever can be explained, leaving drug fever as the diagnosis of exclusion.

Several mechanisms have been implied in the pathogenesis of drug-induced fever, most importantly hypersensitivity reaction involving the formation of antigen-antibody complexes. Common agents associated with hypersensitivity type reaction include penicillins, cephalosporins, phenytoin, methyldopa, procainamide, and antitubercular agents[2,3,10]. Other mechanisms include idiosyncratic reactions, drug effects on the thermoregulatory center, reactions to drug administration, and the extension of drug pharmacological effects as seen in patients who developed cell lysis and release of variable pyrogenic substances following chemotherapy treatment[1,11-13].

Azathioprine suppresses the immune system by inhibiting the activity of T cell lymphocytes. It is a prodrug which, following oral ingestion, is metabolized into active mercaptopurine, a purine synthesis inhibitor that impedes DNA synthesis and inhibits cell proliferation[14]. Azathioprine is known to cause multiple adverse effects, including fever, gastrointestinal symptoms, nervous system symptoms, bone marrow suppression, hepatic symptoms, myalgia, and arthralgia. Discontinuation of azathioprine is seen in about 10%-15% of patients due to side effects[5].

Several cases of azathioprine-induced fever in association with other symptoms have been reported; fever and chills were the only reported symptoms when azathioprine was administered to patients with sarcoidosis and inflammatory bowel disease, and after kidney transplantation[5,7,15,16].

In an observational study, three of 25 patients with RA developed symptoms of fever, chills, skin rash, hepatotoxicity, nausea, and diarrhea 2 wk after starting treatment with azathioprine. One patient developed only fever and chills. Rechallenge was performed in two patients with the appearance of more severe reactions[6]. In these patients, the febrile reaction appeared several days up to several weeks after beginning treatment with azathioprine. In our case the fever appeared within a few hours after restarting the offending agents. Thus the lag period between the initiation of the offending agent and the appearance of fever is highly variable.

In our patient, the sequence of events and the lack of objective evidence of infection are both highly suggestive of azathioprine-induced fever. In addition, previous reports of azathioprine causing drug fever in other disease states support the likelihood of a similar event in this patient[5-7]. With the patient's permission, two rechallenge tests were done in which 50 mg azathioprine was administered. Fever of 38 ℃ was measured about 7 h after administration, without other associated symptoms or objective findings on blood analysis, and without recurrence of the fever upon discontinuation of azathioprine (Figure 1). Thus, fever and chills in our case are the only manifestations of azathioprine and were likely caused by the administration of azathioprine.

In conclusion, drug reaction is an underestimated cause of fever. Clinicians should be aware of the fact that an immunomodulatory drug such as azathioprine, commonly used to treat various autoimmune conditions and to suppress the inflammatory response, can cause fever and chills. It is thus essential to withdraw a suspected medication and perform a rechallenge when fever fails to regress in patients already treated with antibiotics.

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CASE REPORT

Rectal arterio-portal fistula: An unusual cause of persistent bleeding per rectum following a proximal spleno-renal shunt

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Abstract

Gastrointestinal arterio-venous malformations are a known cause of gastrointestinal bleeding. We present a rare case of persistent rectal bleeding due to a rectal arterio-portal venous fistula in the setting of portal hypertension secondary to portal vein thrombosis. The portal hypertension was initially surgically treated with splenectomy and a proximal splenorenal shunt. However, rectal bleeding persisted even after surgery, presenting us with a diagnostic dilemma. The patient was re-evaluated with a computed tomography mesenteric angiogram which revealed a rectal arterio-portal fistula. Arterio-portal fistulas are a known but rare cause of portal hypertension, and possibly the underlying cause of continued rectal bleeding in this case. This was successfully treated using angiographic localization and super-selective embolization of the rectal arterio-portal venous fistula *via* the right internal iliac artery. The patient subsequently went on to have a full term pregnancy. Through this case report, we hope to highlight awareness of this unusual condition, discuss the diagnostic workup and our management approach.

Key words: Portal hypertension; Esophageal varices; Splenorenal shunt; Arteriovenous malformations; Portal vein thrombosis

Core tip: We present a rare case of persistent rectal bleeding due to a rectal arterio-portal venous fistula, in the setting of portal hypertension. Through this case report, we hope to highlight awareness of this unusual condition, and discuss the diagnostic workup and our subsequent management. We believe that this is the first of such cases reported in the literature.

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INTRODUCTION

Arterio-venous malformations occurring in the gastrointestinal tract are a known cause of gastro-intestinal bleeding and can often be missed especially in the presence of other pathology. They can lead to persistent symptoms if left undiagnosed and untreated. We present a patient who had portal vein thrombosis, portal hypertension and continued to have rectal bleeding in spite of a patent spleno renal

shunt to highlight awareness of this unusual condition.

CASE REPORT

A 33-year-old Chinese lady presented with multiple episodes of haematemesis when she was nine years old.She was seen in a secondary hospital and diagnosed to have portal hypertension secondary to portal vein thrombosis (PVT), a possible sequelae of a neonatal umbilical infection. The portal hypertension resulted in her having episodes of bleeding esophageal varices that were treated endoscopically during her childhood years. On clinical examination, she had splenomegaly but no stigmata of chronic liver disease. Investigations revealed that she did not have any liver cirrhosis or prothrombotic disorders. Liver function tests were normal. Radiological investigations confirmed the PVT and splenomegaly. More than twenty years after her initial diagnosis, she started to develop intermittent episodes of rectal bleeding which was attributed to rectal varices. She was anaemic (haemoglobin level 7.0 g/dL) and required intermittent blood transfusions for her symptoms. Her splenomegaly led to consumption thrombocytopenia (platelet levels ranged from 74-103 $\times$ 10^9 g/L) and she subsequently sought a second opinion at a tertiary centre, after much investigation including a contrast enhanced computed tomography scan, upper gastrointestinal endoscopy and a colonoscopy.

Pre-operative investigations were reviewed and showed PVT, splenomegaly and a dilated inferior mesenteric vein all the way down to the pelvis. Colonoscopy reported that there were large rectal varices. Based on these investigations, we presumed that the rectal varices were a result of left sided portal hypertension with the pressure transmitted down the inferior mesenteric vein, and thus performed a splenectomy and an end-to-side proximal splenorenal shunt for portal decompression.

After surgery, the patient continued to have rectal bleeding during her follow-up. A flexible sigmoidoscopy was performed and this revealed almost circumferentially dilated pulsatile submucosal vessels at the lower rectum. These were not reported in the colonoscopy that the patient underwent previously in the secondary hospital. A multiphasic computed tomography mesenteric angiogram confirmed a rectal arterio-venous fistula. On the arterial phase of the scan, there was evidence of arterio-venous shunting as evidenced by the dilated superior rectal vein draining into the superior mesenteric vein, with arterial feeders from the internal iliac arteries (Figure 1). A diagnostic catheter angiogram was performed to confirm the complexity of the feeder vessels and assess the potential collateral damage to rectal mucosa that could occur with angioembolisation. This revealed that there were two major arterial feeders arising from the anterior division of the internal iliac arteries on both sides which were supplying the arterio-portal fistula. There was a single tortuous drainage vein into the inferior mesenteric vein (Figure 2).

A super selective angioembolisation of the arterio-portal fistula was performed *via* a left common femoral artery access. The EV3 Apollo microcatheter (ev3 Endovascular Inc., Plymouth, MA, United States) was introduced and advanced into the arterio-portal fistula nidus and embolisation performed using 2cc of Onyx 18 embolic agent (ev3 Endovascular Inc., Plymouth, MA, United States). Completion angiograms *via* both internal iliac arteries showed successful obliteration of the arterio-portal fistula with cessation of arterio-portal shunting (Figure 3).

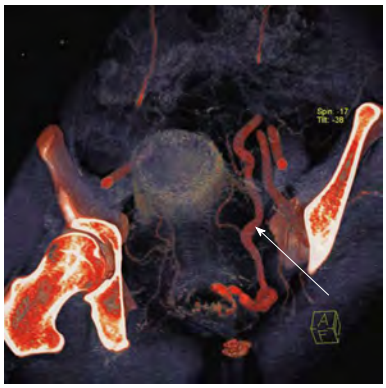

Figure 1 Computed tomography angiography image (post processed with **volume rendering technique) showing rectal arterio-portal fistula draining** into dilated superior rectal vein (arrow).

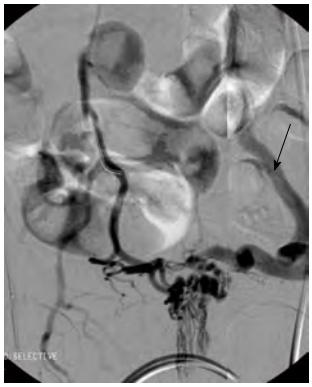

Figure 2 Pelvic angiography image showing the rectal arterio-portal fistula draining into the dilated superior rectal vein (arrow).

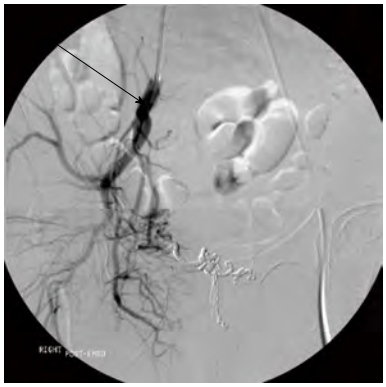

Figure 3 Post embolization angiogram from the anterior branch of the **right internal iliac artery (arrow) showing obliteration of blood flow in the arterioportal fistula.**

Post procedure, clinical examination and a repeat flexible sigmoidoscopy confirmed that there was no rectal ischemia. However, the patient complained of anal pain and further rectal bleeding, although the quantity of bleeding was much reduced. On clinical examination, she was found to have an anal fissure at 3 o'clock position, possibly as a result of the embolisation. This was initially treated with topical methods but did not resolve her symptoms and thus she underwent a lateral anal sphincterotomy. Since surgery, she has successfully delivered a child after a full-term pregnancy and her haemoglobin (12.0 g/dL) and platelet levels have returned to normal.

DISCUSSION

Arteriovenous malformations (AVMs) in the gastrointestinal tract are a known cause of gastrointestinal bleeding, and are usually difficult to diagnose. In the literature, it has been reported that the highest frequency of gastrointestinal AVMs occur in the right sided colon[1]. Arteriovenous malformations in the rectum are rare and it is often difficult to distinguish between bleeding from the AVM and bleeding from haemorrhoids, leading to diagnostic delays and even unnecessary procedures being performed for haemorroids[2].

Arterio-portal fistulas are a type of arteriovenous malformations defined as abnormal communications between the systemic arteries and the portal circulation[3]. They can be congenital or acquired. Congenital causes include arteriovenous malformations, ruptured aneurysms and hereditary telangiectatic diseases where there might be multiple arterio-portal fistulas present[4-7]. The majority of them are a result of penetrating or blunt trauma to the vessels which can be iatrogenic[8]. Most commonly, arterio-portal fistulas originate from the celiac or splanchnic circulation in particular the hepatic artery or splenic artery due to their close proximity to the portal and splenic veins. Rarely, they can be found to arise from the superior mesenteric or inferior mesenteric arteries[3]. In our patient, the arterio-portal fistula arose from the internal iliac arteries and this is believed to be the first such case reported in the literature.

Portal vein thrombosis is a known cause of extrahepatic presinusoidal portal hypertension. In neonates, this can be caused by umbilical infection, often as a result of umbilical vein catheterisation. The infection spreads along the left portal vein to the main portal vein causing thrombosis[8]. Due to the increase in portal resistance, collaterals arise from the high pressure veins in the portal system to the low pressure veins in the systemic circulation. The reversal of blood flow towards the systemic venous circulation leads to formation of varices at the oesophago-gastric region, along the falciform ligament at the umbilicus, in the retroperitoneum *via* the veins of Retzius and at the anorectal region where the superior haemorrhoidal veins decompress into the middle and inferior haemorroidal veins of the systemic circulation. Our patient presented with bleeding esophageal varices at an early age and this lead to her diagnosis of PVT. The leading cause of mortality in portal hypertension is bleeding esophageal varices[9]. Anorectal varices rarely bleed, likely due to the rich plexus of veins around the rectum which shunts away most of the blood.

In our patient, we offer two possibilities to account for her condition. The first is that she has a congenital pre-existing arterio-portal fistula between the rectal artery and the haemorrhoidal veins. However, when the patient first presented with rectal bleeding in the setting of portal hypertension, the most likely diagnosis of bleeding ano-rectal varices was made and she was treated with the aim of reducing her portal pressures, which was the presumed cause of the ano-rectal varices. In retrospect, this was a wrong postulation. The rectal bleeding did not resolve despite the splenorenal shunt and only on further investigations did we discover the arterio-portal fistula which had initially masqueraded as bleeding ano-rectal varices. Awareness of this entity as a possible differential diagnosis for bleeding ano-rectal varices is important. Initial investigation with a dynamic computed tomography scan with arterial and portal venous phase would have brought the diagnosis to light.

The second possibility is that the arterio-portal fistula was formed due to unintended or unnoticed trauma to the rectal wall. The left sided portal hypertension led to the formation of dilated portal vasculature in the rectum. These rectal varices are in close proximity to the branches of the arteries supplying the rectum, like the middle rectal artery in this case. An episode of unsuspecting trauma to the rectal wall possibly during defecation, led to the formation of the abnormal vascular communication between the middle rectal artery and the rectal varices, propagating the formation of an arterio-portal fistula

Multiphasic contrast enhanced computed tomography scan of the abdomen and pelvis helped to clinch the diagnosis of the arterio-portal fistula in this case, as contrast was seen on the draining dilated superior rectal vein in the arterial phase of the scan. This distinguishes it from a simple rectal bleeding secondary to rectal varices. In the setting of portal hypertension, awareness of the possible diagnoses is critical.

In the era prior to interventional radiology, arterio-portal fistulas were usually treated by open surgical excision. With the advent of super-selective angiographic catheterisation, embolisation is now the definitive treatment of choice for patients if the expertise is available[3]. Inadequate imaging especially in the setting of presumed portal hypertension has led to the delayed diagnosis of this arterio-portal fistula and local transanal treatment could have been catastrophic. Endoscopic ablation of the nidus of dilated collaterals in the rectum using sclerosant or banding could have led to massive haemorrhage from high pressure arterial bleeding if it was simply treated as for rectal varices following portal decompression.

In conclusion, arterio-portal fistulas are a known cause of portal hypertension and can also cause a diagnostic dilemma in the setting of portal hypertension. An-

giographic embolisation is the treatment of choice and this can be achieved with minimal morbidity.

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CASE REPORT

Endoscopic retrieval of 28 foreign bodies in a 100-year-old female after attempted suicide

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Abstract

Foreign body ingestion is a common emergency situation in children with one or a few objects having been ingested. Here we report our experience using endoscopic retrieval in a female centenarian with dyspnea and foreign bodies in the esophagus. She attempted suicide by swallowing 26 coins and two other foreign bodies. A gastroscope was used to remove all foreign bodies in the lower esophagus. In total, 26 coins, one ferrous ring and one cylindrical plastic object were retrieved. To our knowledge, this is the first clinical report on retrieval of so many foreign bodies in a single case.

Key words: Foreign body; Esophagus; Endoscopy; Coin; Gastroscope; Retrieval basket

Core tip: Foreign body ingestion is typically a childhood phenomenon, and generally involves one or a few objects. Here, we report our experience using emergency endoscopy in a centenarian with dyspnea who had swallowed 26 coins and other foreign bodies. Rescue measures were applied to maintain stable vital signs and airway patency, while an alligator forceps or basket was inserted through a flexible gastroscope to remove all foreign bodies. The objects removed from the patient included 26 coins, a ferrous ring, and a cylindrical plastic object.

Li QP, Ge XX, Ji GZ, Fan ZN, Zhang FM, Wang Y, Miao L. Endoscopic retrieval of 28 foreign bodies in a 100-year-old female after attempted suicide. *World J Gastroenterol* 2013; 19(25): 4091-4093 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i25/4091.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i25.4091

INTRODUCTION

Ingestion of foreign bodies that lodge in the upper gastrointestinal (GI) tract is common. Most objects pass through the GI tract spontaneously, but some need endoscopic or surgical removal. Here, we report the first case of a centenarian who had swallowed several foreign bodies, which were safely removed by endoscopy.

CASE REPORT

Foreign body ingestion is a commonly encountered clinical problem in pediatric emergency cases, but generally it involves only one or a few objects. Here, we report a case of ingestion of foreign bodies in a centenarian.

A 100-year-old woman complaining of retrosternal pain and tachypnea visited the emergency room. She had swallowed several foreign bodies in a suicide attempt due to intolerable pain induced by a fracture she suffered 3 mo previously. She was bedridden and had a depressed mood, which caused her to attempt suicide. She was admitted to hospital and a routine chest roentgenogram showed the incidental finding of suspicious foreign bodies, which were located in the lower esophagus (Figure 1). Blood

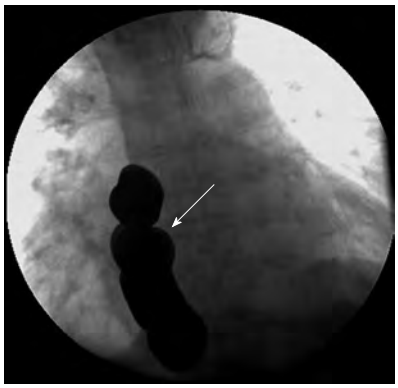

Figure 1 A roentgenogram showing foreign bodies in the lower esophagus (arrow).

Figure 3 **Foreign bodies after removal.** These included 26 coins, a ferrous ring, and a cylindrical plastic object.

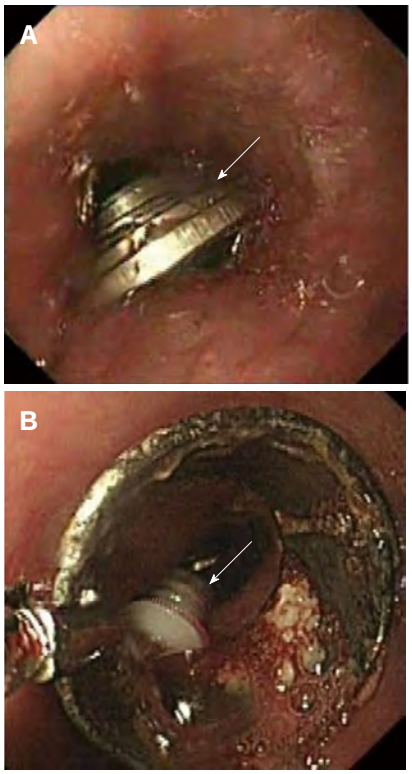

Figure 2 **Gastroscope examination.** A: Gastroscopy displayed several coins (arrow) in the lower esophagus; B: A cylindrical plastic foreign body impacted in the esophageal wall (arrow).

tests were normal, and an electrocardiogram showed premature ventricular extrasystole. Further gastroscopy examination using a flexible endoscope under a conscious state showed that several coins were located in the lower esophagus (Figure 2A). Endoscopic removal of these coins was performed, but failed because of their smooth edges. Therefore, an alligator clamp was employed to secure the coins, and more than 10 coins were successfully removed. Additionally, a cylindrical plastic foreign body was difficult to extract using the alligator clamp (Figure 2B). After several attempts, this foreign body was taken out via a stone retrieval net. Subsequently, additional foreign bodies were found and taken out using the alligator forceps. The foreign bodies removed from the patients included 26 coins, a ferrous ring, and a cylindrical plastic object (Figure 3). The procedure took about half an hour. When we reviewed the gastroscopy procedure, there was no block and no active bleeding in the esophagus.

DISCUSSION

Ingestion of foreign bodies is common in clinical practice. Most foreign body ingestion is accidental, and often occurs in the pediatric population, with a peak incidence between 6 mo and 6 years of age[1]. In contrast to the high frequency of foreign body ingestion in children, the occurrence rate of foreign body ingestion is relatively low in adults. The situation in adults occurs more commonly in patients with psychiatric disorders, mental retardation, or impairment caused by alcohol[2]. This is the first report of a centenarian ingesting foreign bodies in a suicide attempt due to intolerable pain. Additionally, the location of the foreign bodies in this case was in the lower esophagus, which was different from previous reports in which the majority of foreign bodies were located in the upper esophagus[3,4]. Foreign bodies in the upper esophagus may cause mechanical compression of the airway while in the lower esophagus may cause functional narrowing of the cardia. In this case, the patient's dyspnea was caused by both mechanical compression of the airway and functional narrowing of the cardia because of the irritation of so many foreign bodies.

The type of foreign body may influence the risk of complications. The types of foreign objects are different for different ages and cultures. In children, coins are a common type of foreign body[5]. In Asian countries, bone foreign bodies have been regarded as the most common type due to traditional habits of swallowing fish bones or eating soup from which fish bones are not removed[6,7]. The current case was rare in that a centenarian swallowed the coins.

Endoscopic removal of foreign bodies and impacted food boluses is a reliable and safe procedure in the hands of a skilled endoscopists, and it has a high success rate and low level of significant complications[8]. However,

the effectiveness of endoscopic removal of the foreign bodies was challenged by the smooth edges of the coins in the current case. Therefore, an alligator clamp was utilized. Additionally, a stone retrieval net was also employed to extract a cylindrical plastic foreign body in the present case. Many foreign bodies were removed, which is another important point in this case.

According to our experience, the key factor in shortening the procedure time was to catch and remove the foreign bodies in a short time using a powerful retrieval device. If the surface of the foreign body is smooth, it may be much more difficult. Each object is a challenge to the endoscopist. This was the reason why it took us 0.5 h to remove all the foreign bodies using a common stone extraction device. Fortunately, a novel retrieval basket is being developed by Detian Medical (Changzhou, China). Based on our animal and *in vitro* experiments, it is much easier for an endoscopist to catch foreign bodies and polyps and hold them stably in this basket device compared with traditional retrieval devices.

In conclusion, we reported our experience with retrieval of 26 coins and another two foreign bodies from the esophagus of a 100-year-old suicidal patient with dyspnea. To our knowledge, this is the first clinical report of retrieval of so many foreign bodies in a single case.

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CASE REPORT

Epstein-Barr virus negative primary hepatic leiomyoma: Case report and literature review

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Abstract

Primary hepatic leiomyoma is a neoplasm of mesenchymal origin and occurs only rarely. Secondary to benign smooth muscle proliferation, it is usually found in adult women and is associated with Epstein-Barr virus (EBV) infection. Here, we report the 29th case of primary hepatic leiomyoma with its unique features related to diagnosis, treatment and developmental biology. A 48-year-old man, with an immunocompromised status, complained of pain in the upper quadrant of the abdomen. Serological analysis indicated no presence of hepatitis virus, no human immunodeficiency virus, and no EBV infection. The levels of α-fetoprotein and carcinoembryonic antigen were normal. A mass was detected in segment Ⅲ of the hepatic lobe by ultrasonography and an abdominal computed tomography scan. Endoscopy had negative findings. Exploratory laparotomy found no existing extrahepatic tumor and left lateral lobectomy was performed. Pathological examination showed the mass to be a typical leiomyoma. The cells were positive for α-smooth muscle actin and desmin, and negative for the makers of gastrointestinal stromal tumor (GIST), including CD117, CD34 and DOG1 (discovered on GIST1). *In situ* hybridization revealed negative status for EBV-encoded small RNA. After left lateral lobectomy, the patient was not given chemotherapy or radiotherapy. During a 2-year follow-up, no sign of local recurrence or distant metastasis was observed. In conclusion, we report a rare case of primary hepatic leiomyoma in a male patient without EBV infection. Hepatic resection was curative. This case presents data to expand our knowledge concerning the complex and heterogeneous nature of primary liver leiomyoma, indicating that EBV infection is important but neither necessary nor sufficient for the development of primary liver leiomyoma.

Key words: Epstein-Barr virus; Primary hepatic leiomyoma; Cancer diagnosis; Tumor resection; Developmental biology

Core tip: Primary hepatic leiomyoma is usually found in adult women and is associated with Epstein-Barr virus (EBV) infection. We report the 29th case worldwide in a 48-year-old kidney allograft recipient without EBV infection and extrahepatic tumor. He achieved clinical cure by mass resection. The leiomyoma was positive for α-smooth muscle actin and desmin, and negative for gastrointestinal stromal tumor markers, including CD117, CD34 and DOG1 (discovered on gastrointestinal stromal tumor 1). The tumor was negative for EBV-encoded small RNA. The data indicate that EBV infec-

tion is important but neither necessary nor sufficient for development of primary liver leiomyoma.

Luo XZ, Ming CS, Chen XP, Gong NQ. Epstein-Barr virus negative primary hepatic leiomyoma: Case report and literature review. *World J Gastroenterol* 2013; 19(25): 4094-4098 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i25/4094.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i25.4094

INTRODUCTION

Primary hepatic leiomyoma occurs rarely. The first case was described by Demel[1] in a 42-year-old woman. To date, only 28 cases have been reported worldwide in the literature (Table 1). Secondary to benign smooth muscle proliferation, primary hepatic leiomyoma is usually found in adult women, and is associated with Epstein-Barr virus (EBV) infection. Due to its low prevalence, the diagnosis, treatment and biological behavior remain elusive and require further investigation[2-5]. More data to provide essential information concerning this disease are keenly awaited.

CASE REPORT

We report a 48-year-old man who complained of pain in the upper quadrant of the abdomen for 1 year and was admitted 2 years ago. He had received a renal graft 9 years before with the immunosuppressive regimens of cyclosporine A, mycophenolate mofetil and prednisone. Due to the calcineurin inhibitor nephrotoxicity found by fine-needle aspiration biopsy 3 years ago, cyclosporine was changed to tacrolimus. Two years ago, mycophenolate mofetil was replaced by azathioprine due to persistent diarrhea. On the day of admission, he was receiving tacrolimus (4.1 ng/dL), azathioprine (50 mg/d), and prednisone (5 mg/d). Routine blood analysis showed a white blood cell count of 7.2×10^9/L and lymphocyte count of 2.4×10^9/L. His liver function was normal and graded as A (score: 6) by Child-Turcotte-Pugh classification. There was no evidence of hepatitis B or hepatitis C virus infection. Human immunodeficiency virus (HIV) testing was negative. Serological testing for EBV was also negative. α-fetoprotein was 5.27 ng/mL (range: 1.09-8.04 ng/mL), and carcinoembryonic antigen was 2.55 ng/mL (normal range: 0-5 ng/mL). Ultrasonography revealed a mass in the left region of the liver, and an abdominal computed tomography (CT) scan showed a tumor of 3.7 cm × 4.9 cm in segment Ⅲ of the hepatic lobe (Figure 1). No tumor was found by esophagogastroduodenscopy and colonoscopy.

After diagnosis with a liver tumor, the patient underwent exploratory laparotomy. A solitary tumor was found in segment Ⅲ of the liver (Figure 2A). No tumors were present at extrahepatic sites; particularly in the pelvis. Left lateral hepatectomy was performed. The patient recovered with an uneventful postoperative course and abdominal pain disappeared.

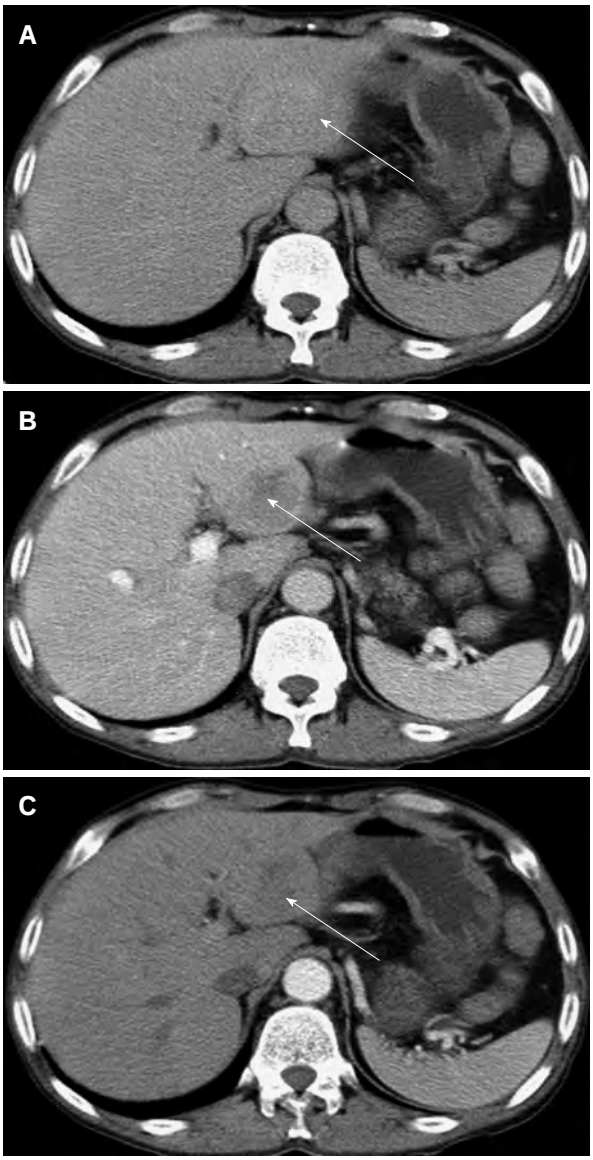

Figure 1 Abdominal computed tomography scan shows a mass in segment Ⅲ of the liver. A: Hepatic equilibrium phase; B: Portal venous phase; C: Hepatic arterial phase. The arrows indicate the tumor in the liver.

Histopathological examination of the resected specimen showed that the tumor consisted of spindle cells with scarce mitotic figures. The cells had elongated nuclei and eosinophilic cytoplasm forming a fabric-like structure, and neither giant cells nor anaplasia were present (Figure 2B). Immunohistochemical staining showed that the cells were positive for α-smooth muscle actin and desmin (Figure 2C and D), and negative for the gastrointestinal stromal tumor (GIST) markers, including CD117, CD34 and DOG1 (discovered on GIST1). *In situ* hybridization revealed that the nuclei of the tumor cells were negative for EBV-encoded small RNA (EBER) (Figure 3).

Diagnosis of primary hepatic leiomyoma was then made. As a benign tumor, neither chemotherapy nor radiotherapy was administered to the patient. During a

Table 1 Summary of the published cases of primary liver leiomyoma

Author	Age/sex	EBV infection	Symptoms	Location/size (cm)	Immunosuppression	Treatment
Demel[1]	42/F	Unknown	RUQ pain	RL/12	NS	Laparotomy
Rios-Dalenz *et al*	87/F	Unknown	RUQ pain/bleeding	LL/-	NS	Autopsy
Ishak *et al*	64/M	Unknown	Abdominal mass	RL/-	NS	Laparotomy
Hawkins *et al*[2]	66/M	Unknown	Abdominal mass	LH/13	NS	Left hepatectomy
Rummeny *et al*	46/F	Unknown	RUQ pain	NS	NS	NS
Hollands *et al*[6]	17/M	Unknown	Abdominal pain	LH/9	NS	Left hepatectomy
Herzberg *et al*	30/F	Unknown	RUQ fullness	RL/19	NS	Partial right hepatectomy
Doyle *et al*[11]	1.5/F	Positive	Incidental	LL/3	Yes	LL segmentectomy
Reinertson *et al*	32/F	Unknown	RUQ pain	LH/10	NS	Left hepatectomy
Hailer *et al*	9/M	Unknown	Incidental	LH/5.6	Yes	Partial hepatectomy
Davidoff *et al*[12]	5/M	Positive	Incidental	RR/15	Yes	Right trisegmentectomy
Yoon *et al*	41/F	Unknown	RUQ discomfort	RL/19	No	Right hepatectomy
Yanase *et al*	59/F	Unknown	Liver dysfunction	RL/13	NS	Right hepatectomy
Mesenas *et al*	59/M	Unknown	NS	RL/3.6	NS	Segmentectomy (S5)
Belli *et al*[7]	67/F	Unknown	Abdominal mass	RL/30	NO	Right extended resection
Sclabas *et al*[13]	30/F	Positive	Epigastric pain	LL/4.4, 0.6	Yes	LL sectionectomy
Cheuk *et al*[14]	37/M	Positive	Abdominal discomfort	LH/3.5, 1	Yes	Conservative management
Kanazawa *et al*	31/M	Unknown	None	LL/3.5	No	LL sectionectomy
Beuzen *et al*	36/F	Unknown	RUQ pain	LL/5	No	LL sectionectomy
Imasato *et al*[3]	61/F	Unknown	None	S1/4.5	No	Right hepatectomy
Urizono *et al*	71/M	Unknown	NS	S1/3	No	Partial hepatectomy
Marin *et al*	64/F	Unknown	None	RL	No	Right hepatectomy
Sousa *et al*	61/F	Unknown	Dyspepsia	LL/9.5	No	Left hepatectomy
Kalil *et al*	44/F	Unknown	Abdominal mass	RL/7	No	Atypical resection
Santos *et al*	28/F	Unknown	Incidental	RL (S6)/5.5	No	Segmentectomy
Raber *et al*	46/F	Unknown	Incidental	RL/2.8	Yes	Conservative management
Perini *et al*[5]	45/M	Positive	Epigastric pain	LL/4.3	Yes	LL sectionectomy
Perini *et al*[5]	45/F	Unknown	RUQ pain	RL (S6)/16.5	No	Segmentectomy

LL: Left lateral; NS: Not stated; RL: Right lobe; RUQ: Right upper quadrant; S: Segment; LH: Left hepatic lobe; EBV: Epstein-Barr virus.

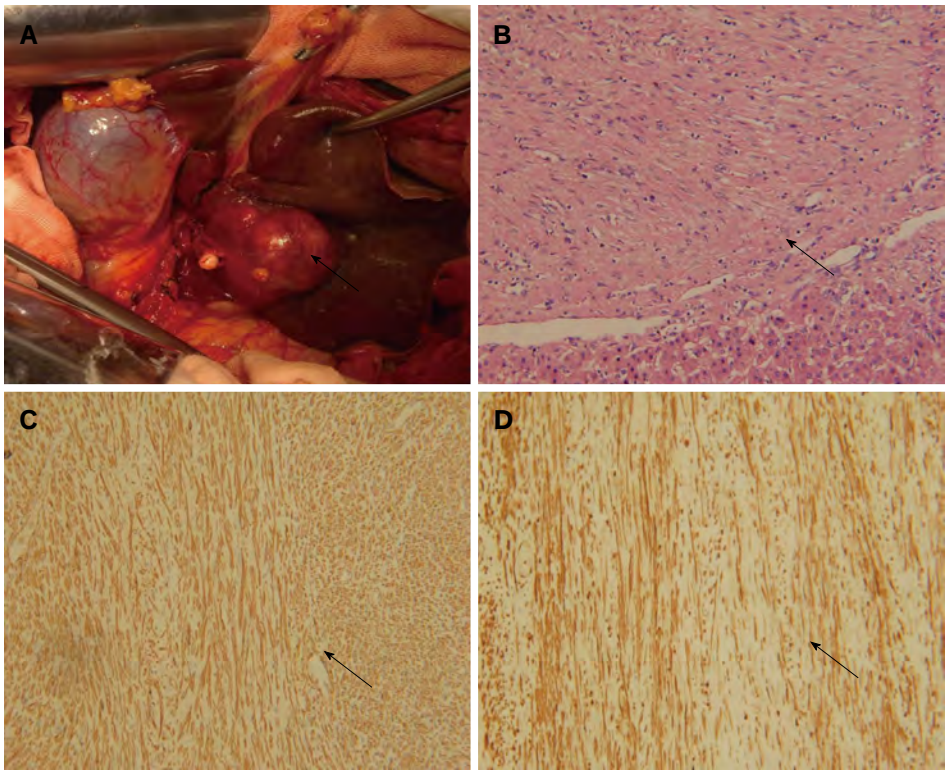

Figure 2 Pathological characteristics of the primary liver leiomyoma. A: Tumor (arrow) located in segment Ⅲ of the liver; B: Tumor (arrow) and normal liver tissue, hematoxylin and eosin staining, × 200; C: α-smooth muscle actin staining (arrow) of tumor tissues, immunohistochemical staining, × 200; D: Desmin staining (arrow) of tumor tissues, immunohistochemical staining, × 200.

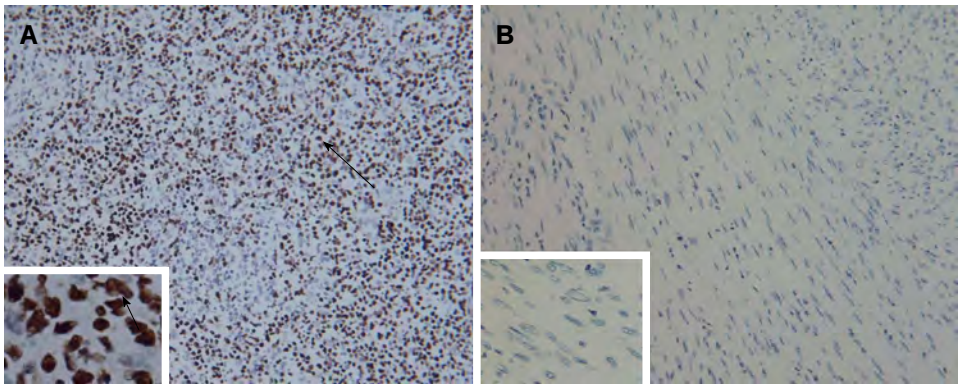

Figure 3 Tumor cells stained negative by *in situ* hybridization with Epstein-Barr virus-encoded small RNA. A: Positive control staining × 200, × 1000; B: Tumor cell staining × 200, × 1000. Arrows indicate positive staining of the nuclei.

24-mo postoperative follow-up, no sign of local recurrence or distant metastasis was observed, indicating a clinical cure in this case.

DISCUSSION

Primary hepatic leiomyoma occurs rarely. The first case was described by Demel[1] in a 42-year-old woman. To date, only 28 cases have been reported worldwide (Table 1). Secondary to benign smooth muscle proliferation, primary hepatic leiomyoma is usually found in adult women, and is associated with EBV infection. Due to its low prevalence, diagnosis, treatment and biological behavior remain elusive and require further investigation.

Leiomyoma is relatively common and tends to originate from the muscularis of the gut or the media of the blood vessels, and usually develops in the urogenital and gastrointestinal tracts. Primary hepatic leiomyoma is rare and has its own particular clinical and biological features.

To diagnose primary hepatic leiomyoma, Hawkins *et al*[2] has proposed the following criteria: (1) the tumor is composed of leiomyocytes; and (2) the presence of a leiomyomatous tumor at other sites can be excluded. Moreover, this liver tumor must be distinguished from GIST[3,4]. In the present case, we excluded the presence of hepatocellular carcinoma, and laboratory tests and histopathological examination were the first step in this process. Then, the diagnosis of leiomyoma was established on the basis of its pathological features. GIST makers (CD117, CD34 and DOG1) were also negative. Combining the findings of ultrasonography, abdominal CT scan, esophagogastroduodenscopy, colonoscopy and exploratory laparotomy, the final diagnosis of primary hepatic leiomyoma was made.

Although no standard therapy is available at present, consistent with the existing reports (Table 1), the tumor was successfully excised and neither chemotherapy nor radiotherapy was applied. Our experience supports that hepatic resection is both diagnostic and curative for primary hepatic leiomyoma.

Some unique characteristics should be noted in this case. First, the patient was male, and primary hepatic leiomyoma is more likely to be found in adult women (18 out of the total 28 cases were female) (Table 1). The relevance of sex may partly be due to the activity of the smooth muscle cells in female urogenital tissue in tumorigenesis and progenesis. The cellular origin of primary hepatic leiomyoma remains unclear and may arise from vessels or the biliary tree[6-8]. In this report, the patient had negative findings in the pelvis and for detection of GIST markers. More observations are required to explore the cellular source of primary hepatic leiomyoma. Second, this case was an adult patient. To date, a total of four pediatric cases (< 18 years) have been identified with primary hepatic leiomyoma (Table 1). Whether or not the developmental mechanisms are different between children and adults requires further investigation. Third, it could be deduced that EBV infection plays a critical role in development of primary hepatic leiomyoma[9,10]. Based on the reported literature, five patients were examined for EBV infection and all of them were positive[11-14]. The relationship between development of primary hepatic and EBV infection and immunocompromised status is also interesting. Seven out of the 28 patients (25%) were immunocompromised (6 transplanted and 1 HIV infection), and five of the seven cases (71.4%) were EBV-positive (4 transplanted and 1 HIV infection). However, in the present case, EBER *in situ* hybridization, which is the gold standard for detection and localization of latent EBV in tissues, showed that the patient did not have EBV infection, which was different from the status of other patients currently being studied.

Our data indicate that EBV infection is important but neither necessary nor sufficient for the development of primary liver leiomyoma. This observation highlights the complex and heterogeneous nature of the disease and raises the question whether EBV is a passenger rather than a causative agent for this tumor. Due to the rare occurrence of the tumor, an international primary hepatic leiomyoma sample bank, which needs worldwide cooperation of the involved institutions, will contribute to untangling the complex pathogenesis using omics- and system-based methodologies, and therefore to clarify the underlying mechanism behind this interesting tumor.

In conclusion, this report of the 29th case of primary hepatic leiomyoma with its unique features related to diagnosis, treatment and developmental biology contributes to our knowledge of the tumor.

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GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access (OA) journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 1352 experts in gastroenterology and hepatology from 64 countries.

Aims and scope

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

WJG is published by Baishideng Publishing Group (BPG) in both electronic and online forms. All *WJG* articles are published in *WJG* website and PubMed Central. The major advantages of OA journals are faster release and delivery, no page or graph restrictions, and increased visibility, usage and impact. Full-text PDF articles and electronic/online versions are freely available to global readers. After the paper is published, the author(s) can obtain high-quality PDF files, which contain the journal cover, a list of editorial board members, table of contents, text, and back cover of the journal. BPG has a strong professional editorial team composed of editorial board members, editors-in-chief, science editors, language editors, and electronic editors. BPG currently publishes 42 OA clinical medical journals, including 41 in English, has a total of 15471 editorial board members or peer reviewers, and is a world first-class publisher.

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The columns in the issues of *WJG* will include: (1) Editorial: The editorial board members are invited to make comments on an important topic in their field in terms of its current research status and future directions to lead the development of this discipline; (2) Frontier: The editorial board members are invited to select a highly cited cutting-edge original paper of his/her own to summarize major findings, the problems that have been resolved and remain to be resolved, and future research directions to help readers understand his/her important academic point of view and future research directions in the field; (3) Diagnostic Advances: The editorial board members are invited to write high-quality diagnostic advances in their field to improve the diagnostic skills of readers. The topic covers general clinical diagnosis, differential diagnosis, pathological diagnosis, laboratory diagnosis, imaging diagnosis, endoscopic diagnosis, biotechnological diagnosis, functional diagnosis, and physical diagnosis; (4) Therapeutics Advances: The editorial board members are invited to write high-quality therapeutic advances in their field to help improve the therapeutic skills of readers. The topic covers medication therapy, psychotherapy, physical therapy, replacement therapy, interventional therapy, minimally invasive therapy, endoscopic therapy, transplantation therapy, and surgical therapy; (5) Field of Vision: The editorial board members are invited to write commentaries on classic articles, hot topic articles, or latest articles to keep readers at the forefront of research and increase their levels of clinical research. Classic articles refer to papers that are included in Web of Knowledge and have received a large number of citations (ranking in the top 1%) after being published for more than years, reflecting the quality and impact of papers. Hot topic articles refer to papers that are included in Web of Knowledge and have received a large number of citations after being published for no more than 2 years, reflecting cutting-edge trends in scientific research. Latest articles refer to the latest published high-quality papers that are included in PubMed, reflecting the latest research trends. These commentary articles should focus on the status quo of research, the most important research topics, the problems that have now been resolved and remain to be resolved, and future research directions. Basic information about the article to be commented (including authors, article title, journal name, year, volume, and inclusive page numbers; (6) Minireviews: The editorial board members are invited to write short reviews on recent advances and trends in research of molecular biology, genomics, and related cutting-edge technologies to provide readers with the latest knowledge and help improve their diagnostic and therapeutic skills; (7) Review: To make a systematic review to focus on the status quo of research, the most important research topics, the problems that have now been resolved and remain to be resolved, and future research directions; (8) Topic Highlight: The editorial board members are invited to write a series of articles (7-10 articles) to comment and discuss a hot topic to help improve the diagnostic and therapeutic skills of readers; (9) Medical Ethics: The editorial board members are invited to write articles about medical ethics to increase readers' knowledge of medical ethics. The topic covers international ethics guidelines, animal studies, clinical trials, organ transplantation, *etc.*; (10) Clinical Case Conference or Clinicopathological Conference: The editorial board members are invited to contribute high-quality clinical case conference; (11) Original Articles: To report innovative and original findings in gastroenterology and hepatology; (12) Brief Articles: To briefly report the novel

and innovative findings in gastroenterology and hepatology; (13) Meta-Analysis: Covers the systematic review, mixed treatment comparison, meta-regression, and overview of reviews, in order to summarize a given quantitative effect, *e.g.*, the clinical effectiveness and safety of clinical treatments by combining data from two or more randomized controlled trials, thereby providing more precise and externally valid estimates than those which would stem from each individual dataset if analyzed separately from the others; (14) Case Report: To report a rare or typical case; (15) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (16) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (17) Autobiography: The editorial board members are invited to write their autobiography to provide readers with stories of success or failure in their scientific research career. The topic covers their basic personal information and information about when they started doing research work, where and how they did research work, what they have achieved, and their lessons from success or failure.

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Acknowledgments

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Format

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1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixudiarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.00000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23243641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
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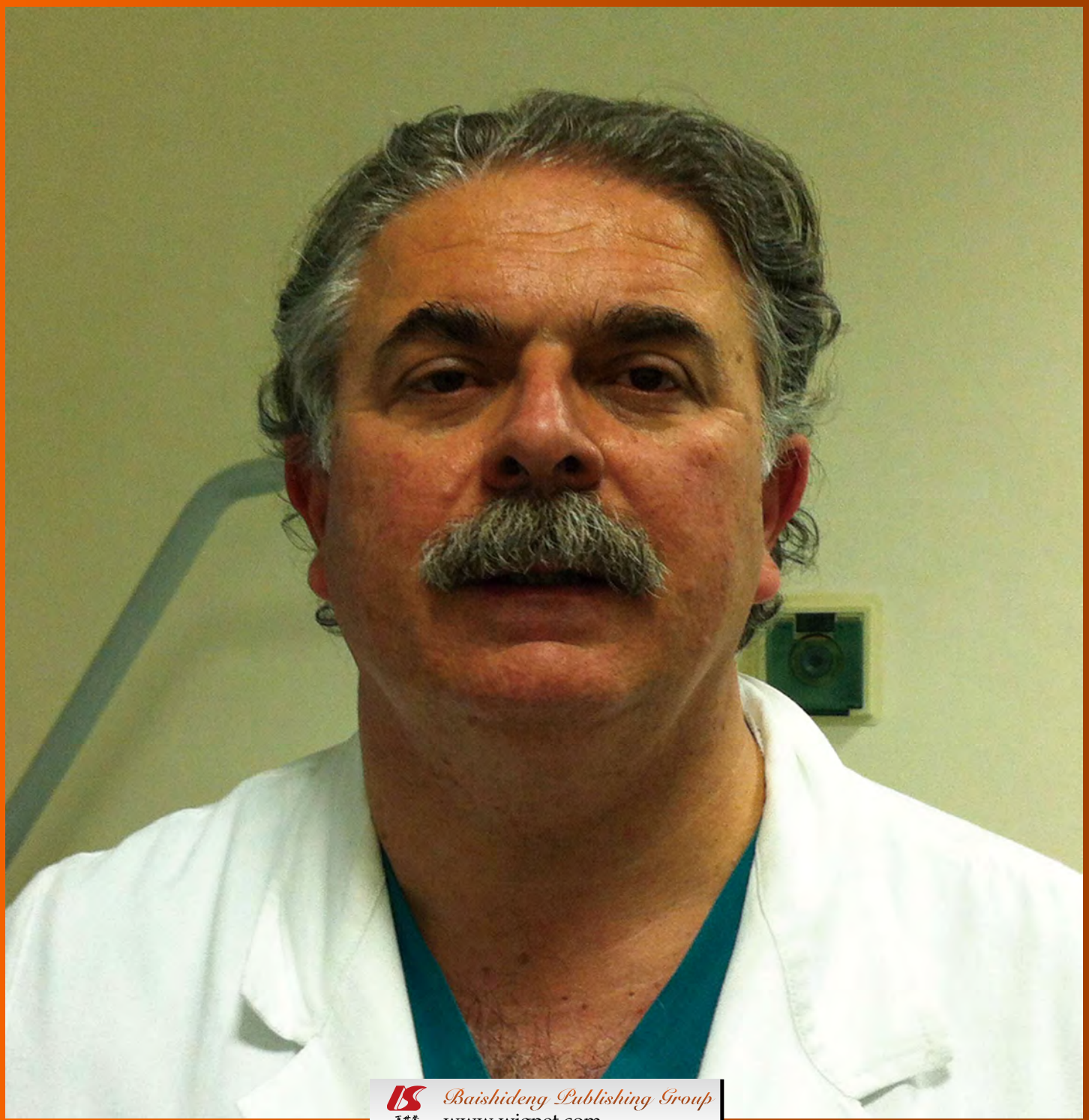

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Weekly Volume 19 Number 26 July 14, 2013

CASE REPORT

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EDITORIAL

Cellular and molecular basis of chronic constipation: Taking the functional/idiopathic label out

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Abstract

In recent years, the improvement of technology and the increase in knowledge have shifted several strongly held paradigms. This is particularly true in gastroenterology, and specifically in the field of the so-called "functional" or "idiopathic" disease, where conditions thought for decades to be based mainly on alterations of visceral perception or aberrant psychosomatic mechanisms have, in fact, be reconducted to an organic basis (or, at the very least, have shown one or more demonstrable abnormalities). This is particularly true, for instance, for irritable bowel syndrome, the prototype entity of "functional" gastrointestinal disorders, where low-grade inflammation of both mucosa and myenteric plexus has been repeatedly demonstrated. Thus, researchers have also investigated other functional/idiopathic gastrointestinal disorders, and found that some organic ground is present, such as abnormal neurotransmission and myenteric plexitis in esophageal achalasia and mucosal immune activation and mild eosinophilia in functional dyspepsia. Here we show evidence, based on our own and other authors' work, that chronic constipation has several abnormalities reconductable to alterations in the enteric nervous system, abnormalities mainly characterized by a constant decrease of enteric glial cells and interstitial cells of Cajal (and, sometimes, of enteric neurons). Thus, we feel that (at least some forms of) chronic constipation should no more be considered as a functional/idiopathic gastrointestinal disorder, but instead as a true enteric neuropathic abnormality.

Key words: Constipation; Enteric glia; Enteric nervous system; Enteric neurons; Interstitial cells of Cajap; Neurogastroenterology

Core tip: Concerning gut motility, in the last years the basic/clinical interplay between gastroenterology and neurology has become stricter, and many pathologic conditions, among which constipation, related to abnormal gastrointestinal motility are now considered and studied by a neurogastroenterological point of view. However, the fact that these conditions are still labelled as "functional" or "idiopathic" is puzzling. We examined the evidence for taking these labels out from constipation, that should be considered as a true neurenteric dysfunction.

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INTRODUCTION

The field of gastrointestinal motor activity has always attracted the researchers' interest; however, in the time course it became apparent that most gut motor disorders are attributable to disordered neural control mechanisms. Thus, by the encounter of gastroenterologists and neurologists a common branch emerged, *i.e.*, neurogastroenterology[1], in which an equal partnership had been recognized concerning gut motility. Recently, ultrastructural morphologists joined and try to bring innovative perceptions on control mechanisms of digestive motility. This had led to interesting and exciting new perspectives in the pathophysiology of some frequent disorders, such as constipation.

Chronic constipation is a frequent symptom in the general population, where is present in 2%-30% of subjects[2]. However, apart from secondary forms, associated to an underlying disease (*e.g.,* neurological[3,4]), commonly used (at least for scientific purposes) classifications still label most cases of constipation as "idiopathic" or "functional"[5-9].

It is worth noting that the concept of functional diseases has been somewhat questioned in the last years[10], since several studies conducted on prototypic functional entities, such as irritable bowel syndrome (IBS) and functional dyspepsia (FD) have revealed that these condition may actually harbor an organic basis[11,12]. In fact, inflammation and neuronal degeneration have been reported in IBS patients[13] and duodenal mastocytosis, eosinophilia and intraepithelial lymphocytosis have been described in both IBS and FD patients[14].

Chronic constipation may be subdivided in two main subtypes, obstructed defecation (OD) and slow transit constipation (STC), that may also co-exist in the same patient[15,16], and it is generally thought (by data originating from both experimental animal models and humans) that colonic sensorimotor dysfunction and abnormal motility play a pivotal pathogenetic role[17-19]. Thus, abnormal colonic and anorectal function had been repeatedly demonstrated in these patients[20-22], and pharmacologic stimulation may help in addressing more targeted therapeutic approaches[23,24]. However, etiological factors are still poorly known[25].

This article will deal with the available evidence for neurobiological abnormalities in chronic constipation, that suggests how this symptom often underlie a true organic enteric disorder.

NEUROENTERIC ABNORMALITIES IN CONSTIPATION

To date, there is mounting evidence that colonic neuromuscular abnormalities may be of paramount importance in this setting[26,27], and there are numerous studies showing that (at least) severely constipated patients may have one or more abnormal features mainly (although not exclusively) linked to elements of the enteric nervous system (ENS)[28].

The ENS, considered as the brain of the gut, integrates secretion and motility into homeostatic patterns of behavior susceptible to disorder[29]. Thus, it is not surprising that some of the enteric circuitries responsible for these activities may be involved in the dysfunction of their basic control mechanisms. The resulting abnormalities are summarized below.

Abnormal colonic neurochemistry

This has been repeatedly shown in constipated patients: several studies showed a decreased content of vasoactive intestinal peptide (VIP) and substance P in tissues obtained from these subjects[30-34]. Moreover, *in vitro* studies confirmed that the diminished contractile response to these substances plays an important role in the impaired motility observed in the colon of constipated patients[35,36]. Of note, these abnormalities seem not to be related to chronic laxative use, since anthranoids cause a reduction in the levels of inhibitory neurotransmitters (VIP, somatostatin), but not of substance P, in the rat colon[37]. Other studies showed that excitatory nerve fibres are present in the circular muscle in STC but they are deficient in tachykinins and enkephalin[38-40]. In addition, investigations conducted on colonic strips showed that a decrease of cholinergic innervation and an increase of non-adrenergic non-cholinergic (NANC) inhibitory innervation play an important role in the impaired motility observed in the colon of patients with slow transit constipation[41]; these effects are mediated by an increase of nitric oxide and a decrease of neurotensin[42-44], as also confirmed by immunohistochemical methods in surgically resected specimens[45].

Enteric nervous system

Earlier studies addressing the ENS have shown the presence of several heterogeneous abnormalities in patients with severe constipation (especially those with STC), including reduced number of argyrophilic neurons[46] and of intraganglionic neurofilaments[47], myenteric plexus hypoganglionosis[48]. More recent studies, with the increasing use of immunohistochemical techniques[49,50], have demonstrated more consistent findings on the main elements of the ENS, such as the decrease of interstitial cells of Cajal (ICC)[51,52] (up to their complete absence in colonic inertia[53]), often associated to a reduced number of enteric neurons[54,55] and/or of enteric glial cells (EGC)[56,57]. Of interest, the expression of c-kit mRNA and c-kit protein was also found to be significantly decreased in the colon of severely constipated patients, suggesting that the c-kit signal pathway may play an important role in ICC reduction in these patients[58].

Colonic smooth muscle

Only a few studies have addressed this issue, often with

discordant findings, probably due to the heterogeneous cohorts of patients evaluated. Some authors reported that the ratio of the thickness of circular to longitudinal muscle was significantly lower in the left colon in constipated subjects[59], whereas other authors described a decreased circular muscle layer thickness in constipated patients[60], but no abnormalities of the colonic muscular layers were described in both studies. Another investigation showed the presence of amphophilic inclusion bodies in the muscularis externa of STC patients[61], even though these findings were found in about half of the patients. Normal actin expression was found in both adults and children with severe constipation[56,62], whereas the use of novel and nonconventional smooth muscle markers may reveal abnormalities linked to the smooth muscle contractile apparatus unnoticed by both routine stainings and alpha-actin, suggesting specific defects of smooth muscle cells involved in the pathogenesis of gastrointestinal motility disorders[63].

SIGNIFICANCE OF NEUROENTERIC ABNORMALITIES IN CONSTIPATION

There are few doubts that the ENS abnormalities repeatedly found in constipated patients play a pivotal role in the genesis of symptoms. In fact, the consistent finding of a significant decrease of ICC, enteric neurons, and (especially) EGC, variously associated each other, justifies the abnormal motor behavior of the large bowel in these patients.

In fact, looking at the physiological properties of these cell populations, it is obvious that the disruption of their number/connections/relationship leads to an impairment of the complex regulation of the well-coordinated colonic motor patterns[64], thus affecting the viscus' motility, due to the strict interplay between ICC, enteric neurons and EGC, with the latter acting as a physiologic bridge (not only by a simple mechanic point of view, but also by means of their neurotransmitter, immunologic, and trophic properties[65]) between the other two cell types.

Unfortunately, to date data are lacking on the possible factors causing neurenteric abnormalities in constipated patients. The current hypothesized mechanisms (often originating from experimental animal models) imply abnormalities in glial trophic factors leading to neural degeneration, and enteric localization of infective agents (bacteria, virus, prions) causing more or less selective degeneration of specific neurenteric cell populations (particularly EGC)[66], whereas genetic factors[67] or neurodegenerative changes due to aging seem to play a lesser role[68].

NEW CELLULAR PLAYERS IN NEUROMOTILITY DISORDERS

In biological sciences, interstitial tissue is seen as the connective tissue that surrounds the cells of a certain tissue while the extracellular matrix elements are known for its great capacity to retain water. However, except for a few described interstitial diseases (*e.g.,* inflammatory bowel disease, interstitial cystitis, tubulointerstitial nephropathy, interstitial lung disease) its role is easily overlooked, as well as the significance of its cellular elements. Recent studies related to biological and histological data revealed, among the known resident (fibroblasts/fibrocytes, adipose cells) and non-resident cells (mast cells, plasma cells, eosinophils, macrophages, *etc.*) of interstitial space, a novel cell type-the telocyte[69,70].

Morphologically, telocytes represent interstitial cells with telopodes-the longest cellular extensions described besides the axons of neurons[71]. This rather unique cell type, difficult to visualize by routine microscopy, displays a particular morphology by electron microscopy: (1) a small cell body (9-15 μm) with scarce cytoplasmic organelles surrounding a moderately euchromatic nucleus; and (2) telopodes are usually tortuous and organized in a 3D network by overlapping and/or by homocellular interactions[72,73]. Telopodes are very long (10-1000 μm), thin (0.1 ± 0.5 μm) and moniliform cytoplasmic extensions; the moniliform aspect is created by the alternation of thin segments-podomers with dilated segments-podoms; the latter accomodate functional units consisting of caveolae, mitocondria and endoplasmic reticulum[74] and occupy a strategic position in relation to stem cell niches, blood capillaries, and/or nerve bundles[75,76]. Telopodes also establish stromal contacts with other cells, such as mast cells, basophils, lymphocytes, eosinophils, plasma cells, or macrophages[77] and non-cellular elements (*e.g.*, collagen and elastic fibers)[78,79].

Telocytes have been described in human and mammalian cavitary and parenchymatous organs, as well as in serous membranes and other tissues (for details see www.telocytes.com). In the last two years telocytes were also described in the gut[77,80,81].

In modern times the significance of the information that could be achieved by signaling molecules found in intercellular fluids is overlooked. There is scarce information on the usefulness of the extracellular organelles (exosomes and shedding microvesicles) released in the extracellular space as mediators of cell-to-cell communication[82]. Such vesicles were recently demonstrated in the proximity of telopodes and even emerging from them in heart[83], lungs[76], skeletal muscle[76], pancreas[73], parotid gland[84] and human uterus[74].

Telocytes are supposed to be involved: (1) in intercellular signaling[72,74,77]; (2) as stem cell adjutants involved in tissue renewal[79,85]; (3) as sensors for steroid hormones[86]; (4) in the guidance of immune cells[77]; (5) as stretch sensors[87]; and (6) as contractility modulators[88]. Even though telocytes seem to be implicated in many important physiological and pathological processes[89,90], their exact functions still remain controversial. Although telocytes have not yet been described at colonic level, their possible involvement in pathophysiological mechanisms

of chronic constipation cannot be overlooked. In favor of this hypothesis there is a possible correlation between the fact that telocytes express receptors for estrogen and progesterone[91,92] and the fact that chronic constipation is linked to sex hormones[93] and is higher in women of reproductive age[94].

CONCLUSION

The improvement of scientific knowledge and the constant, increasing ability to recognize previously unknown pathophysiologic mechanisms is of paramount importance. Thus, labels such as "idiopathic" or "functional", that basically conceal the fact that too little is known of a specific pathologic entity[10], should be hopefully replaced when more knowledge is available, as pointed out several years ago[95]. As such, the recent recognition of neurenteric abnormalities in many patients complaining of constipation should point to reconsidering at least some of these forms (especially STC) as true enteric neuropathies, and to drop the "idiopathic"/"functional" label.

Besides semantic considerations, we feel that a better understanding of possible basic abnormalities in these patients is important, and may have therapeutic implications, addressing the researchers' interest for new options toward more targeted approaches[16].

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REVIEW

Systematic review of surgical resection *vs* radiofrequency ablation for hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) represents one of the most common neoplasms worldwide. Surgical resection and local ablative therapies represent the most frequent first lines therapies adopted when liver transplantation can not be offered or is not immediately accessible. Hepatic resection (HR) is currently considered the most curative strategy, but in the last decade local ablative therapies have started to obtain satisfactory results in term of efficacy and, of them, radiofrequency ablation (RFA) is considered the reference standard. An extensive literature review, from the year 2000, was performed, focusing on results coming from studies that directly compared HR and RFA. Qualities of the studies, characteristics of patients included, and patient survival and recurrence rates were analyzed. Except for three randomized controlled trials (RCT), most studies are affected by uncertain methodological approaches since surgical and ablated patients represent different populations as regards clinical and tumor features that are known to affect prognosis. Unfortunately, even the available RCTs report conflicting results. Until further evidences become available, it seems reasonable to offer RFA to very small HCC (< 2 cm) with no technical contraindications, since in this instance complete necrosis is most likely to be achieved. In larger nodules, namely > 2 cm and especially if > 3 cm, and/or in tumor locations in which ablation is not expected to be effective or safe, surgical removal is to be preferred.

Key words: Hepatocellular carcinoma; Hepatic resection; Surgical therapy; Ablation techniques; Survival; Liver failure

Core tip: The present review shows the lights and shadows of the comparative literature regarding hepatic resection and radiofrequency ablation for hepatocellular carcinoma. Nineteen studies that directly compared these two therapies were found through an extensive literature review; of them, three randomized controlled trial were available for comparison whereas the remaining studies were represented by retrospective observational studies. Results are often conflicting and further randomized controlled trial are warranted; otherwise, retrospective observational studies should include in their analyses statistical approaches aimed at reduce possible confounding sources at a minimum.

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INTRODUCTION

Hepatocellular carcinoma (HCC) represents one of the most common primary malignancies of the liver worldwide, with an incidence that varies in the different geographic areas as a consequence of the regional variations in exposure to risk factors for this tumor[1,2]. The increasing use of surveillance in clinical practice, and the advancements in diagnostic and therapeutic abilities achieved in the last decades have greatly improved patient survival[3-5]. Liver resection and radiofrequency ablation represent the most common first-line therapies adopted when HCC is diagnosed at early stages[6]. Liver resection still remains a mainstay of HCC treatment, and thanks to the considerable improvements in surgical techniques and peri-operative care, the rates of death and complications after liver resection have remarkably decreased over time, giving the procedure added value[7,8]. However, surgery can negatively impact on the already compromised function of cirrhotic livers and, on the other hand, radiofrequency ablation seems safer but its ability to achieve complete and sustained tumor necrosis can be less predictable, and technical feasibility may be sub-optimal. For these reasons, the choice between hepatic resection and radiofrequency ablation for HCC is still a matter of debate. The aim of the present review is to examine the available literature that directly compares these two therapeutic strategies. The qualities and flaws of each included study were highlighted in the attempt to reach conclusions regarding the effectiveness of one treatment with respect to the other and to make suggestions for future research on this debated topic.

LITERATURE STRATEGY SEARCH

A systematic search within the Medline and Embase databases, in the period between 1 January 2000 and 1 December 2012, was performed with the MeSH terms "hepatocellular carcinoma" and ("hepatectomy" or "surgical therapy") and "ablation techniques". The keywords "hepatocellular carcinoma", "partial hepatectomy", "hepatic resection", "radiofrequency ablation" or "percutaneous ablation" and "survival" were used to supplement the literature search. The reference lists of retrieved publications were reviewed for other relevant papers. Only articles involving human subjects and that directly compared radiofrequency ablation *vs* hepatic resection for HCC were considered for the present review. The quality of the selected articles was attributed on the basis of their level of evidence and by means of the Newcastle-Ottawa quality assessment scale for observational studies[9]. The Newcastle-Ottawa Scale (NOS) is a score system that was developed to assess the quality of non-randomized studies, in which a study is judged on three broad perspectives: (1) the selection of the study groups; (2) the comparability of the groups; and (3) the ascertainment of either the exposure or outcome of interest for case-control or cohort studies, respectively.

WHAT GUIDELINES RECOMMEND

Clinical practice guidelines should be evidence-based and should represent the consensus of expert committees. However, it is often very difficult to reach a consensus in the field of HCC, especially as regards the therapeutic approach, given the extremely limited availability of high quality trials. Table 1 reports a summary of the levels of evidence and the strength of recommendations from three published guidelines, namely, the European Association for the Study of the Liver (EASL-EORTC), updated in 2012[10], the American Association for the Study of Liver Diseases (AASLD), updated in 2010[11], and the Asian Pacific Association for the Study of the Liver (APASL), updated in 2010[12]. The EASL and AASLD guidelines are mainly based on the Barcelona Clinic Liver Cancer (BCLC) algorithm for staging and treatment of HCC and represent the most popular treatment algorithms in Western countries[13], however, the BCLC algorithm is not very popular in Asia. There are two important aspects of these guidelines that deserve attention and that are strictly related to each other. The first is represented by the role of the "alternative strategy" of ablation, with respect to resection, and the second is the recommended selection criteria for surgery. It can be immediately noted that radiofrequency ablation is always considered as a strategy alternative, and not competitive, to resection: the EASL recommends ablative therapies "for patients with BCLC 0-A tumours not suitable for surgery", the AASLD suggests that ablative therapy is "effective for patients who cannot undergo resection" and the APASL recommends local ablation as "an acceptable alternative to resection". These recommendations mainly derive from indirect comparisons of the results from the two treatments. In brief, modern standards of HCC resection in cirrhotic patients call for a peri-operative mortality < 3% and an expected 5-year survival rate above 60%[10,14-18], whereas, on the other hand, mortality after RFA has been reported to range between 0.9% and 7.9% and the 5-year survival rate to range between 40% and 70%[19-25]. Most of the uncertainties are related to the efficacy of ablation techniques, since response to ablative therapies is strongly influenced by tumor size and location[19,26-29]. In addition, patients allocated to ablation tend to suffer from a more advanced degree of liver dysfunction in comparison to those undergoing surgery, and this feature can negatively impact the observed results. On the other hand, strict selection criteria for hepatic resection can ameliorate patient survival after surgery and this is especially true as regards liver reserve. These two features are obviously related to each other, since at varying criteria for resection, different patients will be shifted to ablation techniques and this represents the second aspect that deserves attention. For example, a selection of candidates for hepatic resection strictly based on the hepatic vein pressure gradient (HVPG), as recommended by the EASL[10], could exclude several patients from surgery, shifting them to RFA. Specifically, HVPG should be <

Table 1 Proposed evidences and recommendations from international guidelines

Guidelines	Hepatic resection	Radiofrequency ablation
EASL	Resection is the first-line treatment option for patients with solitary tumors and very well-preserved liver function, defined as normal bilirubin with either hepatic venous pressure gradient ⩽ 10 mmHg or platelet count ⩾ 100000 (evidence 2A; recommendation 1B)	Local ablation with radiofrequency or percutaneous ethanol injection is considered the standard of care for patients with BCLC 0-A tumors not suitable for surgery (evidence 2A; recommendation 1B)
EORTC[9]	Additional indications for patients with multifocal tumors meeting Milan criteria (⩽ 3 nodules ⩽ 3 cm) or with mild portal hypertension not suitable for liver transplantation require prospective comparisons with loco-regional treatments. (evidence 3A; recommendation 2C)	In tumors < 2 cm, BCLC 0, Ethanol injection and radio-frequency ablation achieve complete responses in more than 90% of cases with good long-term outcome [evidence 1(i)A; recommendation 1C]
AASLD[10]	Patients who have a single lesion can be offered surgical resection if they are non-cirrhotic or have cirrhosis but still have well preserved liver function, normal bilirubin and hepatic vein pressure gradient < 10 mmHg (recommendation 2)	Local ablation is safe and effective therapy for patients who cannot undergo resection, or as a bridge to transplantation (recommendation 2); Alcohol injection and radiofrequency are equally effective for tumors < 2 cm. However, the necrotic effect of radiofrequency ablation is more predictable in all tumor sizes and its efficacy is clearly superior to that of alcohol injection in larger tumors (recommendation 1)
APASL[11]	Liver resection is a first-line curative treatment of solitary or multifocal HCC confined to the liver, anatomically resectable, and with satisfactory liver function reserve (evidence 2B, recommendation B)	Local ablation is an acceptable alternative to resection for small HCC (< 3 cm) in Child-Pugh A cirrhosis (evidence 2B, recommendation B); Local ablation is a first-line treatment of unresectable, small HCC with 3 or fewer nodules in Child-Pugh A or B cirrhosis (evidence 2B, recommendation B)

Strength of evidence according to study design: Level 1, Randomized controlled clinical trials or meta-analyses of randomized studies; Level 2, Non-randomized controlled clinical trials; Level 3, Case series. Strength of evidence according to end-points: A, Total mortality; B, Cause-specific mortality; C, Carefully assessed quality of life; D, Indirect surrogates. Grading of recommendation: 1, Strong recommendation warranted; 2, Weaker recommendation. Grading of recommendation: A, Further research is very unlikely to change out confidence in the estimate of effect; B, Further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate; C, Further research is very likely to have an important impact on our confidence in the estimate of effect. BCLC: Barcelona Clinic Liver Cancer; HCC: Hepatocellular carcinoma; EASL: European Association for the Study of the Liver; EORTC: European Organisation For Research And Treatment Of Cancer; AASLD: American Association for the Study of Liver Diseases; APASL: Asian Pacific Association for the Study of the Liver.

10 mmHg to allow a safe resection[13], but the evidence for this recommendation is not very strong since it was based on data obtained in a very small cohort studied in the 1990s[30] and surgical techniques have substantially improved since then. Only one recent external validation was conducted on only 39 patients[31], whereas other studies could not confirm the influence of portal hypertension[32]. HVPG measurement can probably help to select surgical candidates, with a very low or null probability of post-operative liver failure, but it probably also excludes patients that can still benefit from surgery and that will be submitted to RFA with a lower chance of cure[32]. Thus, more restrictive criteria for resection result in a better outcome after surgery and a worse outcome after ablation that represents the alternative therapy to be adopted. It can be concluded that such discrepancies, evident even in international guidelines, are attributable to the relatively low level of evidence that can be obtained from the literature, as is pointed out in the following paragraphs.

COMPARATIVE STUDIES ON RESECTION *VS* ABLATION

The literature review retrieved 19 studies that directly compared resection and radiofrequency ablation; of them, three RCTs were available for comparison whereas the remaining studies were represented by retrospective observational studies. Randomized controlled studies were reviewed separately from observational studies. As can be noted from Table 2, the NOS scale of observational studies ranged from 5 to 8, none of them reached the maximum quality assessment of 9 and most of them had a quality scale below 8. In fact, the review of these studies showed that for the two treatment arms patients often have significant differences regarding most clinical and tumor variables, that are able to confound results. Thus, stratification for tumor size was attempted in order to reduce to a minimum potential biases resulting from covariate distribution. Differences observed between the two treatment arms were also highlighted. Characteristics of RCTs are reported in Table 3 and of observational studies in Table 4.

Randomized controlled studies

At December 2012, three RCTs were available for review and all were from Eastern countries[33-35] (Table 3). The first RCT was published by Chen *et al*[33]. Tumor recurrence rate at 2 years after treatment was used as the primary outcome measure to estimate the sample size of the study. After post-randomization exclusion, the study involved 71 patients submitted to ablation and 90 submitted to resection. The results showed that the 3-year overall survival was 71.4% after ablation and 73.4% after surgery. The corresponding disease-free survival rates were 64.1% and 69.0%, respectively. No statistical difference was observed and no differences were observed when patients were stratified by tumor size (*P*-values not provided). The authors concluded that the overall and

Table 2 Summary of published articles that directly compared hepatic resection and radio-frequency ablation identified through literature search

Ref.	Study period	Type of study	NOS
Feng *et al*[35]	2005-2008	RCT	-
Peng *et al*[36]	2003-2008	Retrospective	7
Wang *et al*[37]	2002-2009	Retrospective	6
Ruzzenente *et al*[47]	1995-2009	Retrospective	8
Nishikawa *et al*[42]	2004-2010	Retrospective	7
Hung *et al*[38]	2002-2007	Retrospective	7
Takayama *et al*[39]	2000-2003	Retrospective	5
Huang *et al*[34]	2003-2005	RCT	-
Ueno *et al*[41]	2000-2005	Retrospective	7
Abu-Hilal *et al*[48]	1991-2003	Retrospective	8
Guglielmi *et al*[43]	1996-2006	Retrospective	7
Hiraoka *et al*[40]	2000-2007	Retrospective	7
Hasegawa *et al*[46]	2000-2003	Survey	6
Lupo *et al*[45]	1999-2006	Retrospective	8
Chen *et al*[33]	1999-2004	RCT	-
Ogihara *et al*[49]	1995-2003	Retrospective	7
Montorsi *et al*[50]	1997-2003	Retrospective	6
Hong *et al*[51]	1999-2001	Retrospective	6
Vivarelli *et al*[44]	1998-2002	Retrospective	5

The Newcastle-Ottawa Score (NOS) scale can range from 5 to 9. RCT: Randomized controlled trials.

disease-free survivals were the same for patients with a single tumor ⩽ 5 cm treated with either ablation or resection; however, ablation showed an advantage over surgical resection in causing less post-treatment complications, less pain, and a shorter in-hospital stay[33].

The second RCT was published by Huang *et al*[34]. The 5-year overall survival rate after treatment was used as the primary outcome measure to estimate the sample size of the study. After post-randomization exclusion, the study involved 115 patients submitted to ablation and 115 submitted to resection. Results showed that the 5-year overall survival rates was 54.8% after ablation and 75.7% after surgery ($P = 0.001$). The corresponding recurrence-free survival rates were 28.7% and 51.3%, respectively ($P = 0.017$). The benefit of resection was maintained when patients were stratified by tumor size and number. The authors concluded that surgical resection may provide better survival and lower tumor recurrence rates than ablation for HCC within Milan criteria[34].

The third, and last, RCT was published by Feng *et al*[35]. The 3-year overall survival rate after treatment was used as the outcome measure to estimate the sample size of the study. After post-randomization exclusion, the study involved 84 patients submitted to ablation and 84 submitted to resection. Results showed that the 3-year overall survival rates was 67.2% after ablation and 74.8% after surgery ($P = 0.342$). The corresponding recurrence-free survival rates were 49.6% and 61.1%, respectively ($P = 0.122$). No stratification for tumor stage was provided. The authors concluded that percutaneous radiofrequency ablation may provide therapeutic effects similar to those of hepatic resection[35].

Thus, the available RCTs report different results and only Huang demonstrated a superiority of hepatic resection over ablation. Even if higher survival rates after resection were also observed in the analyses of Chen and Feng, they did not find a statistically significant superiority of surgery over ablation, leaving the question regarding the best therapeutic approach to be adopted unsolved. It should be noted, however, that the different proportions of HCC beyond the very early stage can, at least in part, explain the conflicting results, since it is known that ablation beyond this stage is less able to achieve complete tumor necrosis, thus biasing the final results[19,28,29]. Hence, a further review of the available observational studies is necessary to obtain more clinical, useful information.

Single tumors less or equal to 2 cm

Four observational retrospective studies analyzed outcomes of resection and ablation in single tumors ⩽ 2 cm[36-39] (Table 4) while none of the previous reported RCTs analyzed this specific tumor stage. None of the observational studies reported a convincing comparability between the two treatment arms, and the most frequent differences observed between ablated and surgical patients were that RFA patients were older than surgical patients, had a lower platelet count, belonged more frequently to Child-Pugh class B and were affected by smaller tumors ($P < 0.050$). Thus, results in terms of both patient survival and recurrence rate can be biased by covariate distribution. Three articles deserve some discussion for different reasons. The first article derives from a multi-institutional database of the Liver Cancer Study Group of Japan involving 2550 patients[39]. In this report, disease-free survival (DFS) was significantly better ($P = 0.001$) after resection ($n = 1235$) than after RFA ($n = 1315$), but patient survival was similar ($P = 0.280$). Ablated patients were more frequently in Child-Pugh class B, had higher ICG-R15 and smaller tumor size in comparison to resected patients ($P = 0.001$ in all cases). Therapy and Child-Pugh class were independent prognostic factors of DFS at Cox regression analysis but regression on patient survival was not performed. This report represents the largest series published in the literature that analyzed this specific tumor stage. It can be speculated that patient survival after RFA could be under-estimated, because of more advanced hepatic dysfunction, and, on the contrary, recurrence rate over-estimated because of smaller tumor size. These observations support the hypothesis that patient survival after ablation can be similar to that of surgery for tumors < 2 cm; unfortunately the choice of a composite end-point, as DFS is (in which the event is death or recurrence), does not allow a similar conclusion for just recurrence rate.

In a more recent report by Wang *et al*[37], the authors tried to handle the different covariate distribution by means of propensity score one-to-one match. In their sub-analysis of 104 matched patients with single tumor < 2 cm (52 patients for each arm), the authors reported that resection and RFA provide similar patient survival ($P = 0.296$), but that DFS of surgical patients was significantly better than that of RFA patients ($P = 0.031$). Unfortu-

Table 3 Characteristics of randomized controlled studies that compared hepatic resection *vs* radiofrequency ablation

Ref.	Liver function	Tumor features	Treatment	Study characteristics and main findings
Chen *et al*[33]	Child-Pugh class A ICG-R15 < 30% PLT > 40000/mm^3	Single < 5 cm	HR: 90 RFA: 71	21% of patients randomized to RFA withdrew their consent. The 1-, 3-, and 4-year overall survival rates after RFA and surgery were 95.8%, 71.4%, 67.9% and 93.3%, 73.4%, 64.0%, respectively. The corresponding DFS rates were 85.9%, 64.1%, 46.4% and 86.6%, 69%, 51.6%, respectively. Statistically, there was no difference. The 5-year rates were not reported
		Single tumor ⩽ 3 cm	HR: 42 RFA: 37	Authors stated that patient survival and DFS did not change in tumors < 3 cm but survival rates and *P*-values were not provided (only Kaplan-Meier curves were reported)
		Single 3.1-5.0 cm	HR: 48 RFA: 34	Authors stated that patient survival and DFS did not change in tumors between 3.1 and 5.0 cm but survival rates and *P*-values were not provided (only Kaplan-Meier curves were reported)
Huang *et al*[34]	Child-Pugh class A/B ICG-R15 < 20% PLT > 50000/mm^3	Single ⩽ 5 cm or up to 3 nodules < 3 cm	HR: 115 RFA: 115	Despite randomization, RFA patients had higher prevalence of nodules ⩽ 3 cm (P = 0.021). The 3- and 5-year survival rates for the RFA group and the HR group were 69.6%, 54.8% and 92.2%, 75.7%, respectively (P = 0.001). The corresponding RFS rates were 46.1%, 28.7% and 60.9%, 51.3%, respectively (P = 0.017)
		Single tumor ⩽ 3 cm	HR: 45 RFA: 57	The 3- and 5-year survival rates for the RFA group and the HR group were 77.2%, 61.4% and 95.6%, 82.2%, respectively (P = 0.030). Neither DFS nor RFS for this subgroup were provided
		Single 3.1-5.0 cm	HR: 44 RFA: 27	The 3- and 5-year survival rates for the RFA group and the HR group were 66.7%, 51.5% and 95.5%, 72.3%, respectively (P = 0.046). Neither DFS nor RFS for this subgroup were provided
		Multifocal < 3 cm	HR: 26 RFA: 31	The 3- and 5-year survival rates for the RFA group and the HR group were 58.1%, 45.2% and 80.8%, 69.2%, respectively (P = 0.042). Neither DFS nor RFS for this subgroup were provided
Feng *et al*[35]	Child-Pugh class A/B ICG-R15 < 30% PLT > 50000/mm^3	Up to 2 nodules < 4 cm	HR: 84 RFA: 84	The 1- and 3-year survival rates for HR and RFA groups were 96.0%, 74.8% and 93.1%, 67.2%, respectively (P = 0.342). The corresponding RFS rates were 90.6%, 61.1% and 86.2%, 49.6%, respectively (P = 0.122). Results at 5-year not reported (or not reached). On the basis of this lack of evidence, the authors did not include treatment as a variable in multivariate analysis

Other inclusion criteria common to all randomized controlled trials (RCTs): no radiological evidence of invasion into the portal/hepatic vein branches, no extra-hepatic metastases, no previous treatment of hepatocellular carcinoma (HCC), patient should be suitable to be treated by surgical resection and radiofrequency ablation. HR: Hepatic resection; RFA: Radiofrequency ablation; DFS: Disease-free survival; PLT: Platelet.

nately, the match was unconvincing and the inaccuracy of the match procedure is reinforced by the match provided in the same manuscript for patients with tumors < 3 cm, where covariates were still significantly different, after matching, among the two treatment arms (P < 0.001 in some cases)[37]. This work highlights the need for a rigorous statistical approach in the presence of significant covariate differences; without such an approach, the results can remain difficult to interpret with some degree of certainty.

The third report was published by Peng[36] in 2012, and involved 145 patients, submitted to resection, or ablation, for single tumor ⩽ 2 cm. The authors found that overall survival was better after RFA (P = 0.048) but that recurrence-free survival (RFS) was unaffected (P = 0.548). The results are intriguing since, when looking at the baseline characteristics, the two groups were quite similar as regards clinical and demographical covariates, except for lower prothrombin time and platelet count in the RFA arm. Thus, supposing an effect of worse liver function on survival, this would have to be shown in patients undergoing RFA, returning to an under-estimation of survival after ablation. Multivariate regression analyses showed that treatment allocation was the only significant prognostic factor for overall survival (P = 0.046). If a conclusion, regarding comparative analyses in this HCC stage, is to be drawn, it can be said that there is some evidence that for single nodules, not larger than 2 cm, RFA can provide survival similar to that of resection[24]. An increased recurrence rate, however, has to be expected after RFA even if the tumor is small but this could theoretically be the subject of re-treatment, justifying comparable survivals. For very early HCC, dedicated RCTs are warranted.

Single tumors less than or equal to 3 cm

There is greater experience published in the literature when this size threshold was selected as an inclusion criterion. Overall, seven studies were found to analyze ablation *vs* resection in single tumors ⩽ 3 cm, or that included a sub-analysis in this specific tumor stage (Tables 3, 4)[33,34,40-44]. Two of these studies were the previously cited RCTs by Chen *et al*[33] and Huang *et al*[34], which contained a sub-analysis for this specific tumor stage. In the RCT by Chen *et al*[33], the authors stated that both patient survival and DFS did not change in single tumors < 3 cm, but, unfortunately, both survival rates and *P*-values were not provided. The RCT by Huang *et al*[34] reported a survival advantage of surgery: in the subgroups of 45 resected patients *vs* 57 ablated patients with a solitary nod-

Table 4 Characteristics of observational studies that compared hepatic resection *vs* radiofrequency ablation

Ref.	Liver function	Tumor features	Treatment	Study characteristics and main findings
Peng *et al*[36]	Child-Pugh class A	Single tumor ≤ 2 cm	HR: 74 RFA: 71	RFA patients showed lower prothrombin activity (P = 0.001) and lower platelet count (P = 0.010). Other features were similar between the two groups The 3-, and 5-year survival rates were 87.7% and 71.9%, respectively, after RFA and 70.9% and 62.1% after HR (P = 0.048). The corresponding RFS rates were 65.2% and 59.8% with RFA and 56.1%, and 51.3% after HR (P = 0.548)
Wang *et al*[37]	Child-Pugh class A and B	BCLC early stage	HR: 208 RFA: 254	Patient characteristics were considerably different between the two treatments. RFA patients were significantly older, anti-HCV+, in Child-Pugh class B, with lower platelet count, with smaller and multifocal tumors than HR patients (P = 0.001 in all cases) The 3- and 5-year survival rates were 87.8% and 77.2% for HR, and 73.5% and 57.4% for RFA (P = 0.001). The 3- and 5-year DFS rates were 59.9% and 50.8% for HR and 28.3% and 14.1% for RFA, respectively (P < 0.001)
		BCLC early stage after PS match	HR: 208 RFA: 208	Patient characteristics were different between the two treatment arms. RFA patients were significantly older, anti-HCV+, in Child-Pugh class B, with lower platelet count, with smaller and multifocal tumors than HR patients (P = 0.001 in all cases). Patient and DFS rates not provided for this subgroup
		Single tumor < 2 cm	HR: 52 RFA: 91	Patient characteristics were different between the two treatment arms. RFA patients were significantly older, anti-HCV+, with lower platelet count than HR patients (P < 0.050). No Child-Pugh stratification was provided The 3- and 5-year survival rates were 98% and 91.5% for HR, and 80.3% and 72% for RFA (P = 0.073). The 3- and 5-year DFS rates were 62.1% and 40.7% for HR and 39.8% and 29.3% for RFA, respectively (P = 0.006)
		Single tumor < 2 cm after PS match	HR: 52 RFA: 52	Patient characteristics seem similar between the two treatments. The 3- and 5-year survival rates were 98% and 91.5% for HR, and 82.8% and 82.8% for RFA, respectively (P = 0.269). The 3- and 5-year DFS rates were 62.1% and 40.7% for HR and 46.8% and 38.0% for RFA (P = 0.031)
Ruzzenente *et al*[47]	Child-Pugh class A and B	Up to 3 tumors ≤ 6 cm after PS match	HR: 88 RFA: 88	Patient characteristics seem similar between the two treatments. The 3- and 5-year survival rates were 68.7% and 59.3% for HR, and 50.1% and 27.7% for RFA (P = 0.012). The 3- and 5-year DFS rates were 50.4% and 27.1% for HR and 30.2% and 18.6% for RFA, respectively (P = 0.001)
	Child-Pugh class A and B	Single tumor < 5 cm	HR: 45 RFA: 40	The 3- and 5-year survival rates were 66.1% and 54.5% for HR, and 63.7% and 43.8% for RFA (P = 0.633). The 3- and 5-year DFS rates were 42.4% and 22.6% for HR and 30.7% and 23.0% for RFA, respectively (P = 0.644). Patient and disease-free survival after HR were significantly superior to RFA, in patients with tumors ≥ 5 cm Further stratifications lead to very small groups (n < 10)
Nishikawa *et al*[42]	Child-Pugh class A and B	Single tumor ≤ 3 cm	HR: 78 RFA: 92	RFA patients had smaller tumors (P = 0.001) and lower platelet count (P = 0.004) in comparison to HR patients The 5-year overall survival rates after RFA and HR were 63.1% and 74.6%, respectively (P = 0.259). The corresponding RFS rates were 18.0% and 26.0%, respectively (P = 0.324). In the multivariate analysis treatment was not an independent risk factor for overall and RFS
Hung *et al*[38]	Child-Pugh class A and B	Up to 3 tumors ≤ 5 cm	HR: 229 RFA: 190	RFA patients were significantly older, anti-HCV+, with lower albumin and platelet count (P < 0.050) in comparison to HR patients The 3- and 5-year survival rates were 88.2% and 79.3% for HR, and 77.3% and 67.4% for RFA, respectively (P = 0.009). The 3- and 5-year RFS rates were 56.1% and 40.9% for HR and 29.0% and 20.5% for RFA (P = 0.001)
		Up to 3 tumors ≤ 5 cm after PS match	HR: 84 RFA: 84	Patient characteristics seem similar between the two treatments Patient and DFS rates not provided but only reported in Kaplan-Meier graphs. For patient survival no difference was found (P = 0.519); RFS was significantly worse after RFA (P < 0.001)
		Single tumor < 2 cm	HR: 50 RFA: 66	RFA patients were significantly older, anti-HCV+, with lower albumin and platelet count, higher bilirubin, AST and ICG-R15 and with smaller tumors (P = 0.001) in comparison to HR patients The 3- and 5-year survival rates were 91.1% and 84.6% for HR, and 86.5% and 77.8% for RFA, respectively (P = 0.358). The 3- and 5-year RFS rates were 42.6% and 21.8% for HR and 59.5% and 45.2% for RFA (P = 0.104)
Takayama *et al*[39]	Child-Pugh class A and B	Single tumor ≤ 2 cm	HR: 1235 RFA: 1315	Data from the Liver Cancer Study Group of Japan database. Results were reported in the form of brief communication. RFA patients were significantly more frequently in Child-Pugh class B, had higher ICG-R15 and smaller tumor size (P = 0.001 in all cases) in comparison to HR patients The 1- and 2-year survival rates were 98% and 94% for HR, and 99% and 95% for RFA, respectively (P = 0.280). The 1- and 2-year DFS rates were 91% and 70% for HR and 84% and 58% for RFA, respectively (P = 0.001) Multivariate analysis on DFS confirmed alpha-fetoprotein, therapy and Child-Pugh class as independent factors

Ueno *et al*[41]	Child-Pugh class A and B	BCLC early stage	HR: 123 RFA: 155	RFA patients were significantly more frequently in Liver Damage class B or C, had higher ICG-R15, MELD score and smaller tumor size (P = 0.001 in all cases) in comparison to HR patients The 3- and 5-year survival rates were 92% and 80% for HR, and 92% and 63% for RFA, respectively (P = 0.06). The 3- and 5-year DFS rates were 47% and 38% for HR and 36% and 20% for RFA (P = 0.02)
		Single tumor ⩽ 3 cm	HR: 78 RFA: 92	The 3- and 5-year survival rates were 95% and 95% for HR, and 90% and 60% for RFA, respectively (P = 0.01). The 3- and 5-year DFS rates were 56% and 44% for HR and 37% and 11% for RFA (P = 0.02)
		Single tumor 3.1-5.0 cm	HR: 32 RFA: 9	The 3- and 5-year survival rates were 92% and 72% for HR, and 73% and 73% for RFA, respectively (P = 0.15). The 3- and 5-year DFS rates were 33% and 25% for HR and 14% and 14% for RFA (P = 0.12)
		2 or 3 nodules ⩽ 3 cm	HR: 13 RFA: 54	The 3- and 5-year survival rates were 67% and not reached for HR, and 93% and 63% for RFA, respectively (P = 0.002). The 3- and 5-year DFS rates were 29% and not reached for HR and 35% and 22% for RFA (P = 0.59)
Abu-Hilal *et al*[48]	Child-Pugh class A and B	Single tumor ⩽ 5 cm	HR: 34 RFA: 34	This was a matched analysis for age, sex, tumor size, and Child-Pugh grade The 5-year survival was 56% for HR, and 57% for RFA (P = 0.302). The 5-year DFS was 28% for HR and 21% for RFA (P = 0.028)
Guglielmi *et al*[43]	Child-Pugh class A and B	Up to 3 tumors ⩽ 6 cm	HR: 91 RFA: 109	RFA patients were significantly older, belonged more frequently to Child-Pugh class B and more frequently had multinodular tumors (P = 0.010) in comparison to HR patients The 3- and 5-year survival rates were 64% and 48% for HR, and 42% and 20% for RFA, respectively (P = 0.010). The 3- and 5-year DFS rates were 56% and 27% for HR and 22% and 22% for RFA (P = 0.001) Superiority of HR was confined to patients in Child-Pugh class A. Further stratifications resulted in groups of patients not large enough (n < 10) to obtain realistic comparisons Type of treatment was significantly related to survival and DFS at multivariate analyses
	Child-Pugh class A	Single tumor ⩽ 3 cm	HR: 20 RFA: 11	The 3- and 5-year survival rates were 93% and 71% for HR, and 50% and not reached for RFA, respectively (P = 0.060)
	Child-Pugh class A	Single tumor > 3 cm	HR: 33 RFA: 23	The 3- and 5-year survival rates were 64% and 55% for HR, and 63% and 45% for RFA, respectively (P = 0.700)
Hiraoka *et al*[40]	Child-Pugh class A and B	Single tumor ⩽ 3 cm	HR: 59 RFA: 105	RFA patients belonged more frequently to Child-Pugh class B (P = 0.011), more frequently had tumors < 2 cm (P = 0.001), and had worse ICG-R15 (P = 0.026) in comparison to HR patients The 3- and 5-year survival rates were 91.4% and 59.4% for HR, and 87.8% and 59.3% for RFA, respectively (P = NS). The 3- and 5-year DFS rates were 64.3% and 22.4% for HR and 58.7% and 24.6% for RFA (P = NS) No multivariate analysis provided
Hasegawa *et al*[46]	Child-Pugh class A and B	Up to 3 tumors ⩽ 3 cm	HR: 2857 RFA: 3022	Data were analyzed together with a population of 1306 patients submitted to percutaneous ethanol injection. RFA patients were significantly older, belonged more frequently to Child-Pugh class B, had lower serum albumin, higher bilirubin, worse ICG-R15 and more frequently had multinodular and smaller tumors (P < 0.001 in all cases) in comparison to HR patients Results were limited to 24 mo. The 1- and 2-year survival rates were 98.3% and 94.5% for HR, and 98.5% and 93.0% for RFA, respectively (P = 0.640) The 1- and 2-year recurrence rates were 17.0% and 35.5% for HR and 26.0% and 55.4% for RFA (P < 0.001) At multivariate analysis, type of treatment did not affect overall survival but affected recurrence rate
Lupo *et al*[45]	Child-Pugh class A and B	Single tumor 3-5 cm	HR: 42 RFA: 60	The groups were similar in terms of median age, Child-Pugh score and tumor size The 3- and 5-year survival rates were 57% and 43% for HR, and 53% and 32% for RFA, respectively (P = 0.824). The 3- and 5-year DFS rates were 35% and 14% for HR and 18% and 0% for RFA (P = 0.283) No multivariate analyses were performed
Ogihara *et al*[49]	Child-Pugh class A and B	Single tumor without size limit	HR: 47 RFA: 40	RFA patients were significantly older, belonged more frequently to Child-Pugh class B and had smaller tumors (P < 0.001 in all cases) in comparison to HR patients The 3- and 5-year survival rates were 65% and 31% for HR, and 58% and 39% for RFA, respectively (P = NS). DFS not provided. No multivariate analysis was provided
	Child-Pugh class A and B	Single tumor ⩽ 5 cm	HR: 18 RFA: 26	In these subgroups, RFA patients were still significantly older and belonged more frequently to Child-Pugh class B (P < 0.050) in comparison to HR patients The 3- and 5-year survival rates were 64% and 21% for HR, and 53% and 32% for RFA, respectively (P = NS). The 3- and 5-year DFS rates were 37% and 37% for HR and 31% and 23% for RFA (P = NS) Results did not change in single tumors > 5 cm
Montorsi *et al*[50]	Child-Pugh class A and B	Single tumor ⩽ 5 cm	HR: 40 RFA: 58	All RFA were performed with laparoscopic approach. RFA patients had significantly worse INR and higher AST (P < 0.050). A trend toward higher bilirubin, lower platelet count and higher ALT was also reported (P < 0.10)

				The 3- and 4-year survival rates were 73% and 61% for HR, and 61% and 42% for RFA, respectively (P = 0.139). The RFS rates were not reported and only plotted in a Kaplan-Meier curve reporting a P = 0.024. Five-year rates not reported. Multivariate analysis on survival did not include the primary exposure variable (HR *vs* RFA)
Hong *et al*[51]	Child-Pugh class A	Single tumor ⩽ 4 cm	HR: 93 RFA: 55	RFA patients were significantly older (P < 0.001) but the other characteristics reported were not statistically different between the two groups The 1- and 3-year survival rates were 97.9% and 83.9% for HR, and 100% and 72.7% for RFA, respectively (P = 0.24). The 1- and 3-year RFS rates were 75.9% and 54.7% for HR and 74.1% and 40.2% for RFA (P = 0.54). Five-year rates not reported. Results did not change when patients were stratified by AJCC or CLIP stages No multivariate analyses were performed
Vivarelli *et al*[44]	Child-Pugh class A and B	No inclusion criteria specified	HR: 79 RFA: 79	RFA patients belonged more frequently to Child-Pugh class B and more frequently had multinodular tumors (P < 0.001 in both cases) The 1- and 3-year survival rates were 83% and 65% for HR, and 78% and 33% for RFA, respectively (P = 0.002). The 1- and 3-year DFS rates were 79% and 50% for HR and 60% and 20% for RFA (P = 0.001). Five-year rates not reported. No multivariate analyses were performed
	Child-Pugh class A and B	Single tumor ⩽ 3 cm	HR: 21 RFA: 22	The 1- and 3-year survival rates were 89% and 79% for HR, and 89% and 50% for RFA, respectively (P = NS). The 1- and 3-year DFS rates were 84% and 67% for HR and 70% and 34% for RFA (P = NS). Five-year rates not reported
	Child-Pugh class A and B	Single tumor > 3 cm	HR: 58 RFA: 57	The 1- and 3-year survival rates were 81% and 59% for HR, and 74% and 24% for RFA, respectively (P = 0.007). The 1- and 3-year DFS rates were 77% and 43% for HR and 56% and 12% for RFA (P = 0.003). Five-year rates not reported. These differences were confirmed when the analyses were confined to Child-Pugh class A patients

HR: Hepatic resection; RFA: Radiofrequency ablation; RFS: Recurrence-free survival; DFS: disease-free survival; PS: Propensity score; AST: Aspartate aminotransferase; NS: Not significant; BCLC: Barcelona Clinic Liver Cancer; HCV: Hepatitis C virus; AJCC: American Joint Committee on Cancer; CLIP: Cancer of the Liver Italian Program; MELD: Model for end-stage liver disease.

ule ⩽ 3 cm, the 5-year survival after surgery was 82.2%, significantly higher than the 61.4% after RFA of (P = 0.030). Disease-free or recurrence-free survivals were not analyzed. One limitation is represented by the fact that covariate distribution among the two treatment arms was not provided for these specific subgroups of patients; however, since in the whole study population tumor size was the only variable that proved to be slightly different among the two groups, this sub-analysis seems quite realistic and is, at present, the most robust evidence of the superiority of one treatment (surgery) over the competing one (ablation)[34].

Similar comments regarding covariate distribution, made for single tumors < 2 cm, can be repeated for analyses on single tumors < 3 cm. Of the five retrospective studies found, two series deserve particular discussion. In 2008, Hiraoka published results from a population of 59 surgical and 105 RFA patients: no significant differences were found in terms of both patient survival and DFS[40]. However, the magnitude of the differences observed between the two treatment arms, in terms of Child-Pugh class, ICG-R15, serum albumin, bilirubin, and tumor size that were all in favor of resection, was so large that the comparison was evidently unrealistic. Furthermore, the authors did not provide an inferential analysis, leaving the doubt unsolved[40]. In 2009, Ueno *et al*[41] published a report from the Kagoshima Liver Cancer Study Group reporting that patients with a single nodule ⩽ 3 cm achieved a 5-year survival of 95% after resection (n = 78), significantly higher than that of 60% after RFA (n = 92; P = 0.010), but 75.6% of the resected patients had a Liver damage A whereas 66.3% of ablated patients had a Liver damage B or C (P = 0.001). Stratification of survival for Liver damage returned to non-significant differences in terms of both patient and disease-free survivals and these results did not help clarify, with a convincing degree of evidence, the real superiority of resection over ablation[41]. The remaining studies report results on very small subgroups, often less than 10 patients[42-44], or suffered from wrongful comparison[37], making it hard to consider findings to provide enough degree of evidence.

Single tumors 3-5 cm

Four articles were identified that analyzed comparative results of surgery and ablation in single nodules between 3 and 5 cm or that included a sub-analysis in this specific tumor stage (Tables 3 and 4)[33,34,41,45]. Two of these studies were, again, the RCT by Chen *et al*[33] and the one by Huang *et al*[34], which contained a sub-analysis for this specific tumor stage. In the RCT by Chen *et al*[33], the authors stated that both patient survival and DFS did not change between treatment arms but survival rates and p-values were again not provided. Huang's results reported a 5-year survival after surgery of 72.3% *vs* 51.5% after ablation (P = 0.046); neither DFS nor RFS were provided (Table 3)[34]. Thus, with the limitations of subgroup analyses, the available RCTs reported a limited difference between surgery and ablation for single nodules between 3 and 5 cm. When observational studies were analyzed, the findings became very difficult to interpret. In a subgroup analysis by Ueno *et al*[41] (resection: 32 patients; RFA: 9 patients), no differences were found in terms of either patient survival (5-year rate after resection: 72%; ablation: 73%; P = 0.15) or DFS (5-year rate after resec-

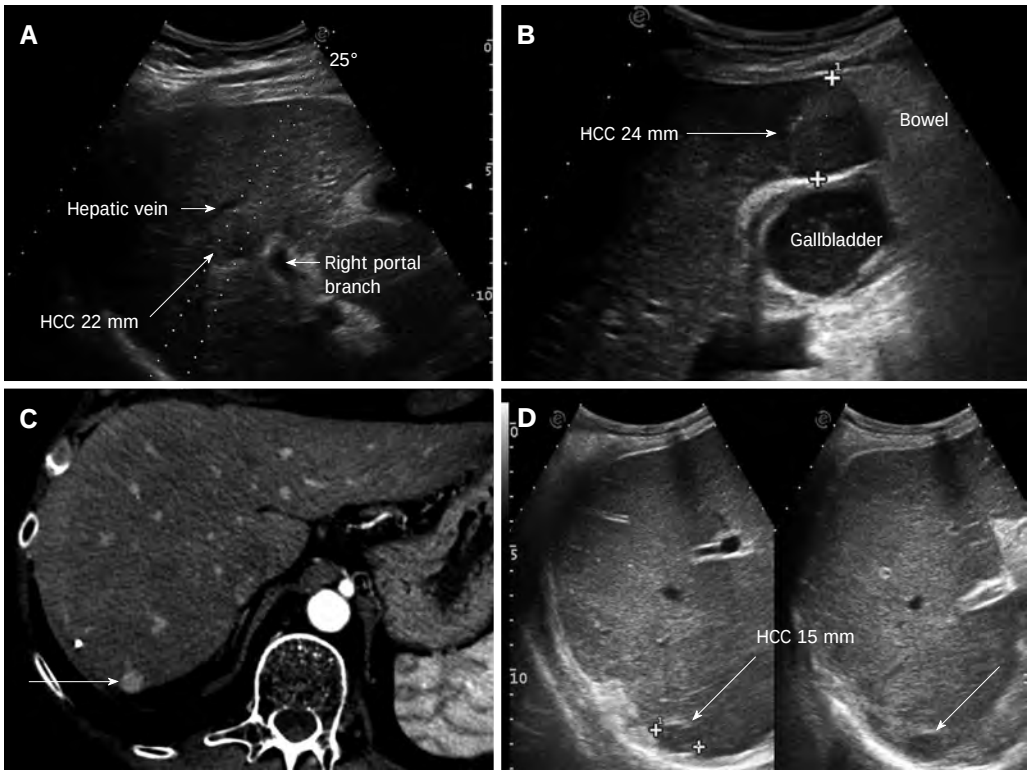

Figure 1 Clinical cases in which performing hepatic resection or radiofrequency ablation had to be decided. A: Small hepatocellular carcinoma (HCC), 22 mm in diameter, located centrally in the right liver lobe in a patient with MELD 10 and clinical signs of portal hypertension. Surgery would have required a right hepatectomy, thus, radiofrequency ablation was preferred even if a reduced rate of complete necrosis could be expected due to the possible heat sink effect of the nearby large vessels; B: The tumor is located sub-capsular, close to the bowel loops and in strict contact with the gallbladder, implying various technical contraindications to percutaneous ablation. Open surgery was the strategy adopted; C: The tumor (long arrow), shown in the arterial phase of contrast enhancement at computed tomography scan, is located sub-capsular at the liver dome; D: Ultrasonography confirms the tumor (long arrow) to lie very deep and without a safe needle track; in fact, these images are taken in deep inspiration, the lesion being hardly visible during normal breathing. The location was considered to contraindicate percutaneous ablation and surgery was performed.

tion: 25%; ablation: 14%; $P = 0.15$) but, as can be immediately noted, the sample size was very small. Another retrospective study published by Lupo *et al*[45] reported that resection and ablation provide very similar results. In particular, the 5-year survival was 43% after resection ($n = 42$) and 32% after ablation ($n = 60$; $P = 0.824$), and the corresponding DFS rates were 14% and 0% ($P = 0.283$). Thus, it must be noted that resection repeatedly leads to better patient survival and recurrence-rate, but the inability to detect a statistical difference between the two treatments leaves the question of the superiority of surgery unsolved. It could be speculated that it is paradoxical for ablation to be inferior to resection for nodules < 3 cm and equivalent for larger tumors, since the ability of RFA to achieve tumor necrosis decreases with the increase in tumor size[19,28,29,46]. Thus, for this single HCC 3-5 cm, it can be said that the literature consistently reports higher patient and disease-free survival rates that do not achieve statistical significance likely only for the small sample size of study populations. This specific tumor stage also probably deserves dedicated studies.

Multiple tumors

The presence of multiple tumors, at diagnostic evaluation prior to treatment, represents the most frequent indication for radiofrequency ablation. Except for the three RCTs and the studies conducted on solitary tumors, multifocal tumor prevalence was almost always higher in ablated patients in comparison to surgical ones[37,43,44,46]. Only two studies reported a subgroup analysis on two or three nodules less than 3 cm, thus within BCLC early stage, excluding single nodules[34,41]. The RCT by Huang reported a survival advantage of surgery ($P = 0.042$): in the subgroups of 26 resected patients *vs* 31 ablated patients with a solitary nodule ⩽ 3 cm, the 5-year survival after surgery was 69.2%, significantly higher than the 45.2% after RFA of[34]. Disease-free or recurrence-free survivals were not analyzed. In the report by Ueno *et al*[41], the 5-year survival was not reached for surgical patients ($n = 13$) and the 3-year survival was in favor of RFA ($n = 54$; $P = 0.002$), while DFS was similar ($P = 0.590$). The difficulty to obtain a comparison within this stage was highlighted by the sub-analysis by Guglielmi *et al*[43] who tried to stratify for Child-Pugh class 11 ablated patients (6 in Child-Pugh class A) *vs* 7 resected patients (all belonging to Child-Pugh class A) without obtaining any reliable result. For multiple tumors, the current comparative literature leaves the impression that the prognosis will be relatively lower despite the treatment adopted.

Other studies

Five studies remain to be briefly discussed[47-51]. The re-

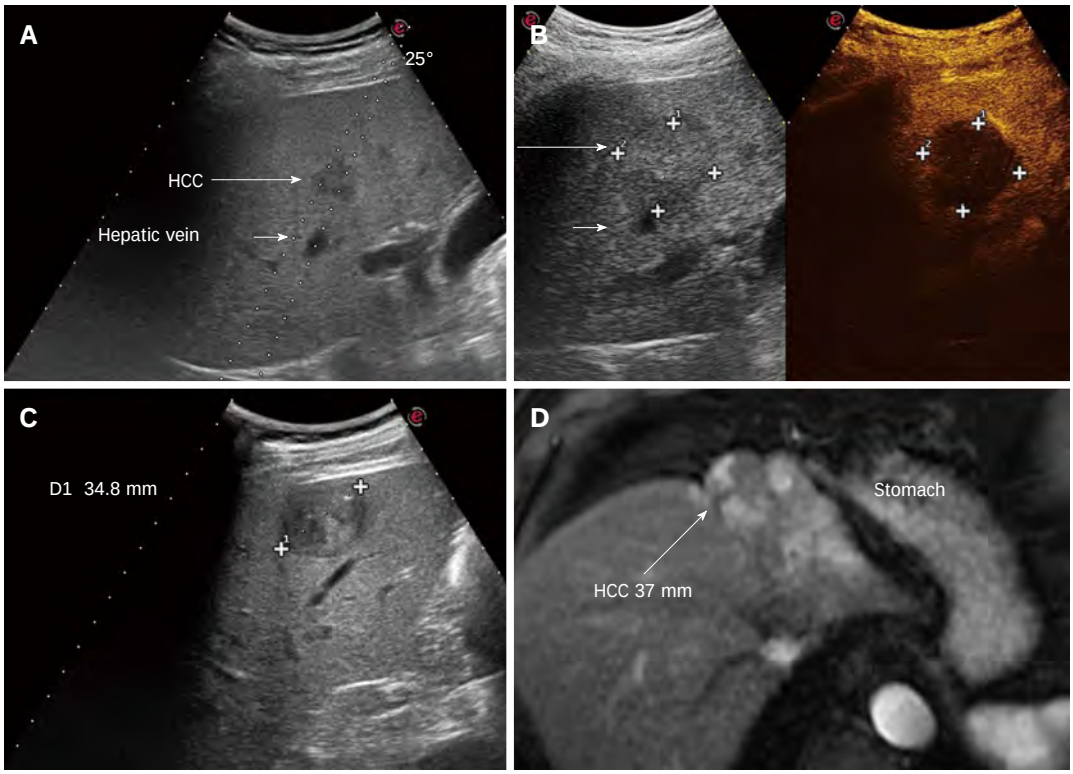

Figure 2 Clinical cases in which performing hepatic resection or radiofrequency ablation had to be decided. A: Ultrasonography through a right inter-costal scan shows a very early hepatocellular carcinoma (HCC) in segment 5 that can be reached with a safe needle track for thermal ablation. Given the small size and easy access, radiofrequency ablation was carried out; B: Post treatment assessment with contrast enhanced ultrasound shows a necrotic devascularized area (34 mm × 35 mm) that includes the tumor with a safety margin > 5 mm; C: Superficial HCC of 35 mm in hepatitis B virus related cirrhosis with preserved liver function. This lesion could be treated by either ablation or resection, but resection is preferable given the superficial location in segment 5 and the size > 3 cm; D: Tumor lesion partially treated by a previous trans-catheter arterial chemoembolization performed in another hospital, in a sub-capsular location close to the stomach. The theoretical path for radiofrequency ablation would lead the needle to puncture the tumor directly and thermal ablation would imply a risk of heat damage to the stomach wall. Laparoscopic resection was the strategy adopted. The long arrow indicates the HCC after treatment.

ports from Ruzzenente *et al*[47], in 2012, and from Abu-Hilal *et al*[48], in 2008, are examples of the attempt to account for confounding variables through matching. The first study used a propensity score match to select patients, submitted to surgery or RFA, having similar covariate distributions[47], and the second used an "a-priori" match based on age, sex, tumor size and Child-Pugh grade[48]. Both studies included tumors larger than 2 cm in both arms and reported an advantage of surgery in determining DFS over ablation but not in terms of patient survival that was similar for single tumors < 5 cm. Of note, the study by Abu-Hilal included only 34 patients for each arm and of the one by Ruzzenente included Child-Pugh B patients. The remaining articles reported results from comparative analyses without tumor size limit[49], or with large (up to 5 cm) size limit, but unfortunately they lack inference analyses[50,51].

DISCUSSION

The present review shows the lights and shadows of the comparative literature regarding hepatic resection and radiofrequency ablation for HCC. It is evident that most studies are affected by questionable methodological approaches since surgical patients and ablated patients represent patient populations that appear quite different as regards clinical and tumor features that are known to affect prognosis. Despite the inconclusive results and the interest in understanding which treatment strategy is best, it is worthwhile pointing out that the situations in which surgery and ablation would be both really equally feasible, and in which they could thus truly compete, occur in less than half of the cases seen in daily clinical practice. In fact, most studies did not report how many patients were excluded from either surgery or ablation, because of the presence of absolute or relative contraindications to one or the other treatment, which might differ in the case of one or the other therapy (thus these patients were most likely offered the alternative therapy). Patients might not be considered suitable for surgery because of liver dysfunction and/or portal hypertension, according to the individual center's strategy, as well as the presence of comorbidities or advanced age contraindicating general anesthesia. Some clinical examples can be found in Figures 1 and 2. In some cases, surgery might not be considered because of the hepatic location of the tumor, which would require very extensive parenchymal sacrifice. Conversely, a subcapsular anterior location exposes the patient to a higher risk of bleeding and/or peritoneal seeding[52], unless a direct puncture of the tumor could be

avoided[53], which is however not always possible. Moreover, complete necrosis of lesions close to the gallbladder is less often possible to achieved because of the potential risk of gallbladder wall damage[26]. Similarly, complete necrosis of lesions abutting the diaphragm may not be possible[27]. Finally, patients with compromised prothrombin time (prolonged International Normalized Radio) are invariably excluded from surgery because this alteration indicates liver dysfunction; similarly, a very low platelet count (< 50.000) is often also considered a contraindication to surgery since it indicates portal hypertension. Such patients should therefore be treated with ablation, as the first alternative therapy, but a clotting impairment might also contraindicate percutaneous ablation or at least increase the risk of adverse events, despite the possibility of preliminary transfusions. All these different variables affecting the choice between resection and ablation most likely justify the difference in the clinical covariates found in the various non randomized studies, as commented above, leading to rather heterogeneous patient populations. This is in keeping with the hypothesis that patients were not randomly allocated to one or the other treatment, but following clear preferences, so that in each case either surgery or ablation was specifically preferred on the basis of clinical variables. Only in rather a few remaining cases of early HCC within the Milan criteria might hepatic resection and radiofrequency ablation be considered truly competitive, and no definitive evidence exists strongly favoring one or the other technique.

However, based on the results reported and commented on above, we can conclude that, until further studies become available, it seems reasonable to offer radiofrequency ablation to very small HCC (< 2 cm) which present an easy access, with no technical contraindications, since in this instance complete necrosis, including the desired safety margin, is most likely to be achieved. At variance, in larger nodules, namely > 2 cm and especially if > 3 cm, and/or in tumor locations in which ablation is not expected to be effective or safe (which often correspond to subcapsular locations, which instead make atypical resections possible), surgical removal is to be preferred in our opinion. For future explorative research, it can be suggested that: (1) intention-to-treat analysis should be included in the studies; (2) further RCTs are warranted, especially for single tumors < 2 cm in which ablation can achieve a sustained pathological response; (3) retrospective observational studies should include in their analyses an inference approach that includes the primary exposure variable (that is resection *vs* ablation) regardless of its statistical difference at univariate analysis; and (4) retrospective observational studies should include stratification for tumor size and liver degree dysfunction together with an attempt at matching, as propensity score can provide.

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REVIEW

Epithelial toll-like receptor 9 signaling in colorectal inflammation and cancer: Clinico-pathogenic aspects

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Abstract

Toll-like receptors (TLRs) recognize specific motifs which are frequently present in bacteria, fungi, prokaryotes and viruses. Amongst TLRs, TLR9 can be activated by such bacterial or viral DNA fragments, immunoglobulin-DNA complexes or synthetic oligonucleotides, which all contain unmethylated cytosine-guanine nucleotide sequences (CpGs). Emerging data indicate that TLR9 signaling has a role in, and may influence, colorectal carcinogenesis and colonic inflammation. CpGs are classified into three groups according to their influence on both the antigen-specific humoral- and cellular immunity, and the production of type 1 interferons and proinflammatory cytokines. TLR9 activation *via* CpGs may serve as a new therapeutic target for several cancerous and various inflammatory conditions. Due to its probable anti-cancer effects, the application possibilities of TLR9-signaling modulation may be extremely diverse even in colorectal tumors. In this review we aimed to summarize the current knowledge about TLR-signaling in the pathogenesis and therapy of inflammatory bowel diseases and colorectal cancer. Due to the species-specific differences in TLR9 expression, however, one must be careful in translating the animal model data into the human system, because of the differences between CpG-oligodeoxynucleotide-responsive cells. TLR9 agonist DNA-based immunomodulatory sequences could also represent a promising therapeutic alternative in systemic inflammatory conditions and chronic colonic inflammations as their side effects are not significant.

Key words: Toll-like receptor 9; Synthetic oligodeoxynucleotide sequences; DNA-based immunomodulatory sequences; Colorectal cancer; Inflammatory bowel diseases

Core tip: Toll-like receptor 9 mediated signaling influences and regulates the severity of mucosal inflammation, and seems to have a protective role against malignant transformation. The modulation of toll-like receptor 9 signaling by synthetic oligodeoxynucleotide agonists or antagonists seems to have beneficial therapeutic effect in inflammatory and cancerous colonic disorders.

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INTRODUCTION

The immunostimulatory effect of DNA was discovered by William Coley, a surgeon from New York[1]. He used living and heat-treated bacteria as a therapeutic option for different kinds of tumors. It has long been known that

microbes contain many immunostimulatory ingredients. In 1980, Tokunaga *et al*[2] identified the bacterial DNA as a main ingredient of the Coley-lysate. Later, they also showed that the same immunostimulatory effect could be achieved by using short synthetic oligodeoxynucleotide sequences (ODNs)[3,4]. In 2000, it was proven that DNA sequences are mainly recognized by the members of the Toll-like receptor (TLR) superfamily[5]. It has also been proven that in TLR9 knock out mice, microbial DNA fragments cannot result in an immune response[5]. It was also shown that the immunomodulating effect of natural and synthetic ODNs is mainly transmitted by TLR9[6].

Peyer's patches (PPs) and isolated lymphoid follicles (ILFs) are immunologic and regenerative organizers of the gut mucosa, and they also represent a unique switch point between colonic inflammation and cancer[7]. Microfold (M) cells are located in the follicle associated epithelium (FAE) of PPs and ILFs, where they mediate the uptake and transcytosis of luminal antigens to the underlying lymphoid tissue. TLR9 was found to be preferentially expressed in M cells[8]. Some TLR polymorphisms are known to be associated with the susceptibility of inflammatory bowel diseases (IBD)[9-11] and sporadic colorectal cancer (CRC) development[12,13], but the current and concrete pathogenic role of TLRs, including TLR9, remains uncertain in these conditions.

In this review we aimed to summarize the current knowledge about TLR-signaling in the pathogenesis of IBD and CRC, focusing especially on TLR9. Recent data indicate that targeting TLR9-signaling may yield new and promising therapeutic approaches to these conditions.

LIGANDS AND SIGNALING OF TOLL-LIKE RECEPTORS

TLRs belong to the type 1 transmembrane glycoproteins, which contain extracellular leucin-reach repeated sequences and Toll/interleukin-1 receptor signaling domains. TLR4 was the first to be identified, and currently 10 TLRs have been identified in humans, while 13 have been identified in mice[14]. TLRs are mainly expressed in the cells of the innate and adaptive immunity (*i.e.*, monocytes, macrophages, lymphocytes, mast cells, dendritic cells), however, some (TLR4, -5 and -9) may be expressed by modified epithelial cells as well[15]. Apical epithelial TLR9 activation by bacterial DNA fragments has been reported to maintain colonic homeostasis[16].

TLRs usually recognize microbial wall components, DNA and ribonucleic acid (RNA) fragments. TLR1, -2, -4, -5, and -6 are localized to the cell surface, while TLR3, -7, -8, and -9 are present in the intracellular compartment[17-20]. TLRs bind specific motifs, which frequently appear in bacteria, fungi, protozoa, and viruses[21,22]. These motifs can be lipids and lipopeptides (TLR1, -2, -4, -6), bacterial flagelline (TLR5), and fragments of nucleic acids (TLR3, - 7, -8, -9). TLR3 binds double stranded RNA from viruses, while TLR7 and -8 can recognize single stranded RNAs. Moreover, TLR7 recognizes immunoglobuline-self-RNA complexes in autoimmune disease conditions. Imiquimod is a specific ligand for TLR7. TLR9 could be activated by bacterial and viral DNA, immunoglobuline-DNA complexes, and synthetic ODNs, which contain unmethylated CpG sequences[21,22].

The signals transmitted by TLRs activate both innate and adaptive immunity. Due to the immune evasion nature of tumor cells, the dysregulated activation of adaptive and innate immune systems could result in cytotoxic effects. This could in turn eradicate the diseased cells or even control the tumorous progression. TLRs recognize pathogen-associated molecular patterns (PAMPs) originating from microbiota and could also bind endogenous ligands, such as danger-associated molecular patterns (DAMPs)[23]. Both bacterial DNA and synthetic ODNs activate the innate and adaptive immune system *via* plasmacytoid dendritic cells (pDCs) and macrophages[24].

TOLL-LIKE RECEPTOR 9 SIGNALING

Due to TLR9-associated activation, pDCs produce interferon-α which influences the cytokine production of B cells[24] resulting in pro- (*e.g.*, interleukin 6, tumor-necrosis factor-α) and anti-inflammatory (*e.g.*, interleukin 10) cytokine release and co-expression of MHC Ⅱ type surface antigens.

The activation of TLR9 is a complex pathway. The uptake of DNA sequences is the most unclear process, which is influenced by the structure of the DNA fragments. Many cell types can easily take up single stranded DNA, but the uptake of double stranded DNA may be more effective if a cationic lipid is used for packing it in, because TLR9 is located in the intracellular compartment of endosomes[24,25]. It was shown that fluorescein isothiocyanate labelled CpG DNA is transferred to the intracellular compartment by non-specific endocytosis[25]. This transport is non-specific, because DNA sequences lacking CpG dinucleotides may also be recognized by TLR9, and this way of immune activation can be competitively inhibited by non-CpG sequences[25]. After transportation to the intracellular compartment, endosomal acidic maturation occurs. This process may be inhibited by pH raising agents (*e.g.*, chloroquine, bafilomycin A)[25]. Finally pro- and anti-inflammatory cytokines may be released and B cell proliferation may be enhanced[25]. The main steps of TLR9-signaling are summarized in Figure 1.

The signal molecules of this pathway [*e.g.*, myeloid differentiation primary response gene 88 (Myd88), tumor necrosis factor receptor-associated factor 6 (TRAF6), interleukin receptor associated kinase (IRAK)-1, -4; p50/p65 heterodimer of nuclear factor (NF)-κB] are non-specific and are also involved in the signaling of other TLRs. Interferons may also be released by a mitogen activated protein kinase (MAPK)-associated pathway, which is also intensively being investigated[26].

CPG OLIGODEOXYNUCLEOTIDE CLASSES

The immunostimulatory effect of unmethylated CpG

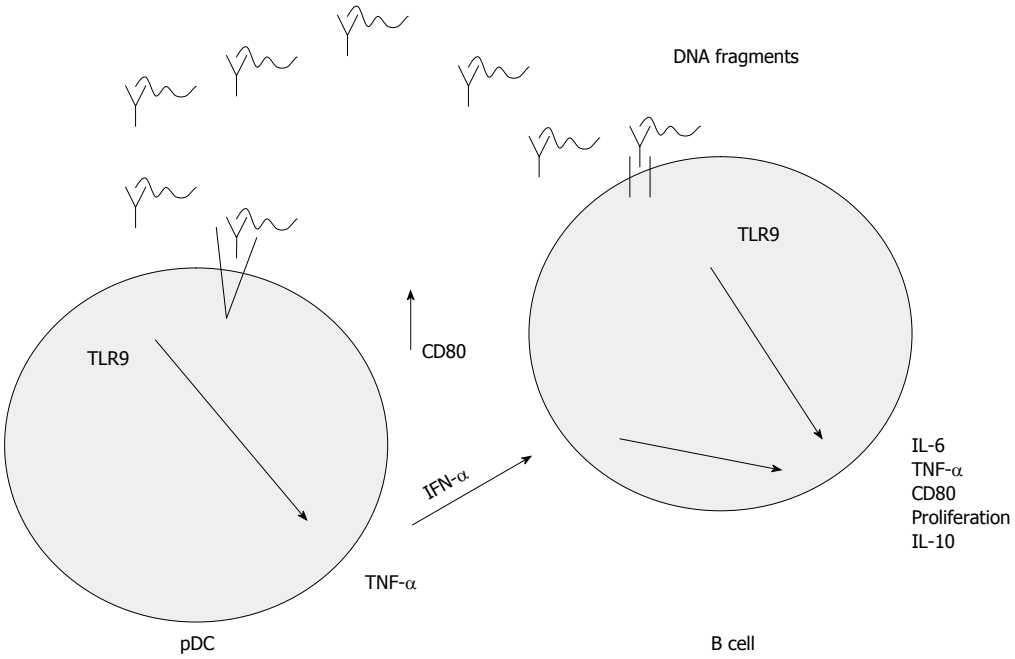

Figure 1 Toll-like receptors 9-mediated cytokine release in the colonic mucosa. Binding DNA fragments by toll-like receptors (TLRs) of plasmacytoid dendritic cells (pDCs) results in pro-inflammatory cytokine release and subsequent B cell activation together with both CD80 overexpression and B cell proliferation. CD80 provides a costimulatory signal necessary for T cell activation and survival. IFN: Interferon; TNF: Tumor necrosis factor; IL: Interleukin.

sequences has been proven in mice and in other species, as well as in *in vitro* human cell line experiments[27,28]. The CpG DNA sequences can be classified into three classes based on the different immune cell-mediated immune responses and their chemical structure. It has already been documented how these differences in the chemical structure may determine the immunostimulatory effect of these sequences on immune cells[29]. Liu *et al*[29] demonstrated in mice that three CpG-ODN classes can differentially affect antigen-specific humoral and cellular immune responses. Specifically, the B- and C-class CpG-ODNs induced a potent Th1-mediated immunity with comparable antibody levels as well as CD4+ and CD8+ T cell responses. In contrast, although the A-class CpG-ODNs weakly enhanced antibody titers and CD8+ T cell response regarding cytotoxic activity, they were not able to change the IgG1/IgG2a ratio or elicit antigen-specific, interferon γ-secreting CD4+ and CD8+ T cells. Consistent with this, three CpG-ODN classes provided differential antigen-specific protection against an intracellular bacterial infection (*i.e.*, Listeria monocytogenes). These three classes of CpG-ODNs did not show significant differences regarding the interleukin 12 producing effect[30,31]. These results may provide not only better understanding of the adjuvant activities of three CpG-ODN classes, but also of implications for the rational design of CpG-ODN adjuvants.

CONTRIBUTION OF TOLL-LIKE RECEPTOR 9 POLYMORPHISM TO DISEASE DEVELOPMENT

Components of Gram-negative bacterial cell walls alert the host to invading bacteria and activate innate immunity. These responses are usually effective in combating infection and restoring normal host function. However, in individuals susceptible to IBD, they may become excessive and lead to mucosal damage.

In genes for all the contributing proteins, single nucleotide polymorphisms (SNPs) have been identified that may increase IBD susceptibility[32]. There are several lines of sensing bacterial components (described earlier), all of which result in activation of NF-κB, and thereby stimulate the innate immune response. In genes of TLR9 signaling, SNPs have been found that may increase IBD susceptibility[9,33]. Török *et al*[9] reported that a SNP in the promoter region of the TLR9 gene was associated with increased risk of Crohn's disease in a German cohort. These genetic findings confirm an important role for innate immunity, pro- and anti-inflammatory immune responses for both gut homeostasis and the development of chronic inflammation in IBD.

Regarding the connection between polymorphisms in TLR9 genes and the risk of colorectal cancer no data are currently available.

ROLE OF TOLL-LIKE RECEPTOR 9 SIGNALING IN COLONIC INFLAMMATION AND CARCINOGENESIS

The commensal microbiota of the intestinal tract confer multiple health benefits to the host, including amelioration of IBD. It was recently identified that TLR9-induced type 1 interferons mediate the anti-inflammatory effects in experimental colitis[34]. The addition of neutralization antibodies to type 1 interferons abolished the anti-inflam-

matory effects, whereas the administration of recombinant interferon-β mimicked the anti-inflammatory effects induced by TLR9 agonists.

The relapse of IBD may occur following an infection with *Campylobacter jejuni* (*C. jejuni*). In a murine model of dextran sulfate sodium (DSS) induced colitis, the infection of the animals by *C. jejuni* disrupted TLR9-induced reinforcement of the intestinal epithelial barrier and colonization by *C. jejuni* increased the severity of DSS-induced colitis[35].

In humans, the gene expression and protein expression level of not just TLR2, -4, and -8, but also TLR9 increased in the biopsy samples of active ulcerative colitis patients. Furthermore, the levels of these TLRs positively correlated with the severity of intestinal inflammation as well as with inflammatory cytokine production[36]. Based on these results, it is plausible that TLR9 mediated signaling influences and regulates the severity of the mucosal inflammation.

In colonic carcinogenesis the role of TLR9 signaling is not well studied. It was recently published that ODNs targeting TLR9 oppositely modulate DNA repair genes in tumor versus immune cells and enhance the biologic effects of chemotherapy[37]. The first publication about the relation of TLR9 expression to colonic carcinogenesis was also published nowadays[38]. Eiró *et al*[38] found TLR9 expression to be higher in hyperplastic or adenomatous polyps compared to other polyp types. TLR9 expression was decreased in hyperplastic and villous polyps from patients who developed colorectal cancer. Their findings suggest a possible protective role of TLR9 expression against malignant transformation in the colorectal mucosa.

THERAPEUTIC POTENTIAL OF TOLL-LIKE RECEPTOR AGONISTS

The therapeutic targeting of TLRs may be useful in diseases such as tumors, allergies or viral infections. In these disorders, TLR agonists and antagonists result in a different immune response. In allergic diseases, like asthma or inflammatory conditions, such as IBD, these agents have an important effect on T cells. For a wider spectrum of anti-tumoral immune response TLR agonists in tumorous diseases require the involvement of the innate immunity, pDCs, monocytes and macrophages, as well as the activation of Th1-dependent immunity and induction of apoptosis[28].

Toll-like receptor agonists in colorectal cancer

In 2011, Rosa *et al*[39] demonstarted that an immunomodulatory oligonucleotide sequence (IMO) in combination with cetuximab has an antitumorous effect on a K-ras mutated colorectal carcinoma model. This is probably based on the alteration of MAPK phosphorylation and results in structural and functional changes in the relationship between epidermal growth factor receptor (EGFR) and TLR9[39]. They used a synthetic IMO having free 5' end. The CpG DNA sequence had dimer structure, where the 3-3' ends were connected by glycerin or 2'-deoxy-7-deazaguanosine modification. Mutation of the K-ras gene has a critical role in colon, lung and pancreatic cancers, and may cause a resistance to anti-EGFR therapy[40,41]. This is the reason why panitumumab and cetuximab therapy do not show a positive effect on the control of proliferation and metastasis of K-ras mutated colon cancer. This kind of biologic therapy could be only useful in the case of patients carrying the wild type K-ras gene[40].

It was shown in an *in vivo* murine xenograft model and *in vitro* human cancer cell lines (GEO, SW48 and LS174T) that IMOs can restore the therapy sensitivity for K-ras mutant colon and pancreatic cancers[40]. These cell lines, except GEO, were resistant for EGFR inhibition therapy, if they had a K-ras mutation. A small number of GEO cells carrying K-ras mutations showed sensitivity to anti-EGFR antibodies. This demonstrates that cells could carry a different K-ras mutation and could respond to EGFR inhibition therapy in a different way based on their K-ras status[39-41].

TLR9 agonists were also tested on a breast cancer cell line which was estrogen receptor positive[42]. After estrogen-TLR9 agonist combination the test showed significant reduction of transactivation *via* the estrogen receptor. Estrogen receptors may also take part in colorectal carcinogenesis[43,44], therefore, this interaction may have further therapeutic importance in colorectal cancer as well.

Currently, TLR9 agonist therapy has been tested clinically on colon, pancreatic and breast cancers[45-48], and experiments are running on oesophageal squamous cell cancer[49], melanomas[50], lymphomas[51,52], non-small cell lung carcinomas[53], renal tumors[47] and androgen resistant prostate cancers[54].

Toll-like receptor agonists in inflammatory bowel disease

It has long been known that in IBD patients, antibodies against own or microbial antigens can be detected. Antibodies against Saccharomyces cerevisiae, outer membrane porin, Pseudomonas fluorescens, pancreas, bacterial flagelline as well as anti-chitobioside-, anti-laminaribioside-, and anti-mannobioside antibodies[55] have all been identified. These antibodies are recognized by PAMP and DAMP receptors. The most important members of these receptor families are the nucleotide oligomerization domain (NOD) - caspase recruitment domain (CARD) system (mainly NOD2 receptor in Crohn's disease) and TLRs. These receptors are localized in the intestinal mucosa, and by increased activation and genetic polymorphisms these receptors create an excessive immune response. At the end of the pathway pro-inflammatory cytokines are released, regulatory T cells are thought to loose their control function and the Th1/Th17 cell subpopulation becomes over-expressed[55,56-58].

Rachmilewitz *et al*[56] used IMOs in a DSS-induced

colitis mouse model and found decreased IL-6, IL-12 and interferon mRNA levels. The levels of matrix metalloproteinases were found to be proportionally decreased. The immunological, clinical, biochemical and histological results showed decreased activity index of the inflammation. From these data one could suggest that the continuous presence of bacteria and bacterial DNA, which densely contain non-methylated CpG sequences, may act as a physiological factor. Furthermore, they could influence the release of inflammatory cytokines in IBD and thus may serve as a therapeutic tool[57].

A newly developed therapeutic agent is a synthetic DNA-based immunomodulatory sequence (DIMS0150), which acts through TLR9 signaling[57]. Based on the results of clinical trials, DIMS0150 seems to restore the steroid sensitivity of the mucosa in steroid-resistant ulcerative colitis patients. In the third phase of clinical trials, 71% of patients achieved remission after 12 weeks of administration of this drug[57]. Although it has no notable side effects, the mode of its administration, namely it has to be spread over the inflamed mucosa with the help of a spray catheter during colonoscopy, makes its use widely intolerable for patients. New ways of drug administration (*i.e.*, colon solvent capsules) must be developed in the near future.

Based on the results of clinical trials[45-54], TLR9 agonists are therapeutically safe *in vivo*. Only some minor side effects, mainly a dose-dependent local inflammation of the connective tissue were observed.

THERAPEUTIC POTENTIAL OF TOLL-LIKE RECEPTOR 9 ANTAGONISTS

Due to complex signaling of oligodeoxynucleotide binding TLRs (including TLR9) a dynamic regulation of pro- and anti-inflammatory cytokines is present[59]. Therefore, TLR9 antagonists and inhibitory oligodeoxynucleotides (inh-ODNs) also may represent new therapeutic options[60] in the treatment of autoimmune diseases. The mechanism of their action is by controlling and blocking the dangerous immune response activated by the self-antigen recognizing receptors. Interestingly, inh-ODNs have TLR9 (and TLR7) antagonist activity, but this effect is sequence dependent. These inhibitory oligonucleotides competitively inhibit TLR9 activation in a manner that competitively antagonizes the binding of ligands to the active, proteolytically cleaved TLR9 sequence. Their therapeutic use shows promise in systemic autoimmune diseases, DNA-mediated sepsis, and chronic inflammatory conditions (*e.g.*, IBD) in which TLR9 plays an important role[60].

SPECIES-SPECIFIC DIFFERENCE IN TOLL-LIKE RECEPTOR 9 EXPRESSION

In the majority of *in vivo* studies mice were used as animal models for showing that CpG-ODNs are effective both as adjuvants and for therapeutic intervention in infectious and tumour model systems. However, one must be careful in translating the murine data into the human system, because of the differences between CpG-ODN-responsive cells in mice and humans. One major and important difference between mice and humans refers to the expression pattern of TLR9. In humans, only pDC and B cells express TLR9 and respond directly to TLR9 activation. All other effects of TLR9 ligands on human immune cells seem to be indirect and depend on factors produced by pDCs and B cells[61].

The situation in mice is different because not only pDCs and B cells, but other dendritic cell subsets and macrophages express TLR9 and thus respond directly to TLR9 activation[61]. Given this important species-specific difference in TLR9 expression, mice are not ideal animal models for establishing TLR9-based therapeutic strategies. The natural ligands for TLR9 can be mimicked by special CpG-ODNs[62].

Besides rodent models, a few studies have analysed the immune response of CpG-ODNs in other animals[62]. Guzylack-Piriou *et al*[63] demonstrated that pig pDCs are the main producers of interferon-α in response to certain CpG-ODNs. Importantly, they additionally showed that myeloid DCs and monocytes/macrophages are refractory to CpG-ODNs. Thus, the CpG-ODN responsiveness in pigs seems to mimic the situation in humans, and therefore recommends the pig as an animal model for preclinical studies with CpG-ODN.

CONCLUSION

Since the immunomodulatory effects of TLRs are known, they are the center of biological, immunological and therapeutic research. Most of the research teams are dealing with the potential therapeutic use of TLR9 agonists and antagonists because their use is not restricted to a specific group of patients. They can be widely applied in almost all diseases where dysregulated immunity plays a central role *via* antibody production or phagocytosis by macrophages.

TLR9 plays a central role in both innate and adaptive immunity. The signalling cascade mediated by CpG ODNs is a complicated pathway and contains many steps, including the synthesis of proinflamatory cytokines and the production of interferons, and thus significant activation of pDCs and T-lymphocytes. The activation of TLR9 acts as a new therapeutic modality in bacterial, viral, inflammatory and neoplastic diseases. In inflammatory circumstances, TLR9 agonists act by both decreasing the enormous immune activation, especially in IBD, and setting the balance of the Th1/Th2 immune response. They may have an effect on the suppression of Th1/Th17 overexpression as well. In tumorous conditions, especially in colorectal cancer, these agents were able to restore anti-EGFR therapy sensitivity caused by a K-ras mutation. They also seem to be effective therapeutic agents in estrogen receptor positive breast

cancers, androgen-resistant prostate tumors, melanomas, lymphomas, large cell lung cancers and renal tumors. The side effects of TLR9 agonists are not significant. Further investigations of these new therapeutic modalities may have promising results in the near future.

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ORIGINAL ARTICLE

Hugl-1 induces apoptosis in esophageal carcinoma cells both *in vitro* and *in vivo*

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Abstract

AIM: To determine whether the human giant larvae homolog 1 gene (Hugl-1/Llg1/Lgl1) exerts tumor suppressor effects in esophageal cancer.

METHODS: We constructed a Hugl-1 expression plasmid, pEZ-M29-Hugl1, for gene transfection. We transfected the pEZ-M29-Hugl1 plasmid into Eca109 esophageal cancer cell lines with Lipofectamine 2000 to overexpress Hugl-1. Real-time reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting were performed to determine the effects of the plasmid on Hugl-1 expression. *In vitro* cell proliferation and apoptosis were examined separately by cell counting Kit-8 (CCK-8) assay, flow cytometry, and Western blotting before and after the transfection of the plasmid into Eca109 cells. Cell cycle distribution was assessed with flow cytometry. The effect of Hugl-1 overexpressing on tumor growth *in vivo* was performed with a xenograft tumor model in nude mice. Expression of Hugl-1 in xenograft tumor was analyzed by immunohistochemistry. The transferase-mediated dUTP nick end-labeling (TUNEL) technique was performed to detect and quantitate apoptotic cell.

RESULTS: The transfection efficiency was confirmed with real-time RT-PCR and Western blotting. Our results show that compared with control groups the mRNA levels and protein levels of Hugl-1 in pEZ-M29-Hugl1-treated group were remarkably increased ($P < 0.05$). The CCK-8 assay demonstrated that the growth of cells overexpressing Hugl-1 was significantly lower than control cells. Cell cycle distribution showed there was a G_0/G_1 cell cycle arrest in cells overexpressing Hugl-1 (64.09% ± 3.14% *vs* 50.32% ± 4.60%, 64.09% ± 3.14% *vs* 49.13% ± 2.24%). Annexin V-fluorescein isothiocyanate revealed that apoptosis was significantly increased in cells overexpressing Hugl-1 compared with control group (17.33% ± 4.76% *vs* 6.90% ± 1.61%, 17.33% ± 4.76% *vs* 6.27% ± 0.38%). Moreover, we found that Hugl-1 changes the level of the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax and the activation of both caspase-3 and caspase-9. With a TUNEL assay, we found that Hugl-1 markedly increased the apoptosis rate of Eca109 cells *in vivo* (60.50% ± 9.11% *vs* 25.00% ± 12.25%). It was shown that Hugl-1 represents a significantly more effective tumor suppressor gene alone in a xenograft tumor mouse model. This data suggest that Hugl-1 inhibited tumor growth and induced cell apoptosis *in vivo*.

CONCLUSION: These results suggest that Hugl-1 induces growth suppression and apoptosis in a human esophageal squamous cell carcinoma cell line both *in vitro* and *in vivo*.

Key words: Esophageal squamous cell carcinoma; Human giant larvae homolog 1; Proliferation; Apoptosis

Core tip: In this paper, we constructed a plasmid to express Hugl-1 which has significant homology to the Drosophila tumor suppressor gene lethal giant larvae. The human esophageal squamous cell carcinoma cell line Eca109 was used as the object of study. We found a positive correlation between Hugl-1 expression and cell apoptosis in Eca109 cells both *in vitro* and *in vivo*. These data suggest that Hugl-1 is a tumor suppressor gene in esophageal cancer and may provide a novel target for the treatment of esophageal cancer patients.

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INTRODUCTION

Esophageal cancer has two major histological types: squamous cell carcinoma (ESCC) and adenocarcinoma (EAC)[1,2]. ESCC is one of the most frequently diagnosed cancers in China[3]. It has been well established that surgical treatment can prolong the survival time of cancer patients, yet the 5-year survival rate for ESCC after surgery is still low (ranging 14%-22%)[4]. Most esophageal cancers are diagnosed in the advanced stages[5]. Thus, detecting gene alternations that promote the carcinogenesis process leading to esophageal cancer will have a profound impact on the diagnosis and treatment of the disease.

Lethal giant larvae (lgl), an evolutionarily conserved and widely expressed cytoskeletal protein, is indispensable for the establishment and maintenance of cell polarity and is a regulator of cell proliferation[6,7]. In Drosophila, mutations in three neoplastic tumor suppressor genes, discs large (dlg), scribble (scrib) and lgl, have revealed a link between the regulation of cell polarity and cell proliferation[8-13]. The human homologs of lgl are Hugl-1 (Llgl1) and Llgl2. The Hugl-1 protein shares 62.5% similarity with Lgl[14]. Several studies have shown that Hugl-1 transcripts are reduced or absent in a high proportion of breast cancers, lung cancers, prostate cancers, ovarian cancers, colorectal cancers, melanomas, endometrial cancers and hepatocellular carcinomas[15-19]. These studies have also shown that Hugl-1 may function as a tumor suppressor gene in various cancer types. In ESCC tissue samples, Hugl-1 is notably lower than in normal tissues[20]; however, the effect of Hugl-1 on tumor progression and prognosis in ESCC is not clear.

In the present study, we analyzed Hugl-1 expression in the esophageal carcinoma cell line Eca109 as well as in tissue samples. By using a forced overexpression technique, we explored the biological activity of Hugl-1 and the underlying mechanism *in vitro* and *in vivo*. We demonstrated that Hugl-1 inhibits proliferation in the esophageal carcinoma cell line as well as in ESCC tissue samples and that it promotes apoptosis in esophageal carcinoma cells and xenograft tumors through a mitochondria-related pathway.

MATERIALS AND METHODS

Cells, cell culture

The human ESCC cell line, Eca109, purchased from the China Center for Type Culture Collection (Wuhan Province, China) and cultured in RPMI-1640 medium (Gibco, United States) containing 10% fetal bovine serum (Gibco, United States), in a humidified atmosphere of 5% CO_2 at 37 ℃.

Plasmid construction and purification of cultured Eca109 cells

Hugl-1 expression plasmids were constructed with pEZ-M29 as the vector, Hugl-1 as the expression gene and ampicillin resistance for antibiotic selection (GeneCopoeia, United States). An empty expression plasmid of the same type was used as a control. Eca109 cells were seeded into a 6 cm dish at a density of 5×10^5 cells per well and incubated overnight with 5% CO_2 at 37 ℃. For each transfection, 9 μL of lipofectamine 2000 (Invitrogen, United States) and 3 μg of the Hugl-1 expression plasmid were added to 1 mL of Opti-MEM (Invitrogen, United States) and incubated for 5 min at room temperature. The diluted plasmid and lipofectamine were mixed together and incubated for 30 min before adding them directly to the cells. Eca109 cells overexpressing Hugl-1 were grown in RPMI-1640 medium with 200 μg/mL of G418 for stable clone selection.

Real-time RT-PCR

Total RNA was prepared from the Eca109 cells with TRIzol reagent (Invitrogen, United States) according to the manufacturer's protocol. First-strand cDNA was synthesized using the PrimeScript™ RT reagent kit (Takara, Japan). The isolated RNA (1 μg) was used as template to perform one-step RT-PCR according to the protocol, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. All reactions were conducted in a 25 μL volume.

Real-time RT-PCR was conducted on the resulting cDNA with the SYBR Green method and the AB7500 Real-time RT-PCR system. The sequences of the primer sets used were as follows: forward 5'-AGAAGGCTGGGGCTCATTTG-3' and reverse 5'-AGGGGCCATCCACAGTCTTC-3' for GAPDH (258 bp); forward 5'-GCTGCTTCGATCCCTACAGTGAC-3' and reverse 5'-CGGCACATCCTAAGCTCCAG-3' for Hugl-1 (131 bp). PCR was performed by initial denaturation at 95 ℃ for 30 s followed by 40 cycles of 5 s at 95 ℃, 30 s at 60 ℃ and 1 min at 72 ℃. The threshold cycle (Ct) values of each sample were used in the $2^{-\Delta\Delta CT}$ data analysis method.

Western blotting

Cells were harvested from 6-well culture plates, and

aliquots of cell extracts were separated on an 8%-12% SDS-polyacrylamide gel. The proteins were then transferred to a polyvinylidene difluoride membrane (Millipore, United States) and incubated overnight at 4 ℃ with the following rabbit polyclonal antibodies: anti-Hugl1 (ab39292, Abcam), anti-Bcl2 (SC-492, Santa Cruzc), anti-Bax (5023, Cell Signaling), anti-p21 (2947, Cell Signaling), anti-cyclin D1 (2978, Cell Signaling), anti-survivin (2808, Cell Signaling), anti-caspase9 (9502, Cell Signaling), anti-caspase3 (9662, Cell Signaling), anti-p65 (3037, Cell Signaling), anti-p-p65 (3033, Cell Signaling) or anti-GAPDH (2118, Cell Signaling).

The blots were rinsed three times in TBST and incubated with a 1:10000 diluted goat-anti-rabbit secondary antibody (LICOR, United States) conjugated to horseradish peroxidase for 1 h at room temperature before they were washed extensively with TBST. Finally, the membranes were scanned with a two-color infrared imaging system (Odyssey, LICOR, United States). Membranes were also probed for GAPDH as an additional loading control.

Cell proliferation analysis

Cells were seeded into 96-well plates at a density of 3000 cells per well 48 h after transfection. The effects of let-7a on cell proliferation were examined with CCK-8 (Dojindo, Japan) according to the manufacturer's instruction 0, 24, 48, 72 and 96 h after seeding.

Cell cycle analysis

Cell cycle analysis was performed with flow cytometry (BD FACS Aria Ⅲ, United States). Cultured cells were harvested 48 h after transfection with pEZ-M29-eGFP and pEZ-M29-Hugl1, respectively, washed with ice-cold phosphate buffered solution (PBS), and fixed in 70% ethanol overnight at 4 ℃. After centrifugation at 500 × *g* for 5 min at 4 ℃, the cell pellets were stained with 10 μg/mL propidium iodide (PI) and 10 μg/mL RNase A in phosphate buffered saline (PBS) buffer for 20 min at room temperature in the dark. Cell cycle analysis was performed with three independent experiments.

Flow cytometric analysis of apoptotic cells using Annexin V-fluorescein isothiocyanate kit

The cultured cells were harvested after treatment with pEZ-M29-eGFP and pEZ-M29-Hugl1, respectively, washed with ice-cold PBS and centrifuged for 5 min at 500 × *g* at 4 ℃. The supernatants were discarded, and the cell pellets were resuspended in ice-cold binding buffer. Double staining with Annexin V-fluorescein isothiocyanate (FITC) and PI was performed using the Annexin V-FITC kit (Beyotime, China) according to the manufacturer's recommendations, and the cells were then analyzed by FACS (BD FACS Aria Ⅲ, United States).

Nude mice xenograft experiments

BALB/c nude mice (5-6 week old) were obtained from the Beijing HFK Experimental Animal Center and were quarantined for one week before tumor implantation. Animal welfare and experimental procedures were performed in strict accordance with guidelines. Mice were randomly divided into two groups (six mice per group). A xenograft tumor model was established by subcutaneously injecting either Hugl1-overexpressing cells or PBS-treated cells (2×10^6) suspended in 0.1 mL of PBS into the right flank of mice, and the tumor volume was measured every week until the mice were sacrificed. At the end of the experiment (day 21), tumors were harvested for additional analyses. Differences in tumor growth were tested for statistical significance.

Immunohistochemistry analysis

The xenograft tumors were embedded in paraffin, cut into 4 μm sections, and either stained with hematoxylin and eosin or treated with Hugl-1 antibody for immunohistochemical evaluation. The results were captured by microscopy (Olympus, Japan).

Transferase-mediated dUTP nick end-labeling assay

The transferase-mediated dUTP nick end-labeling (TUNEL) technique was performed to detect and quantitate apoptotic cell death using the in situ Cell Death Detection Kit (Roche, United States) according to the manufacturer's instructions. Chamber slides were fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton X-100. The slides were then incubated with the TUNEL reaction mixture for 1 h at 37 ℃. After the slides were washed with PBS, they were incubated with peroxidase-conjugated antibody for 30 min at 37 ℃ and were developed with the DAB system. A minimum of 3 fields were randomly selected, and the total cells were counted in each field to achieve a minimum number of 100 total cells. Apoptotic rates (the number of apoptotic cells/total cells) were expressed as mean ± SD from different fields.

Statistical analysis

The statistical analysis was performed using SPSS software (version 17.0 for Windows). Data were presented as means ± SD and comparisons were made using Student's *t* test. A probability of 0.05 or less was considered statistically significant.

RESULTS

Overexpression of Hugl-1 in vitro

The eGFP was used as an marker to detect whether the pEZ-M29-Hugl1 plasmid vectors were successfully transduced *in vitro*. The transfection efficiency is shown in Figure 1A, *in vitro* approach to 60%.

To analyze the effect of pEZ-M29-Hugl1 on the expression of cancer genes, we assessed the mRNA levels of Hugl-1 in the treated cells by Real-time RT-PCR. Our results demonstrated that, compared with group 1 (PBS-

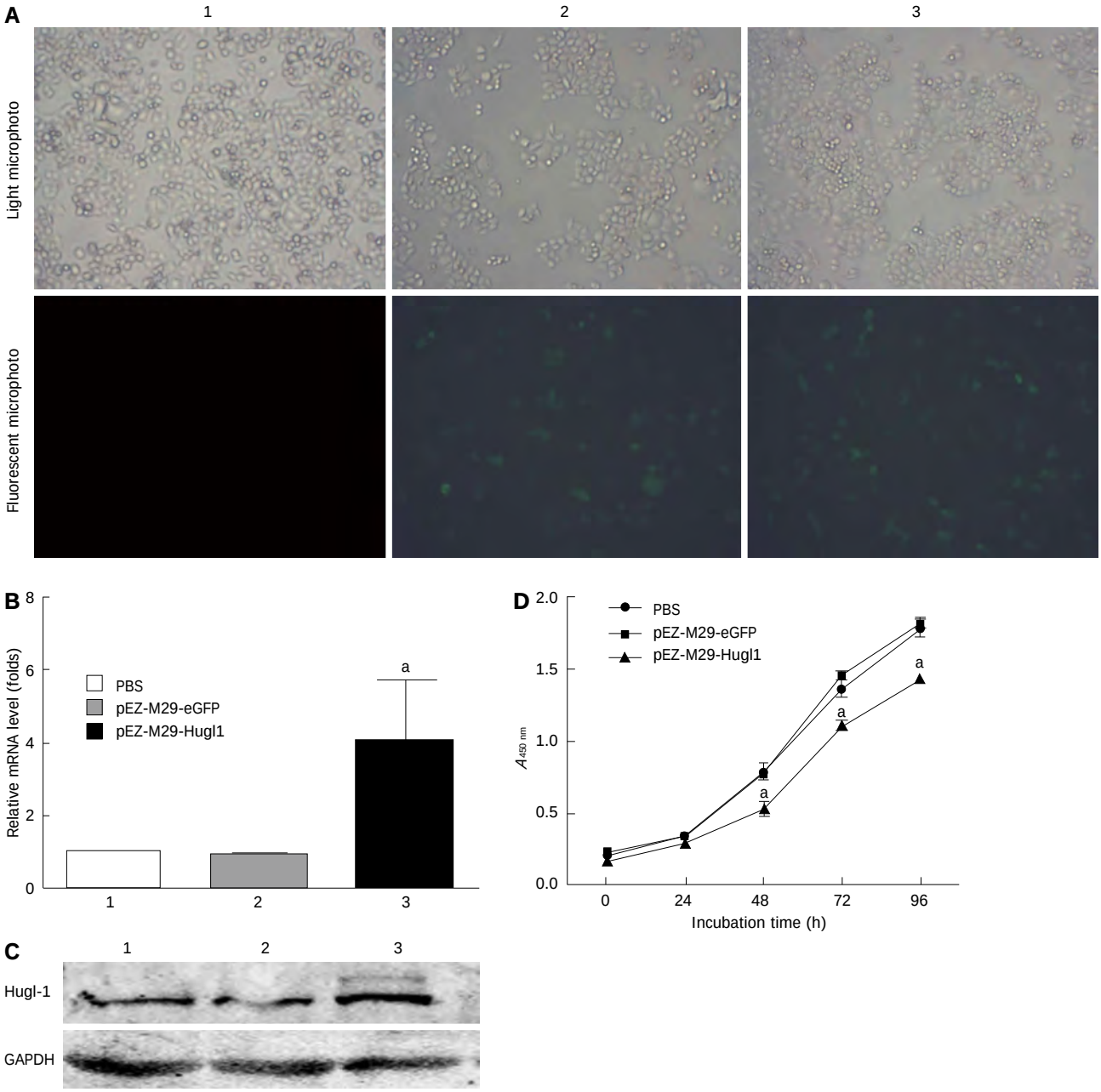

Figure 1 Transfection with pEZ-M29-Hugl1 increased Hugl-1 expression and inhibited the proliferation in Eca109 cells. A: Fluorescent expression in Eca109 cells (× 200); B: Real-time reverse transcription-polymerase chain reaction data of Hugl-1 mRNA levels following transfection with pEZ-M29-Hugl1 plasmids (group 3), pEZ-M29-eGFP (group 2), or treatment with phosphate buffered saline (PBS) (group 1); C: Western blotting data showing Hugl-1 protein expression levels following pEZ-M29-Hugl1 transfection compared with control groups; D: The effect of Hugl-1 on cell proliferation was assessed by cell counting Kit-8. Results represent mean values of three experiments and are indicated as mean ± SD. $^{a}P < 0.05$ *vs* the pEZ-M29-eGFP-treated and PBS-treated groups. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

treated), the mRNA levels of Hugl-1 in group 3 (pEZ-M29-Hugl1-treated) were remarkably increased ($P < 0.05$), but the mRNA levels in group 2 (pEZ-M29-eGFP-treated) were not noticeably different (Figure 1B).

We next assessed the expression of Hugl-1 protein by Western blotting. The expression of Hugl-1 was consistent with results from real-time RT-PCR, and compared with group 1, the protein level of group 3 was increased (Figure 1C).

Effect of Hugl-1expression on the proliferation of Eca109 cells

Cell proliferation assays were performed with the cell counting Kit 8 assay 48 h after transfection. The proliferation of Eca109 cells 48 h after being transfected with pEZ-M29-Hugl1 was slower than that of the other two control groups ($P < 0.05$). Therefore, Hugl-1 inhibited the proliferation of Eca109 cells (Figure 1D).

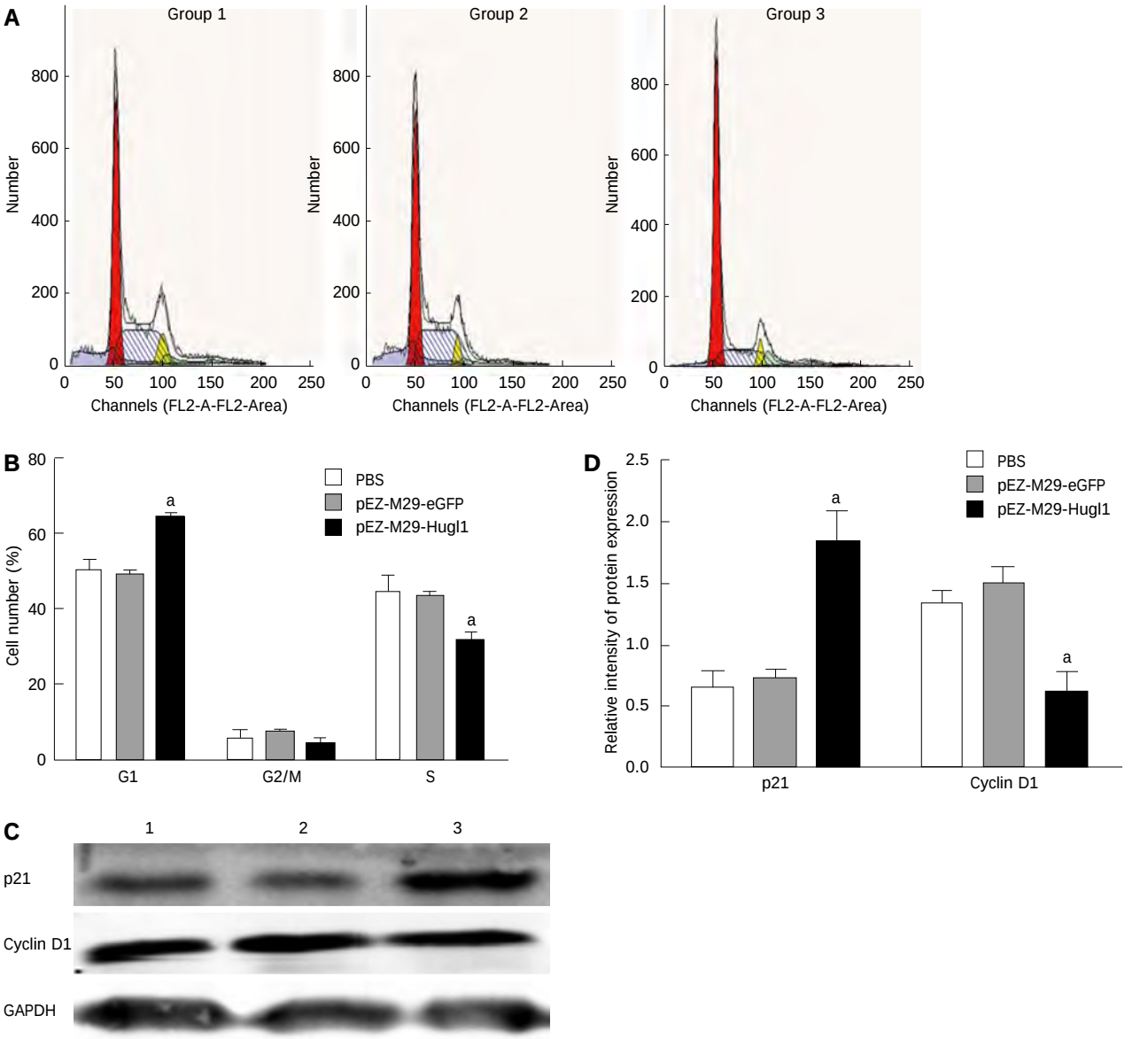

Figure 2 Effect of Hugl-1 on cell cycle distribution of Eca109 cells *in vitro*. A: Cells were treated with pEZ-M29-Hugl1, pEZ-M29-eGFP or phosphate buffered saline (PBS) for 48 h and were then prepared for fluorescence-activated cell sorting analysis; B: Data are presented as mean ± SD of three independent experiments; C: Western blotting data of p21 and cyclin D1 protein expression levels following transfection with pEZ-M29-Hugl1 or controls; D: Analysis of the expression of proteins. $^{a}P < 0.05$ *vs* the pEZ-M29-eGFP-treated and PBS-treated groups. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

Effect of Hugl-1 protein on the cell cycle of Eca109 cells

The mechanism underlying the inhibition of cell proliferation in Eca109 cells was investigated by analyzing the cell cycle with FACS following pEZ-M29-Hugl1 transfection. It was observed that Hugl-1 overexpression arrested the cell cycle in the G_1 phase (Figure 2A). The pEZ-M29-Hugl1 transfected cells were found to contain 64.09% ± 3.14% of cells in the G_1 phase and 31.47% ± 4.90% of cells in the S phase, whereas in the PBS-treated group, 50.32% ± 4.60% of cells were in the G_1 phase and 49.30% ± 4.98% of cells were in the S phase (Figure 2B). There was no difference between the group 3 and the group 2 (49.13% ± 2.24% in the G_1 phase and 43.47% ± 2.09% in the S phase).

Figure 2C shows that cells overexpressing Hugl-1 exhibited down-regulation of cyclin D1 and up-regulation of p21; these results suggest that the G_0/G_1 cell cycle arrest induced by Hugl-1 involved a reduced level of cyclin D1 and an increased level of p21. The protein levels of p21 was upregulated (1.83 ± 0.25 *vs* 0.64 ± 0.14, 1.83 ± 0.25 *vs* 0.72 ± 0.08, $P < 0.05$) and cyclin D1 was down-regulated (0.61 ± 0.18 *vs* 1.33 ± 0.12, 0.61 ± 0.18 *vs* 1.48 ± 0.15, $P < 0.05$) in the pEZ-M29-Hugl1 transfected cells (Figure 2D).

Effect of Hugl-1 protein on Eca109 cell apoptosis

After transfection, cells were incubated with Annexin V-FITC in a buffer containing PI and were then analyzed by flow cytometry (Figure 3A). The results show that group 3 had a higher apoptosis rate (17.33% ± 4.76%)

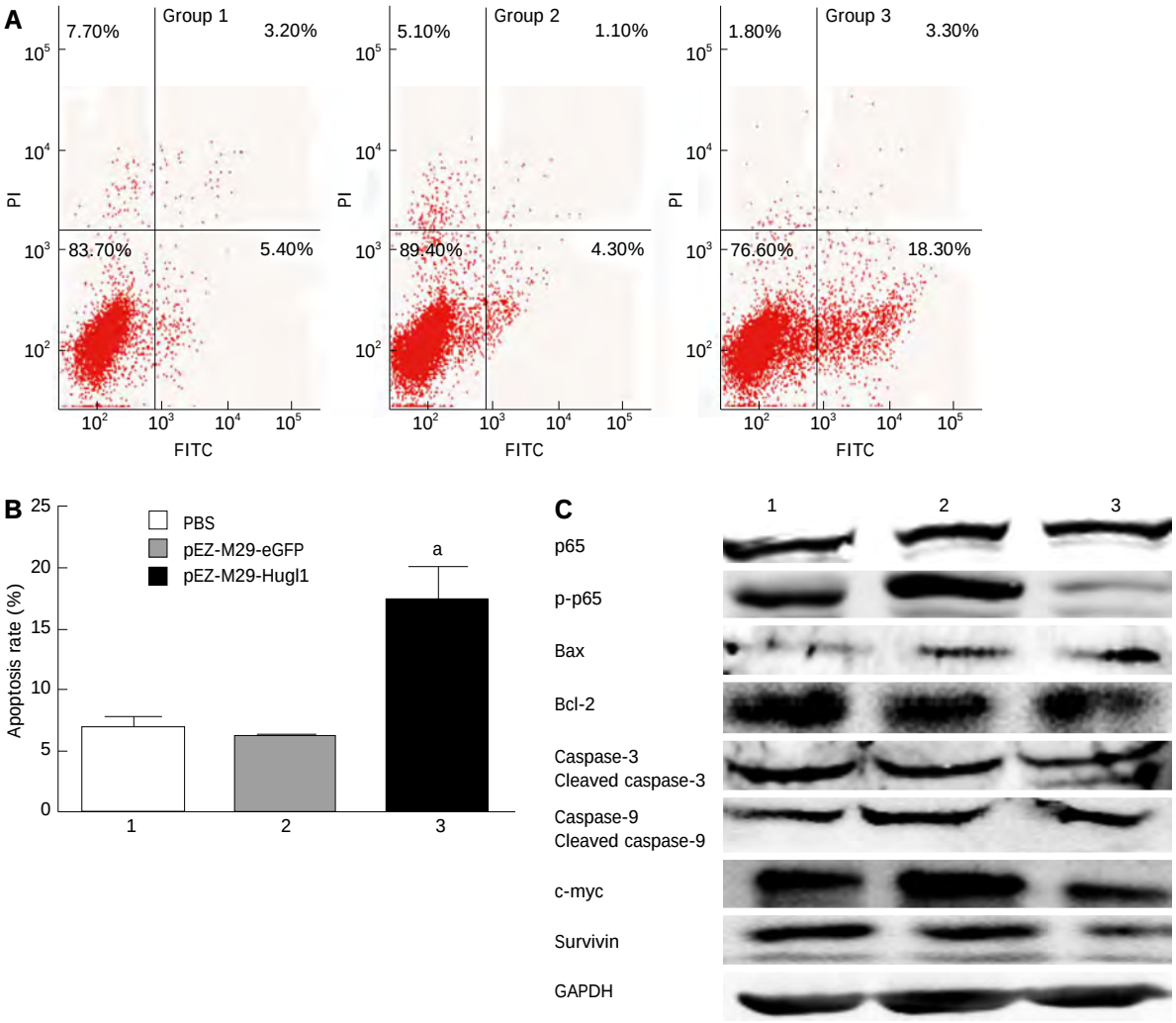

Figure 3 Effect of Hugl-1 on apoptosis of Eca109 cells *in vitro*. A: Cells were treated for 48 h and were then processed for FACS by staining with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI); B: After transfection with pEZ-M29-Hugl1, a significant number of cells were in an early state of apoptosis, and a population of cells had progressed to a later stage of apoptosis; C: Up-regulation of Hugl-1 led to a change of the protein levels of p65, p-p65, Bax, Bcl-2, caspase-3 and -9, survivin and c-myc among the three cell lines. All experiments were performed three times independently. $^{a}P < 0.05$ *vs* the pEZ-M29-eGFP-treated and phosphate buffered solution (PBS)-treated groups. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

compared with group 1 (6.90% ± 1.61%) ($P < 0.05$), and as we expected, no difference was observed between the apoptosis rates of group 1 and group 2 (6.27% ± 0.38%) ($P > 0.05$) (Figure 3B).

To identify the mechanisms that were affected by Hugl-1 expression, Eca109 cells expressing Hugl-1 were analyzed by Western blotting for changes in the levels of various cell-signaling proteins. Figure 3C shows that phospho-p65 was essentially absent in Hugl-1-overexpressing cells, but the total p65 level decreased only slightly. These results suggest that Hugl-1 down-regulated the nuclear factor kappa B (NF-κB) signaling pathway through inhibition of IKKα/β and p65 phosphorylation.

To establish that Hugl-1 induced apoptosis, we examined the activation of the classical caspases and the Bcl-2 family of proteins by Western blotting. Figure 3C shows that Hugl-1 up-regulated the expression of Bax, of cleaved caspase-3, and of cleaved caspase-9, and it down-regulated Bcl-2, survivin, and c-myc expression. These results suggest that Hugl-1 induced apoptosis in Eca109 cells through activation of the mitochondrial apoptotic pathway.

Effect of Hugl-1 expression on xenograft tumor growth

To study the effect of Hugl-1 on tumor growth, nude mice were inoculated with Hugl-1-overexpressing cells, and the resulting tumor growth was compared to that in a control group of mice injected with PBS-treated cells. The difference in tumor growth between the two groups of mice was statistically significant at $P < 0.05$. In the control group, tumors displayed rapid and continued outgrowth during the course of the experiment, and the mean tumor volume was 1126.56 ± 141.70 mm^3. In contrast, the mean tumor size for the experimental group was 606.03 ± 22.49 mm^3 (Figure 4A).

Hematoxylin and eosin staining revealed a significant level of cell death in tumor tissues treated with the pEZ-M29-Hugl1 plasmid compared to the control group (Fig-

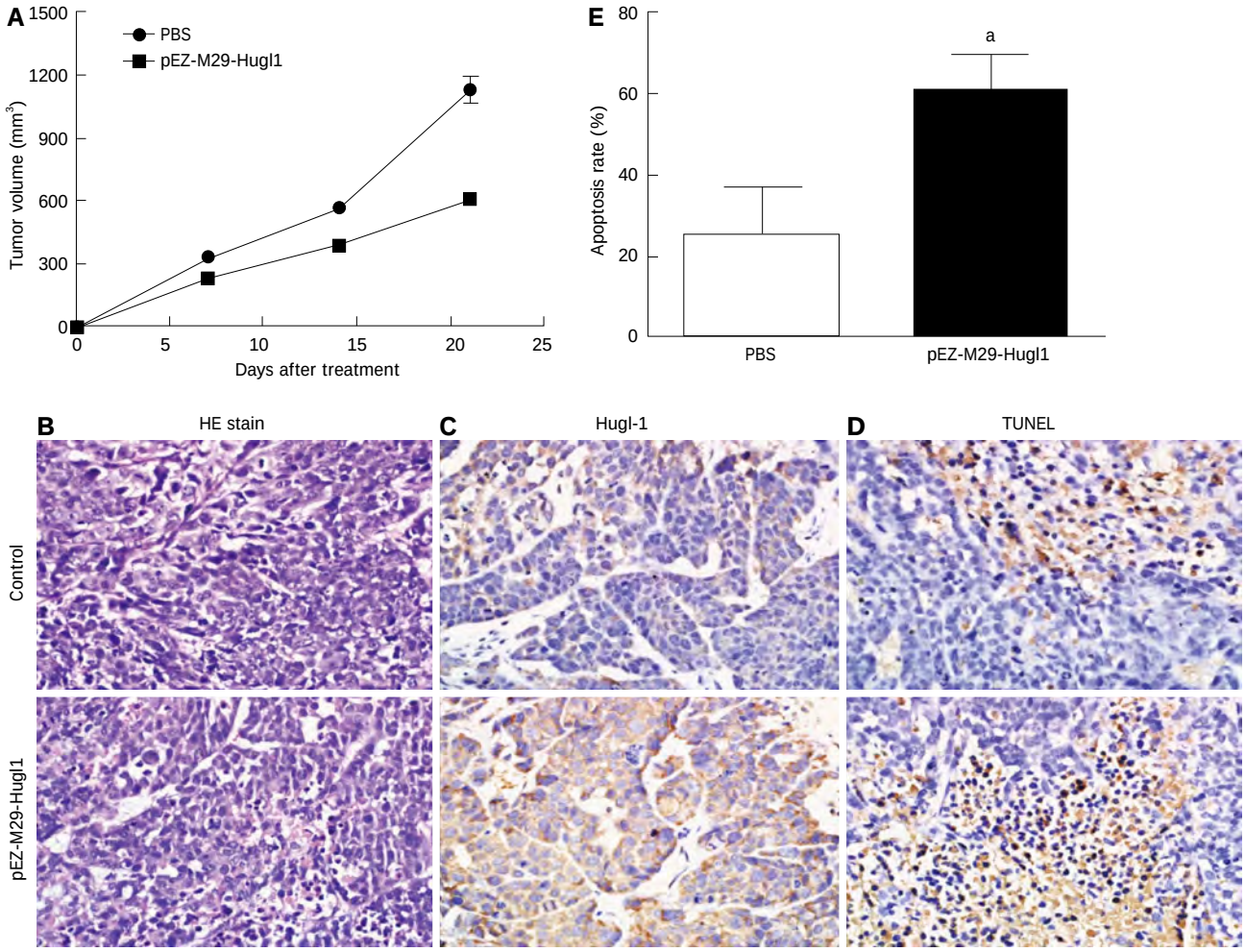

Figure 4 Effect of Hugl-1 on xenograft tumor *in vivo*. A: Cells were injected subcutaneously into nude mice, and with one group of mice receiving pEZ-M29-Hugl1-treated cells and another receiving phosphate buffered saline (PBS). Tumor volume was measured at 7-d intervals for 21 d; B: Tumor sections were observed by hematoxylin and eosin (HE) staining (× 400); C: Expression of Hugl-1 in tumor tissues was analyzed by immunohistochemistry. Many cells were strongly positive for Hugl-1 in the pEZ-M29-Hugl1-treated tumor sections (× 400); D: Representative photomicrographs showing transferase-mediated dUTP nick end-labeling (TUNEL) staining for evidence of apoptosis in transplantation tumors undergoing various treatments (× 400); E: Quantitative analysis of apoptotic cells in tumors treated with PBS or pEZ-M29-Hugl1. Apoptotic cells, shown by TUNEL, were significantly increased in tumors treated with pEZ-M29-Hugl1. Data are presented as mean ± SD (× 400). $^{a}P < 0.05$ *vs* the pEZ-M29-eGFP-treated and PBS-treated groups.

ure 4B). As shown in Figure 4C, many cells were strongly positive for Hugl-1 in the pEZ-M29-Hugl1-treated tumor sections. Apoptotic cells in the tumor sections were analyzed by TUNEL staining (Figure 4D), which showed markedly more positive cells in the pEZ-M29-Hugl1-treated group (60.50% ± 9.11%) than in the PBS-treated group (25.00% ± 12.25%) (Figure 4E).

DISCUSSION

The oncogenesis of esophageal cancer involves accumulated alternations of oncogenes, tumor suppresser genes and other epigenetic regulations[21,22]. Hugl-1 gene is one of the tumor suppresser genes involved in tumor cell proliferation. In this study, we reported that Hugl-1 was a potent anticancer gene for Eca109 cells through inducing G_0/G_1 cell cycle arrest and apoptosis. We concluded that up-regulation of Hugl-1 suppressed esophageal cancer cell proliferation by CCK-8 assay. Flow cytometry showed that overexpression of Hugl-1 reduced the number of cells in S-phase while increasing the number of cells in G_0/G_1-phase, indicating a G_0/G_1 arrest. To understand the mechanism by which Hugl-1 induces esophageal cancer apoptosis, we analyzed the expression of p65, p-p65, Bcl-2, Bax, survivin, c-myc, cyclin D1, p21, and caspase-3 and -9 between cells transfected with the Hugl-1-expressing plasmid and those transfected with the control plasmid. Immunoblotting analysis revealed that Hugl-1 overexpression significantly decreased the expression of p-p65, cyclin D1, Bcl-2, survivin, and c-myc and that it increased the expression of p21 and Bax. In addition, Hugl-1 significantly increased the expression and activity of caspase-3 and caspase-9. More importantly, Hugl-1 potently suppressed the growth of Eca109 cells xenografted in nude mice by inducing cell apoptosis.

Cell cycle arrest and apoptosis are two main ways by which cell growth can be inhibited. In higher eukaryotes, multiple cyclin-dependent kinases associate with multiple cyclins to regulate cell cycle progression[23,24]. Cyclin D1, a member of the G_1 cyclins, controls the cell cycle tran-

sit from G_1 to S phase[25]. The activities of CDKs and CDK/cyclin complexes are known to be regulated by the CIP/KIP family member p21[26]. P21 is, in turn, under transcriptional control of the tumor suppressor p53 and is required for p53-dependent cell cycle arrest[27]. In this study, we found that up-regulation of Hugl-1 in Eca109 cells resulted in a G_0/G_1 cell cycle arrest that was accompanied by down-regulation of cyclin D1 and up-regulation of p21. These data suggest that the mechanism of Hugl-1-induced cell cycle arrest involves down-regulation of cyclin D1 and up-regulation of the CDK inhibitor p21, causing inhibition of CDK activity.

Furthermore, the increase in Hugl-1 expression induced down-regulation of the anti-apoptotic gene Bcl-2 and up-regulation of the pro-apoptotic gene Bax. The central cast of players in the mitochondrial pathway of programmed cell death is the extended Bcl-2 family of proteins[28,29]. Therefore, the balance between the levels of Bcl-2 and Bax is critical in determining the fate of cells in terms of survival or death[30]. Homo-oligomerization of Bax leads to permeabilization of the mitochondrial membrane and subsequent release of cytochrome C to activate apoptosis[31]. Bcl-2 interacts with Bax, preventing its homo-oligomerization and, ultimately, apoptosis[31]. In this case, we found that Hugl-1 induced apoptosis in Eca109 cells, as evidenced by the increase in Annexin V-positive cells, the reduced cell proliferation, and the changes in caspase activation. Currently, there are two known pathways that activate the apoptotic caspase cascade, the intrinsic (mitochondrial) and extrinsic pathways[32]. Our results disclosed that the caspase-9-regulated intrinsic pathway was involved in Hugl-1-induced cell apoptosis. In Hugl-1-treated cells, we observed an increase in the cleaved caspases-9 and caspases-3. These results suggest that Hugl-1 induces apoptosis in Eca109 cells through activation of the mitochondrial pathway.

Considerable evidence indicates that NF-κB is constitutively active in esophageal cancer and that its activation is correlated with tumor progression[33]. The relationship between the NF-κB signaling pathway and tumor cell apoptosis has been extensively studied[34]. It has been presumed that the NF-κB pathway was involved in suppressing apoptosis[35]. The NF-κB family is composed of homodimers and heterodimers of the Rel family of proteins, including p65 (RelA), c-Rel, RelB, p52 and p50[36]. The most abundant form of NF-κB is a heterodimer with two subunits: p50 and p65. Our study showed that phospho-p65 was essentially absent in Hugl-1-overexpressing cells, but the total level of p65 was only slightly reduced. It has been suggested that Hugl-1 down-regulates the NF-κB signaling pathway through inhibition of the IKKα/β and p65 phosphorylation.

In addition, in the nude mice xenografted with Eca109 cells, we found that up-regulation of Hugl-1 reduced tumor growth (606.03 ± 22.49 mm^3 *vs* 1126.56 ± 141.70 mm^3). With a TUNEL assay, we found that Hugl-1 markedly increased the apoptosis rate of Eca109 cells *in vivo* (60.50% ± 9.11% *vs* 25.00% ± 12.25%). Thus, our results indicate that Hugl-1 may be a tumor suppressor of esophageal cancer.

In summary, our study has demonstrated that Hugl-1 exerts tumor suppressor effects by inducing growth suppression and apoptosis both *in vitro* and *in vivo*. G_0/G_1 cell cycle arrest induced by Hugl-1 occurs through a pathway that is mediated by p53-dependent p21 and cyclin D1 and that apoptosis induced by Hugl-1 occurs through the mitochondria pathway. The data presented here also indicate that Hugl-1 interferes with cell proliferation by affecting the NF-κB signaling pathway. The observations that Hugl-1 expression led to the loss of activated IKKα/β and p65 suggest that Hugl-1 is a negative regulator of NF-κB signaling. More importantly, Hugl-1 induced growth suppression and apoptosis in a human esophageal carcinoma cell line *in vivo*. Taken together, we show that Hugl-1 induces growth suppression and apoptosis in a human esophageal squamous cell carcinoma cell line both *in vitro* and *in vivo*. These data suggest that Hugl-1 may provide a novel target for treatment of esophageal cancer patients.

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COMMENTS

Background

Esophageal squamous cell carcinoma (ESCC) is one of the most frequently diagnosed cancers in China. Most esophageal cancers are diagnosed in the advanced stages. Thus, detecting gene alternations that promote the carcinogenesis process leading to esophageal cancer will have a profound impact on the diagnosis and treatment of the disease. The human homologs of lethal giant larvae (lgl) are Hugl-1 (Llgl1) and Llgl2. The Hugl-1 protein shares 62.5% similarity with lgl. Several studies have shown that Hugl-1 transcripts are reduced or absent in a high proportion of breast cancers, lung cancers, prostate cancers, ovarian cancers, colorectal cancers and hepatocellular carcinomas. However, the effect of Hugl-1 on tumor progression and prognosis in ESCC is not clear. The authors aimed to determine whether the Hugl-1 exerts tumor suppressor effects in esophageal cancer.

Research frontiers

Targeted molecular therapy is a new effective treatment for cancer including esophageal cancer. Hugl-1 is a potential tumor suppressor in several cancers, but the role of Hugl-1 remains controversial and its exact role in ESCC remains unknown.

Innovations and breakthroughs

Authors constructed a Hugl-1 expression plasmid, pEZ-M29-Hugl1, for gene transfection. Authors transfected the pEZ-M29-Hugl1 plasmid into Eca109 esophageal cancer cell lines to overexpress Hugl-1. The results showed that overexpression of Hugl-1 could inhibit the growth of Eca109 cells and promote cell apoptosis, and modulate the expression of Bcl-2, Bax, caspase-3, caspase-9, *etc.* Hugl-1 may serve as a potential therapeutic target in ESCC. It suggested that Hugl-1 is a tumor suppressor and interact with the mitochondrial pathway in ESCC.

Applications

The results showed that Hugl-1 is a tumor suppressor and interact with the mitochondrial pathway in ESCC. It may contribute to the future research of ESCC and be a promising target for therapeutic intervention in ESCC.

Terminology

Lethal giant larvae homolog 1 (Human): This gene encodes a protein that is similar to a tumor suppressor in Drosophila.

Peer review

The function of Hugl-1 as a tumor suppressor has been studied in other cancers, but not in ESCC. Thus authors examined the effects of Hugl-1 up-regulation on cell growth and apoptosis in the ESCC cell line Eca109 and determined the conclusion. This conclusion is meaningful in the sense characterized the function of Hugl-1 in ESCC cells.

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ORIGINAL ARTICLE

Effects of rhein on intestinal epithelial tight junction in IgA nephropathy

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Abstract

AIM: To investigate the effects of rhein on intestinal epithelial tight junction proteins in rats with IgA nephropathy (IgAN).

METHODS: Twenty-eight female Sprague-Dawley rats were randomly divided into four groups (7 per group): Control, IgAN, Rhein-treated, and Rhein-prevented. Bovine serum albumin, lipopolysaccharide and CCl_4 were used to establish the rat model of IgA nephropathy. The Rhein-treated group was given rhein from week 7 until the rats were sacrificed. The Rhein-prevented group was given rhein from week 1. Animals were sacrificed at the end of week 10. We observed the changes in the intestinal epithelial tight junctions using transmission electron microscopy, and expression of intestinal epithelial tight junction proteins zona occludens protein (ZO)-1 and occludin by immunofluorescence using laser confocal microscopy. Changes in mRNA and protein expression of ZO-1 and occludin were measured by reverse transcriptase polymerase chain reaction and Western blotting. The ratio of urinary lactulose/mannitol was measured by high performance liquid chromatography (*HPLC*) for assessing the intestinal permeability.

RESULTS: In the control group, the tight junctions lied between epithelial cells on the top of the outer side of the cell membrane, and appeared in dense dotted crystal structures, the neighboring cells were binded tightly with no significant gap, and the tight junction protein ZO-1 and occludin were evenly distributed in the intestinal epithelial cells at the top of the junction. Compared with the control group, in the IgAN group, the structure of the tight junction became obscured and the dotted crystal structures had disappeared; the fluorescence of ZO-1 and occludin was uneven and weaker (5.37 ± 1.27 *vs* 10.03 ± 1.96, $P < 0.01$; 4.23 ± 0.85 *vs* 12.35 ± 4.17, $P < 0.01$); the mRNA expression of ZO-1 and occludin decreased (0.42 ± 0.19 *vs* 0.92 ± 0.24, $P < 0.01$; 0.40 ± 0.15 *vs* 0.97 ± 0.25, $P < 0.01$); protein expression of ZO-1 and occludin was decreased (0.85 ± 0.12 *vs* 1.98 ± 0.43, $P < 0.01$; 0.72 ± 0.15 *vs* 1.38 ± 0.31, $P < 0.01$); and the ratio of urinary lactulose/mannitol increased (3.55 ± 0.68 *vs* 2.72 ± 0.21, $P < 0.01$). In the Rhein-prevented and Rhein-treated groups, compared with the IgAN group, the intestinal epithelial tight junctions were repaired; fluorescence of ZO-1 and occludin was stronger (11.16 ± 3.52 and 8.81 ± 2.30 *vs* 5.37 ± 1.27, $P < 0.01$; 10.97 ± 3.40 and 9.46 ± 2.40 *vs* 4.23 ± 0.85, $P < 0.01$); mRNA of ZO-1 and occludin increased (0.81 ± 0.17 and 0.64 ± 0.16 *vs* 0.42 ± 0.19, $P < 0.01$; 0.82

± 0.22 and 0.76 ± 0.31 *vs* 0.40 ± 0.15, $P < 0.01$); protein expression of ZO-1 and occludin was increased (2.07 ± 0.41 and 1.57 ± 0.23 *vs* 0.85 ± 0.12, $P < 0.01$; 1.34 ± 0.21 and 1.15 ± 0.17 *vs* 0.72 ± 0.15, $P < 0.01$); and the ratio of urinary lactulose/mannitol decreased (2.83 ± 0.43 and 2.87 ± 0.18 *vs* 3.55 ± 0.68, $P < 0.01$).

CONCLUSION: Rhein can enhance the expression of ZO-1 and occludin, repair damaged tight junctions, and protect the intestinal barrier.

Key words: Intestine; Tight junction; Rhein; IgA nephropathy; Rat

Core tip: It has been reported that the incidence and aggravation of IgA nephropathy (IgAN) are often accompanied with intestinal mucosal damage. We speculate that various factors cause the destruction of the intestinal mucosal barrier, food proteins activate the mucosal immune system, and a large amount of secretory IgA is deposited in kidney and causes IgAN. Rhubarb has a protective effect on the intestine. Rhein is isolated from rhubarb and we speculate that it also has a protective effect, although this has not been reported to date. We used various biochemical approaches to confirm this.

Peng SN, Zeng HH, Fu AX, Chen XW, Zhu QX. Effects of rhein on intestinal epithelial tight junction in IgA nephropathy. *World J Gastroenterol* 2013; 19(26): 4137-4145 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4137.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4137

INTRODUCTION

IgA nephropathy (IgAN) is the most common primary glomerular disease worldwide[1]. Although the etiology and pathogenesis of IgAN are still not clear, and it lacks effective treatment, the incidence and aggravation of IgAN are often accompanied with intestinal mucosal damage[2]. We speculate that various virulence factors cause destruction of the intestinal mucosal barrier, the permeability of the intestinal mucosa increases, food proteins activate the intestinal mucosal immune system, and a large amount of secretory IgA is produced and deposited in the kidney, which causes renal damage and IgAN. We suggest that the protection of the intestinal mucosal barrier can decrease the permeability of intestinal mucosa and prevent or reduce the occurrence of IgAN.

It has been reported that rhubarb has a protective effect on the intestinal mucosal barrier[3]. Rhein (1.8-dihydroxy-3-carboxy-anthraquinone, CAS number: 478-43-3) is an anthraquinone monomer isolated from rhubarb, and we speculate that it may also have a protective effect on the intestinal mucosal barrier and delay or prevent the course of IgAN. The function of the intestinal mucosal barrier mainly depends on the integrity of the tight junction proteins in the intestinal epithelial cells. A decrease in tight junction proteins increases intestinal permeability and leads to dysfunction of the intestinal mucosal barrier[4]. As far as we are aware, a protective effect of rhein on the intestinal epithelial tight junction proteins in rats with IgAN has not yet been reported. Therefore, we used various biochemical approaches to determine how rhein regulates the expression of intestinal epithelial tight junction proteins in IgAN.

MATERIALS AND METHODS

Materials

Rhein (> 95% purity) was extracted and identified by Chengdu Mansite Pharmaceutical Co. Ltd. (batch number: MUST-11032801; China). Antibodies against occludin were purchased from Abcam (Cambridge, United Kingdom). Antibodies against zonula occludens protein (ZO)-1 were purchased from Invitrogen (Carlsbad, CA). Bovine serum albumin (BSA) was purchased from Roche (Mannheim, Germany). Lipopolysaccharide (LPS), lactulose and mannitol were purchased from Sigma (St Louis, MO). Carbon tetrachloride and castor oil were purchased from Shanghai Reagents (China). Antibodies against β-actin, horseradish-peroxidase-conjugated secondary antibodies and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were purchased from Beijing Zhongshan (China). Trizol and reverse transcriptase polymerase chain reaction (RT-PCR) kit were purchased from Transgen (Beijing, China).

Animal model

Twenty-eight female Sprague-Dawley rats weighing 180-220 g were obtained from the Animal Center of Nanchang University. They were housed in the animal facilities of the Nanchang University, with free access to food and water. Animals were treated humanely by use of protocols that were approved by the Institutional Animal Use and Care Committee of Nanchang University. Rats were divided randomly into the control group, IgAN group, Rhein-prevented group, and Rhein-treated group ($n = 7$ each). The IgAN experimental animal model was established by treatment with BSA, LPS and CCl_4[5], and specific implementation was as follows: BSA (400 mg/kg, oral every other day) for 6 wk plus LPS (0.05 mg, intravenous injection at wk 6 and 8) and CCl_4 (0.1 mL dissolved in 0.5 mL castor oil, subcutaneous injection weekly for 9 wk). The Rhein-treated group was given rhein (100 mg/kg per day)[6] from week 7 until sacrifice. The Rhein-prevented group was given rhein (100 mg/kg per day) from week 1. The control and IgAN groups were given the same volume of normal saline. All the rats were sacrificed at week 10.

Transmission electron microscopy

Seven rats per group were analyzed by transmission elec-

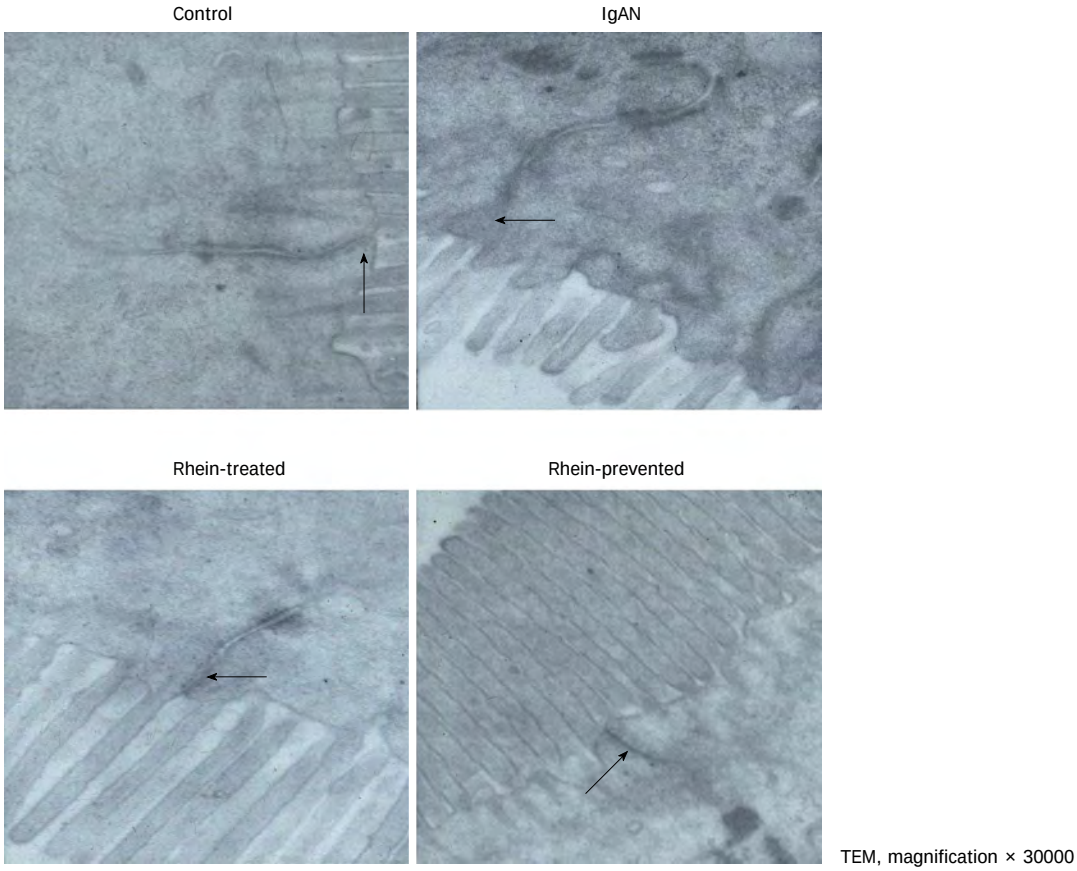

Figure 1 Electron micrograph of intestinal epithelial cells showing tight junction. A: In the control group, the tight junction appeared as an electron-dense belt at the apex of the intestinal epithelial cells (arrow), indicating an intact intestinal mucosal barrie; B: In the IgA nephropathy (IgAN) group group, the intercellular space was widened, the tight junction was indistinct, and the density was reduced (arrow); C and D: In the Rhein-treated and Rhein-prevented groups, the density of the tight junctions was increased compared with that in the IgAN group (arrows). TEM: Transmission electron microscopy.

tron microscopy (TEM) (H-600). Pieces of ileum, 2 mm × 2 mm, were fixed in 2.5% glutaraldehyde overnight at 4° C. The fixed tissues were then post-fixed in 1% osmium tetroxide for 2 h and then rinsed and stored in 0.1 mol/L sodium cacodylate buffer containing 6% sucrose for 12 h. The pieces of ileum were dehydrated through a graded acetone series and embedded in epoxy resin. Semi-thin sections (1.5 μm) were cut and stained with toluidine blue. Ultra-thin sections were stained with 4% uranyl acetate solution in 50% ethanol and lead citrate and then the intestinal epithelial tight junctions were examined by TEM.

Immunofluorescence analysis of occludin and ZO-1

Seven rats per group were analyzed by immunofluorescence. Pieces of ileum, 5 mm × 5 mm, were frozen in liquid nitrogen and 10-μm frozen sections were cut. The frozen sections were fixed with cold acetone for 10 min at 4 ℃. After extensive washing three times (5 min per wash) with cold PBS, the frozen sections were blocked with 10% normal sheep serum in PBS and then incubated with the antibodies against occludin (1:200, Abcam) and ZO-1 (1:100, Invitrogen) at 4 ℃ overnight, followed by staining with FITC-conjugated secondary antibodies. Stained frozen sections were examined with a laser confocal microscope equipped with a digital camera, identifying occludin and ZO-1 by light green color (excitation light wave length of 490 nm). Stained frozen sections were analyzed by a morphological analysis system to determine semi-quantitatively the expression of occludin and ZO-1. Five visual fields were randomly observed under high magnification, with two sections selected from each specimen. The integrated optical density of the positive material in each visual field and its area were measured by morphological analysis system; the ratio of which showed the relative content of occludin and ZO-1.

RT-PCR

Five rats per group were analyzed by RT-PCR. RT-PCR was used for mRNA detection and semi-quantitative assessment. Total RNA was extracted from the small intestine using Trizol reagent (Transgen), measured and verified with a UV spectrophotometer. cDNA was synthesized using an One-Step RT-PCR kit (Transgen) from 1 μg total RNA. Primers were designed by the Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA) according to mRNA sequences (by GenBank) of occludin, ZO-1 and β-actin (as control). The sequences of primers were as follows: forward primer of β-actin gene was 5'-TCAGGTCATCACTATCGGCAAT-3'

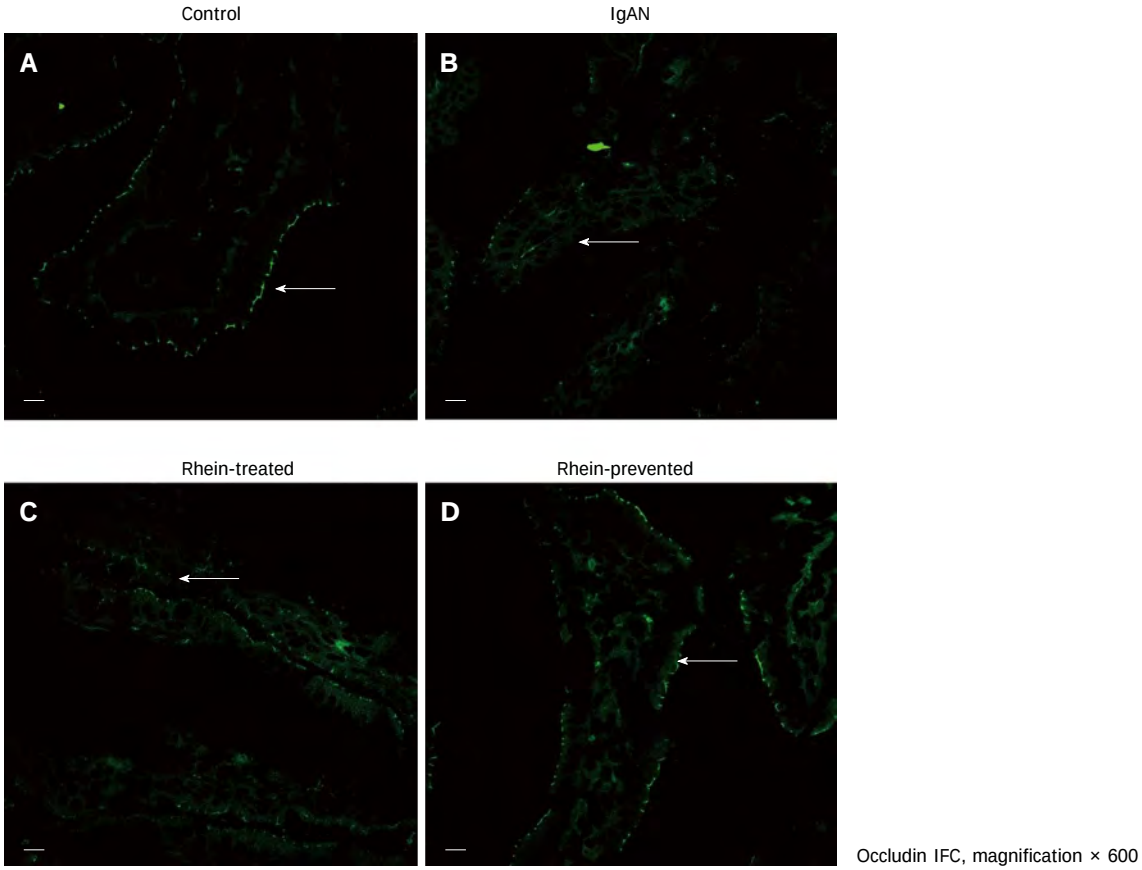

Figure 2 Location of tight junction protein occludin in rat ileum. Laser confocal microscope immunofluorescence staining of ileum from all four groups of rats. A: Cross-section of a normal intestinal villus. Immunoreactive occludin was localized at the apex of intestinal epithelial cells, consistent with the site of the intestinal mucosal barrier (arrow); B: Cross-section of an intestinal villus in the IgA nephropathy (IgAN) group. Occludin immunofluorescence staining became weak and discontinuous (arrow); C and D: Cross-section of an intestinal villus in the Rhein-treated group and cross-section of an intestinal villus in the Rhein-prevented group. Compared with the IgAN group, occludin immunofluorescence staining became stronger and continuous (arrows). Scale bars = 10 μm. IFC: Integrated fluidic circuit.

and its reverse primer was 5'-AAAGAAAGGGTGT AAAACGCA-3'. The forward primer of the occludin gene was 5'-TGCGTGGCTTCCACACTT GCT-3' and its reverse primer was 5'-TTTGCCGCTCTGGGGTCT-GT-3'. The forward primer of the ZO-1 gene was 5'-TGCCCGGCCATTTGAACGCA-3' and its reverse primer was 5'-TCAGG CGGCTGTGTGGAACT-3'. The PCR products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide to confirm that products of the expected size were detected. The electrophoretic bands were analyzed using a gel image analysis system. The results were normalized to the respective β-actin expression. RT-PCR experiments were repeated twice.

Western blotting

Five rats per group were analyzed by Western blotting. For Western blotting, the small intestines were frozen in liquid nitrogen until further use. Protein extraction was carried out using the RIPA lysate (Solarbio, Beijing, China). Protein (20-50 μg per lane) was separated by SDS-PAGE. Occludin was separated on 10% gel and ZO-1 on 8% gel. Proteins were transblotted to polyvinylidene difluoride membranes (Solarbio) in standard Tris-glycine transfer buffer, pH 8.3, containing 0.5% SDS. After transfer, membranes were blocked for 1 h at room temperature in TBST (10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 0.2% Tween-20) containing 5% non-fat milk powder, and incubated overnight at 4 ℃ with either anti-occludin (Abcam) or anti-ZO-1 (Invitrogen) diluted 1:200 in TBST containing 1% non-fat milk powder. Membranes were then washed in TBST for 30 min, incubated with horseradish-peroxidase-conjugated goat anti-rabbit IgG, diluted 1:5000 (Beijing Zhongshan, China) in TBST, washed in TBST for 30 min, and resolved by chemiluminescence (Thermo, Waltham, MA). All membranes were stripped and reprobed with anti-β-actin antibodies (Beijing Zhongshan) as loading controls. Intensities of immunoreactive bands were quantified by densitometry, and normalized to the respective β-actin content. Western blotting experiments were repeated twice.

Measurement of intestinal permeability

Intestinal permeability was determined using two non-metabolized sugars. Three grams lactulose and 1.5 g mannitol were dissolved in 60 mL distilled water. After a fasting period of 12 h, all animals received 2 mL lactulose/mannitol solution by orogastric tube. One hour

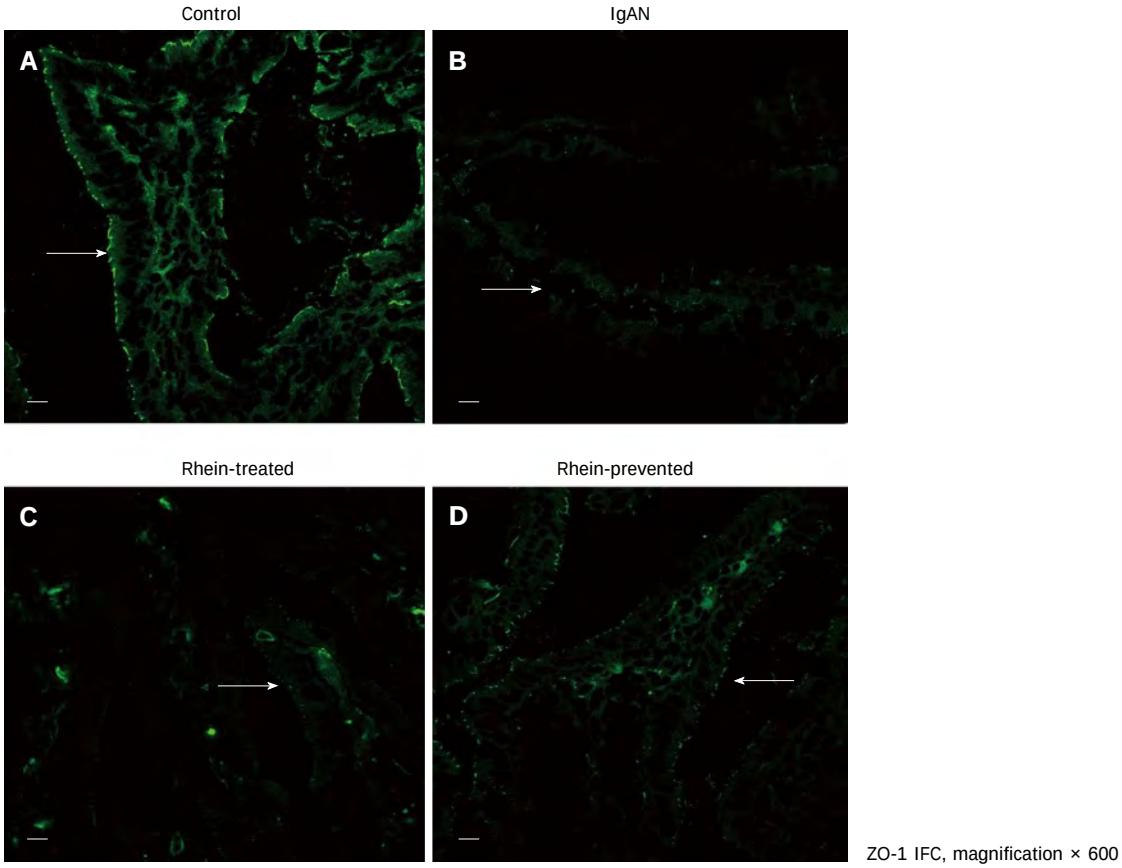

Figure 3 Location of tight junction protein zona occludens protein-1 in rat ileum. Laser confocal microscope immunofluorescence staining of ileum from all four groups of rats. A: Cross-section of a normal intestinal villus. Immunoreactive zona occludens protein (ZO)-1 was localized at the apex of intestinal epithelial cells, consistent with the site of the intestinal mucosal barrier (arrow); B: Cross-section of an intestinal villus in the IgA nephropathy (IgAN) group. ZO-1 immunofluorescence staining became weak and discontinuous (arrow); C and D: Cross-section of an intestinal villus in the Rhein-treated group and cross-section of an intestinal villus in the Rhein-prevented group. Compared with the IgAN group, ZO-1 immunofluorescence staining became stronger and continuous (arrows). Scale bars = 10 μm. IFC: Integrated fluidic circuit.

Table 1 Average optical density value of occludin and zona occludens protein-1

	Control group	IgAN group	Rhein-treated group	Rhein-prevented group
In immunofluorescence ($\times 10^{-2}$)				
Occludin	12.35 ± 4.17	4.23 ± 0.85[b]	9.46 ± 2.40[b,d]	10.97 ± 3.40[d]
ZO-1	10.03 ± 1.96	5.37 ± 1.27[b]	8.81 ± 2.30[d]	11.16 ± 3.52[d,f]
In reverse transcriptase polymerase chain reaction				
Occludin	0.97 ± 0.25	0.40 ± 0.15[b]	0.76 ± 0.31[a,d]	0.82 ± 0.22[d]
ZO-1	0.92 ± 0.24	0.42 ± 0.19[b]	0.64 ± 0.16[b,d]	0.81 ± 0.17[d,e]

The results were normalized to the respective β-actin expression. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group; [d]$P < 0.01$ *vs* IgA nephropathy (IgAN) group; [e]$P < 0.05$ *vs* Rhein-treated group; [f]$P < 0.01$ *vs* Rhein-prevented group.

after feeding, the 6-h urine was collected using metabolic cages before sacrifice. The ratio of urine concentrations of lactulose and mannitol was measured to assess the intestinal permeability.

Statistical analysis

All measurement data were expressed as mean ± SE. Statistical analysis was performed using SPSS 17.0 software. Comparison between groups was made using one-way analysis of variance followed by Student-Newman-Keuls test. $P < 0.05$ was considered to be statistically significant.

RESULTS

TEM results

ZO-1 and occludin are important components in tight junctions, so we performed a morphological analysis of the junctions (Figure 1). Tight junctions are belt-shaped and expand around the apex of epithelial cells. TEM indicated that the cell membrane was intact, and distinct junction complexes were observed in the Control Group. However, in the IgAN Group, the structure of the tight junctions became obscured and the dotted crystal structures disappeared. The microvilli were sparse with irregular length and arrangement. The situations in rhein-treated group and rhein-prevented group were improved compared with IgAN group. The intestinal epithelial tight junctions were repaired with respect to structural integration, with close intercellular connection and high electron density.

Laser confocal microscopy

Indirect immunofluorescence staining for occludin (Figure 2; intestinal epithelium by occludin immunofluorescence staining) and ZO-1 (Figure 3; intestinal epithelium

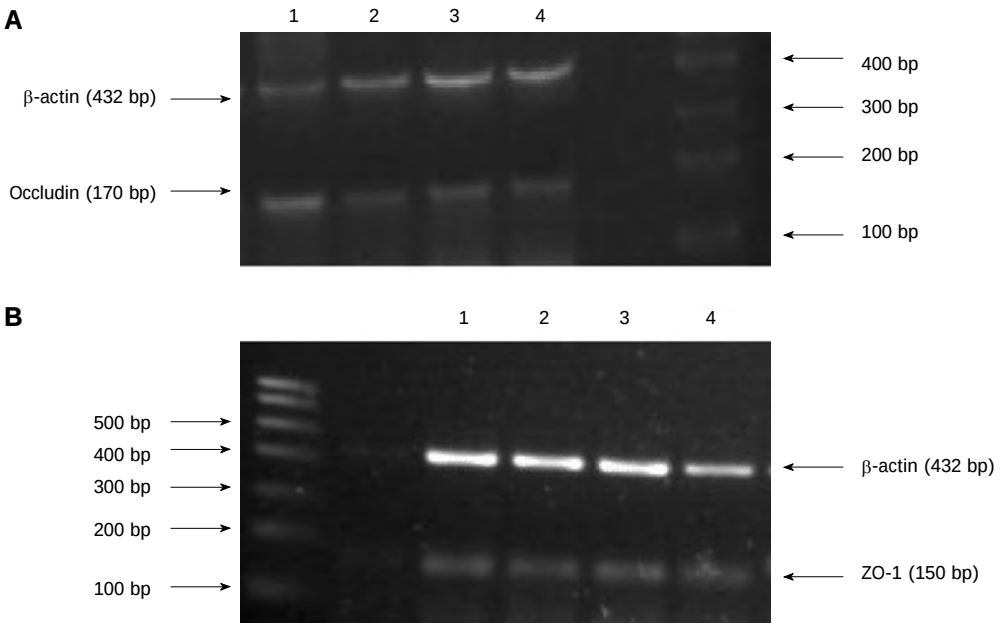

Figure 4 Reverse transcriptase polymerase chain reaction analysis for zona occludens protein-1 and occludin mRNA in rat ileum. By reverse transcriptase polymerase chain reaction, amplification products of expected size [150 bp for zona occludens protein (ZO)-1 and 170 bp for occludin] were obtained in the ileum in all four groups of rats. β-actin was the housekeeping protein. The levels of occludin and ZO-1 expression in the IgA nephropathy (IgAN) group were lower than in the control group. In the Rhein-treated and Rhein-prevented groups, the levels were higher than in the IgAN group. 1: Control; 2: IgAN; 3: Rhein-treated; 4: Rhein-prevented.

by ZO-1 immunofluorescence staining) was performed. In the control group, occludin and ZO-1 staining was found at the apical part of the lateral membranes of the polar epithelial cells and distributed continuously, similar to an intestinal mechanical barrier. In the IgAN Group, the green signals were intermittent and markedly weaker than those in the Control group ($P < 0.01$), and the integrity of the barrier was damaged. The condition of the Rhein-treated and Rhein-prevented groups was ameliorated compared with the IgAN group ($P < 0.01$). The green signals of ZO-1 in the Rhein-prevented group were stronger than in the Rhein-treated group ($P < 0.01$). In contrast, no change in the green signals of occludin was observed between these two groups (Table 1).

Rhein upregulated expression of occludin and ZO-1 mRNA in the small intestine

Occludin, ZO-1 and β-actin RNAs were 170, 150 and 432 bp long, respectively. RT-PCR semi-quantitative analyses showed that the levels of occludin and ZO-1 expression in the IgAN group were significantly lower than in the control group ($P < 0.01$). In the Rhein-treated and Rhein-prevented group, the levels were markedly higher than in the IgAN Group ($P < 0.01$). The level of ZO-1 expression in the Rhein-prevented group was higher than in the Rhein-treated group ($P < 0.05$). In contrast, no change in occludin expression was observed between these groups (Figure 4 and Table 1).

Rhein upregulated expression of occludin and ZO-1 protein in the small intestine

Western blotting analysis showed that occludin and ZO-1 protein expression decreased significantly in the IgAN group compared with the control group ($P < 0.01$). In the Rhein-treated and Rhein-prevented groups, occludin and ZO-1 protein expression was higher than in the IgAN group ($P < 0.01$). ZO-1 protein expression in the Rhein-prevented group was higher than in the Rhein-treated group ($P < 0.01$). However, no change in occludin protein expression was observed between the groups (Figure 5 and Table 2). These findings are consistent with the immunofluorescence results.

Rhein decreased intestinal permeability

The intestinal permeability was assessed by differential uptake of lactulose and mannitol in all four groups. Measurement of mannitol and lactulose by HPLC showed that the ratio of urinary lactulose/mannitol increased in the IgAN group compared with the control group ($P < 0.01$), indicating an increase of intestinal permeability. In the Rhein-treated and Rhein-prevented groups, the ratio of urinary lactulose/mannitol decreased compared with the IgAN group ($P < 0.05$), indicating decreased intestinal permeability. The decrease in intestinal permeability in the Rhein-prevented group was more obvious than that in the Rhein-treated group ($P < 0.05$) (Table 3).

DISCUSSION

IgAN is defined as the predominant deposition of IgA in the glomerular mesangium[7]. The etiology of IgAN has not been completely clarified, but one hypothesis involves the stimulation of antigen by an intestinal route causing an increase in IgA production in the intestinal

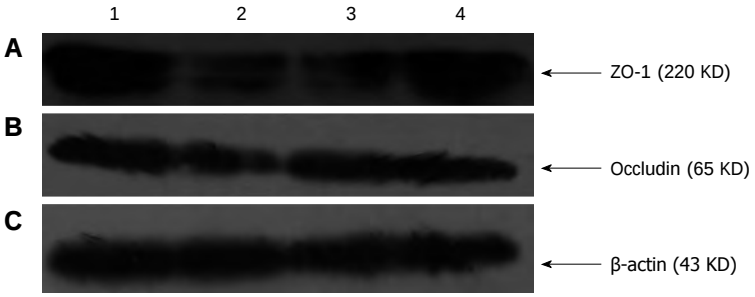

Figure 5 Western blotting analysis for zona occludens protein-1 and occludin of rat ileum. Western blotting analysis revealed zona occludens protein (ZO)-1 immunoreactivity by a band of 220 kDa in the control group and by a weaker band at the same level in the IgA nephropathy (IgAN) group. Compared with the IgAN group, there were stronger bands at the same level in the Rhein-treated and Rhein-prevented groups. Occludin immunoreactivity was revealed by a band of 65 kDa in the control group and by a weaker band at the same level in the IgAN group. Compared with the IgAN group, there were stronger bands at the same level in the Rhein-treated and Rhein-prevented groups. β-Actin was the housekeeping protein. 1: Control; 2: IgAN; 3: Rhein-treated; 4: Rhein-prevented.

Table 2 Grey level of occludin and zona occludens protein-1 in Western blotting

	Control group	IgAN group	Rhein-treated group	Rhein-prevented group
Occludin	1.38 ± 0.31	0.72 ± 0.15[b]	1.15 ± 0.17[d]	1.34 ± 0.21[d]
ZO-1	1.98 ± 0.43	0.85 ± 0.12[b]	1.57 ± 0.23[a,d]	2.07 ± 0.41[d,f]

The results were normalized to the respective β-actin expression. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group; [d]$P < 0.01$ *vs* IgA nephropathy (IgAN) group; [f]$P < 0.01$ *vs* Rhein-treated group.

Table 3 Ratio of urinary lactulose/mannitol

	Concentration of lactulose	Concentration of mannitol	Ratio of them
Control	5.73 ± 0.37	2.10 ± 0.05	2.72 ± 0.21
IgAN group	7.38 ± 1.42[b]	2.08 ± 0.11	3.55 ± 0.68[b]
Rhein-treated group	6.02 ± 0.31[d]	2.10 ± 0.05	2.87 ± 0.18[d]
Rhein-prevented group	6.06 ± 0.97[c]	2.15 ± 0.07	2.83 ± 0.43[d]

[b]$P < 0.01$ *vs* control group; [d]$P < 0.01$ *vs* IgA nephropathy (IgAN) group; [c]$P < 0.05$ *vs* Rhein-treated group.

mucosa, such as ulcerative colitis or Crohn's disease[8,9]. In recent years, an IgAN animal model induced by oral immunization has supported this hypothesis[10].

It has been reported that the permeability of the intestinal mucosa of IgAN patients is significantly higher than normal[11]. This is consistent with the experimental results, but its mechanism is not yet clear. Intestinal mucosal permeability is closely related to the integrity of the intestinal barrier. Mucosal barrier plays an important role in protecting the body from food antigens, microorganisms and their harmful metabolites[12]. The mucosal barrier includes mechanical, immune, chemical and biological barriers, among which, the mechanical barrier is essential for maintaining the integrity of the intestinal barrier. It is mainly composed of the intestinal epithelial cells and cellular junctions among them. The function of the intestinal barrier is affected by the morphology and number of epithelial cells and cellular junctions[13]. The cellular junctions include tight junctions, intermediate junctions, desmosomes, and gap junctions, and tight junctions are closely related to the mechanical barrier[14]. Tight junctions, or ZO, expand around the apex of epithelial cells and form a semipermeable barrier in the paracellular pathway in most vertebrate epithelia[15]. Disruption of the tight junctions can cause increased permeability and leakiness[16,17]. Three groups of macromolecules are considered as integral components of the tight junctions: occludins, claudins and junction adhesion molecules[18]. ZO-1 is the major tight junction protein that binds to the intracellular domain of occludins[19]. The interaction between occludin and ZO-1 plays a crucial role in maintaining the structure of tight junctions and epithelial barrier function[20,21]. Therefore, detection of occludin and ZO-1 reflects the condition of the tight junctions and intestinal mucosa barrier.

Our results showed that expression of intestinal epithelial tight junction proteins occludin and ZO-1 was significantly reduced in the IgAN Group. Therefore, we hypothesized that increased intestinal permeability in that group might be related to the decrease in expression of intestinal epithelial tight junction proteins. This decrease may be due to excess secretion of inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, NO, and oxygen free radicals, which is caused by intestinal antigen stimulation in the modeling process[22,23]. It has been reported that TNF-α affects the interaction of occludin and ZO-1 with components of the actin cytoskeleton[24]. Moreover, a series of recent reports has indicated that TNF-α disrupts tight junction assembly and decreases expression of ZO-1[25,26]. The synergy between TNF-α and IFN-γ can downregulate occludin expression of the occludin promoter[27].

Increased expression of tight junction proteins improves intestinal mucosal barrier function[28,29]. It has been reported that rhubarb can promote intestinal mucosal barrier function recovery and alleviate intestinal bacterial translocation in animal models of burns[30]. Therefore, we speculated that rhein, as the main pharmacological component of rhubarb, would also have a protective effect on the intestinal mucosal barrier and delay or prevent progression of IgAN. Western blotting showed that occludin and ZO-1 protein expression in the Rhein-treated

and Rhein-prevented Groups was higher than that in the IgAN Group, which was consistent with the immunofluorescence and RT-PCR results. The protective effect of rhein on tight junction proteins may be associated with the following aspects. First, rhein lowers the activity of macrophages and inhibits the secretion of TNF-α and other inflammatory cytokines that damage the structure and function of tight junctions[31]. Second, rhein has a positive effect on the peristaltic reflex of the small intestine[32], speeds up the excretion of intestinal bacteria and LPS, and reduces LPS-induced secretion of inflammatory cytokines. Third, reactive oxygen species destroy tight junction proteins by affecting the signal transduction pathway[33]. Rhein can remove reactive oxygen species and alleviate oxidative damage[34]. Lastly, dysfunction of the intestinal microcirculation results in structural damage of tight junctions[35]. Rhein inhibits intestinal microvascular endothelial cell secretion of NO, endothelin-1 and other vasoconstrictor substances, which improves the intestinal microcirculation[36].

In summary, rhein reduces intestinal permeability by protecting intestinal epithelial tight junction proteins ZO-1 and occludin, which alleviates the damage to the intestinal mucosa in IgAN. In this regard, rhein may be a potential therapeutic agent for protecting the intestinal mucosa in IgAN.

COMMENTS

Background

IgA nephropathy (IgAN) is the most common primary glomerular disease worldwide. The etiology and pathogenesis of IgAN are still not clear, and it lacks effective treatment. The incidence and aggravation of IgAN are often accompanied with damage to the intestinal mucosa. Rhein is an anthraquinone monomer isolated from rhubarb. It has been reported that rhubarb has a protective effect on the intestinal mucosal barrier in burns and pancreatitis, but a protective effect on the intestinal mucosal barrier in IgAN has not yet been reported.

Research frontiers

The intestinal mucosal barrier is an important area in research related to the etiology and pathogenesis of IgAN. The research hotspot is how to protect the intestinal mucosal barrier to prevent the occurrence of IgAN.

Innovations and breakthroughs

It has been reported previously that rhubarb has a protective effect on the intestinal mucosal barrier in burns and pancreatitis. Rhein is one of the anthraquinone monomers isolated from rhubarb. The protective effect of rhein on the intestinal mucosal barrier in IgAN has not yet been reported. The authors observed the protective effect of rhein on the intestinal mucosa in a rat model of IgAN; further demonstrated the pathogenesis of intestinal mucosal barrier injury in IgAN; and showed that intestinal protection and repair play an important role in the prevention and treatment of IgAN.

Applications

The results suggest that rhein is a potential therapeutic material that could be used in protecting intestinal mucosa barrier and preventing and treating IgAN.

Terminology

Rhein (1.8-dihydroxy-3-carboxy-anthraquinone, CAS number: 478-43-3) is a substance in the anthraquinone group obtained from rhubarb. Originally the rhubarb plant was used as a laxative, and it was believed that rhein along with other anthraquinone glycosides imparted this activity.

Peer review

This was a good descriptive study in which the authors analyzed the preventive effect of rhein on the intestinal mucosa barrier in rats with IgAN. The results are interesting and suggest that rhein is a potential therapeutic substance that could be used in protecting the intestinal mucosa and preventing IgAN.

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ORIGINAL ARTICLE

Restoring the Treg cell to Th17 cell ratio may alleviate HBV-related acute-on-chronic liver failure

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Abstract

AIM: To investigate the role of T helper 17 cells (Th17) and regulatory T cells (Treg) in hepatitis B virus (HBV)-related acute-on-chronic liver failure (ACLF).

METHODS: We enrolled 79 patients with HBV infection into the study, 50 patients with HBV-related ACLF and 29 patients with chronic hepatitis B (CHB), from the First Affiliated Hospital of Medical College from January 2009 to June 2012. The ACLF patients were diagnosed according to the criteria recommended by The 19th Conference of the Asian Pacific Association for the Study of the Liver in 2009. Twenty healthy individuals with a similar gender and age structures to the two patient groups were also included as the normal controls (NC). Of the 50 ACLF patients, 28 were subsequently classified as non-survivors: 19 patients died from multi-organ failure, 3 underwent liver transplantation, and 6 discontinued therapy during follow-up because of financial reasons. The remaining 22 ACLF patients whose liver and anticoagulation function recovered to nearly normal levels within the next 6 mo were classified as survivors. The number of circulating Treg and Th17 cells was determined upon diagnosis and during the 8th week of follow-up through flow cytometry.

RESULTS: The percentage of circulating Treg cells in the ACLF group was significantly higher than that in the CHB group (5.50% ± 1.15% *vs* 3.30% ± 1.13%, $P < 0.01$). The percentages of circulating Th17 cells in the ACLF and the CHB groups were significantly higher than that in the NC group (6.32% ± 2.22% *vs* 1.56% ± 0.44%, $P < 0.01$; 3.53% ± 1.65% *vs* 1.56% ± 0.44%, $P < 0.01$). No significant difference in Treg cell to Th17 cell ratio was observed between the ACLF group and the CHB group (0.98 ± 0.44 *vs* 1.12 ± 0.64, $P = 0.991$), whereas those in the two HBV infection groups were significantly lower than that in the NC group (1.85 ± 1.22; both $P < 0.01$). The percentage of Treg cells in the survivors during the 8th week of follow-up was significantly lower than that during peak ACLF severity [total bilirubin (TBIL) peak] (3.45% ± 0.97% *vs* 5.18% ± 1.02%, $P < 0.01$). The percentage of Th17 cells in survivors during the 8th week of follow-up was significantly lower than that during the peak TBIL (2.89% ±

0.60% *vs* 5.24% ± 1.46%; $P < 0.01$). The Treg cell to Th17 cell ratio during the 8th week of follow-up was significantly higher than that during the TBIL peak (1.22 ± 0.36 *vs* 1.10 ± 0.54; $P < 0.05$).

CONCLUSION: Restoring the Treg cell to Th17 cell ratio during the follow-up phase of ACLF could maintain the immune system at a steady state, which favours good prognosis.

Key words: Hepatitis B virus; Acute-on-chronic liver failure; Regulatory T cells; T helper 17 cells; Treg cell to Th17 cell ratio

Core tip: In this study, the expression of circulating Treg and Th17 cells in hepatitis B virus-related acute-on-chronic liver failure (ACLF), chronic hepatitis B (CHB), and normal controls (NC) was measured using flow cytometric analysis. The percentages of circulating Treg and Th17 cells in ACLF group increased significantly compared with that in CHB group and NC group. Furthermore, the ratio of Treg to Th17 cells increased significantly upon recovery. Our study suggests that the reverting ratio of Treg to Th17 cells at the follow-up phase of ACLF could maintain the immune system at a steady state in favour of good prognosis.

Niu YH, Yin DL, Liu HL, Yi RT, Yang YC, Xue HA, Chen TY, Zhang SL, Lin SM, Zhao YR. Restoring the Treg cell to Th17 cell ratio may alleviate HBV-related acute-on-chronic liver failure. *World J Gastroenterol* 2013; 19(26): 4146-4154 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4146.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4146

INTRODUCTION

Acute-on-chronic liver failure (ACLF) is defined as an acute hepatic insult that manifests as jaundice and coagulopathy complicated by ascites and/or encephalopathy within four weeks among patients with chronic liver disease. The major aetiologic agents of ACLF are alcohol and drugs in the West and infectious agents in the East[1]. A characteristic feature of ACLF is its rapid progression and high incidence of short- and medium-term mortality, ranging from 50% to 90%[2]. Recent advances in medical therapy have only slightly decreased the mortality rate of HBV-related ACLF. Antiviral treatment improves the survival of patients with HBV-related ACLF[3,4]. Administering granulocyte-colony stimulating factor improves survival of patients with ACLF[5]. Liver transplantation can also improve outcomes, even in critically ill patients with multi-organ failure[6]. However, these advances have not significantly decreased the mortality associated with HBV-related ACLF.

In China, hepatitis B virus (HBV) infections account for 82% of all ACLF[7]. The exact mechanism of HBV-related ACLF is currently unclear. HBV is not directly cytopathic[8] and the hepatocellular injury caused by HBV infection is predominantly immune-mediated[9,10]. Wasmuth *et al*[11] demonstrated that the immunopathology of ACLF is similar to "sepsis-like" immune paralysis. Cytokines also play an important role in ACLF[12,13]. Evidence shows that circulating IL-17$^+$ T cells accumulate in large numbers in the liver of CHB patients, increasing with progression from CHB to ACLF[14,15]. By contrast, Treg cells suppress immune responses and inflammatory diseases[16,17], and they regulate chronic inflammatory responses that contribute to the pathologic events in the liver during HBV infection[18]. Several research groups have demonstrated increased Th17 cells in the peripheral blood and liver tissues, as well as changes in the balance between Th17 and Treg cells in ACLF patients[19,20].

In this study, we focus on the balance between CD4$^+$CD25$^+$FoxP3$^+$ Treg cells and CD4$^+$IL17$^+$ Th17 cells in HBV-related ACLF and examine the effects of this balance on patient responses to therapy and outcomes.

Table 1 Clinical characteristics of subjects in hepatitis B virus-related acute-on-chronic liver failure, chronic hepatitis B, and normal control groups *n* (%)

	ACLF (n = 50)	CHB (n = 29)	NC (n = 20)
Male/female	42/8	24/5	16/4
Age (yr)	40.2 ± 12.0	34.4 ± 12.0	34.5 ± 9.2
ALT (IU/L)	176.6 ± 430.0	328.4 ± 386.8	22.3 ± 6.7
TBIL (μmol/L)	566.3 ± 133.5	68.8 ± 90.9	12.2 ± 2.3
INR	1.83 ± 0.44	1.09 ± 0.10	0.99 ± 0.03
HBeAg	17 (34)	18 (62.1)	NA
Anti-HBe	30 (60)	8 (27.6)	NA
HBVDNA (Log10 IU/mL)	5.25 ± 1.30	6.11 ± 1.28	NA
MELD score	22.5 ± 4.7	4.6 ± 6.5	-2.7 ± 1.9

This table shows the mean ± SD of age, alanine aminotransferase (ALT), total bilirubin (TBIL), international normalized ratio (INR), and hepatitis B virus (HBV) DNA of all patients. Model for End-stage Liver Disease (MELD) scores are shown for the HBV-related acute-on-chronic liver failure (ACLF) group, chronic hepatitis B (CHB) group, and normal control (NC) group. The proportion of patients positive for hepatitis B e antigen (HBeAg) or hepatitis B e antibody (anti-HBe) in the ACLF and CHB groups is also displayed. NA: Not available.

MATERIALS AND METHODS

Subjects

We enrolled 79 patients with HBV infection into the study, 50 patients with HBV-related ACLF and 29 patients with CHB, from the First Affiliated Hospital of Medical College, Xi'an Jiaotong University (Xi'an, China) from January 2009 to June 2012. The ACLF patients were diagnosed according to the criteria recommended by The 19th Conference of the Asian Pacific Association for the Study of the Liver in 2009[1]. Patients were excluded if their liver disease was caused by conditions other than HBV infection. No patient received steroids or other immunosuppressive drugs within 6 mo before sampling.

Table 2 Predisposing factors of hepatitis B virus-related acute-on-chronic liver failure non-survivors and survivors *n* (%)

	Variceal bleeding	Infection			Drug	Alcohol	Indeterminant reasons
		Gastrointestinal tract	Upper respiratory tract	Ascites			
Non-survivors (n = 28)	2 (7.1)	5 (17. 8)	4 (14.3)	2 (7.1)	7 (25.0)	2 (7.1)	8 (28.6)
Survivors (n = 22)	0	7 (31.8)	5 (22.7)	0	5 (22.7)	1 (4.5)	4 (18.2)

Table 3 Clinical characteristics of non-survivors and survivors in hepatitis B virus-related acute-on-chronic liver failure group

	Non-survivors (n = 28)	Survivors (n = 22)	P value
Male/female	24/4	18/4	ND
Age (yr)	40.8 ± 11.0	39.5 ± 13.3	0.570
TBIL (μmol/L)	607.6 ± 117.5	513.7 ± 136.7	0.006
INR	2.00 ± 0.44	1.62 ± 0.33	0.088
MELD scores	23.7 ± 4.6	20.9 ± 4.4	0.021

This table shows the mean ± SD of age, total bilirubin (TBIL), international normalized ratio (INR) and Model for End-stage Liver Disease (MELD) scores for non-survivors and survivors in the hepatitis B virus (HBV)-related acute-on-chronic liver failure (ACLF) group. The P values of each parameter between groups are also displayed. ND: Not done.

The normal controls (NC) consisted of 20 healthy individuals with similar gender and age structures to the two patient groups.

Blood samples were collected from the ACLF patients 1 wk after diagnosis and again on the 2nd, 4th, 6th, 8th, and 12th week. The blood samples of the CHB patients were collected upon diagnosis and before receiving antiviral therapy. The clinical and biochemical details of ACLF patients at the time of their highest total bilirubin (TBIL), as well as those of the CHB patients are listed in Table 1. The Model for End-stage Liver Disease (MELD) score was calculated using the following formula: 3.8 × $\log_e$[bilirubin (mg/dL)] + 11.2 × $\log_e$(INR) + 9.6 × $\log_e$[creatinine (mg/dL)] + 6.4 × (aetiology: 0 if cholestatic or alcoholic, 1 otherwise)[21].

We classified 28 patients as non-survivors: 19 patients died from multi-organ failure, 3 underwent liver transplantation, and 6 discontinued therapy during follow-up because of financial reasons. The remaining 22 patients whose liver function and coagulation recovered to nearly normal within the next 6 mo were classified as survivors.

The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Medical College, Xi'an Jiaotong University, and written informed consent was obtained from each subject prior to enrolment in the study.

Cell stimulation and culture

Heparinized whole blood (200 μL) from study subjects was incubated for 4 h in phorbol 12-myristate 13-acetate (PMA) (final concentration 25 ng/mL) and ionomycin (final concentration 1 μg/mL) with monensin (end concentration 1.4 μg/mL) at 37 ℃ under a 5% CO_2 atmosphere.

Then, the whole blood was separated and incubated with anti-CD4-fluorescein isothiocyanate (FITC) and anti-CD25-phycoerythrin conjugate (PE) or anti-CD4-FITC. After simultaneous fixation and permeabilization, the cells were incubated for 30 min with anti-Foxp3-phycoerythrin-cyanine 5 conjugate (PE-Cy5) or anti-IL17A-PE. Then, the cells were washed again and were resuspended in PBS for flow cytometric analysis.

Flow cytometric analysis

To detect the expression of circulating Th17 and Treg cells, the whole blood was subjected to flow cytometry on a CyFlow® SL machine (PARTEC Company, Germany) using FloMax software.

Virologic and immunologic assessment

The levels of HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HCV, anti-HDV, anti-HEV, and anti-HIV antibodies were detected *via* qualitative enzyme immunoassays. The serum HBV DNA levels were measured using a real-time polymerase chain reaction (PCR) assay with a detection limit of 1 × 10^3 IU/mL. All tests were performed in a clinical laboratory according to standardized methods.

Statistical analysis

Results are expressed as mean ± SD. Statistical comparisons between groups were performed using a Mann-Whitney non-parametric U test. A Spearman's correlation analysis was performed to evaluate the relationship between variables. The data were analyzed using SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL). Differences with P values < 0.05 were considered statistically significant in all analyses.

RESULTS

Clinical characteristics of different short-term outcomes of ACLF patients

The characteristics of survivors and non-survivors in the ACLF group are listed in Table 2. The biochemical parameters and MELD scores at the time of peak TBIL level of the survivors and non-survivors are listed in Table 3.

Peripheral Th17 and Treg cells expression between groups

The percentages of $CD4^+CD25^+FoxP3^+$ (Treg) cells and $CD4^+IL\text{-}17^+$ (Th17) cells and the ratio of Treg to Th17 cells between groups are relative to $CD4^+$ T cells (Figure 1A). The percentages of circulating Treg cells in the two HBV infection groups were significantly higher than that in the NC group [2.46% ± 0.78%, P = 0.000 (*vs* ACLF),

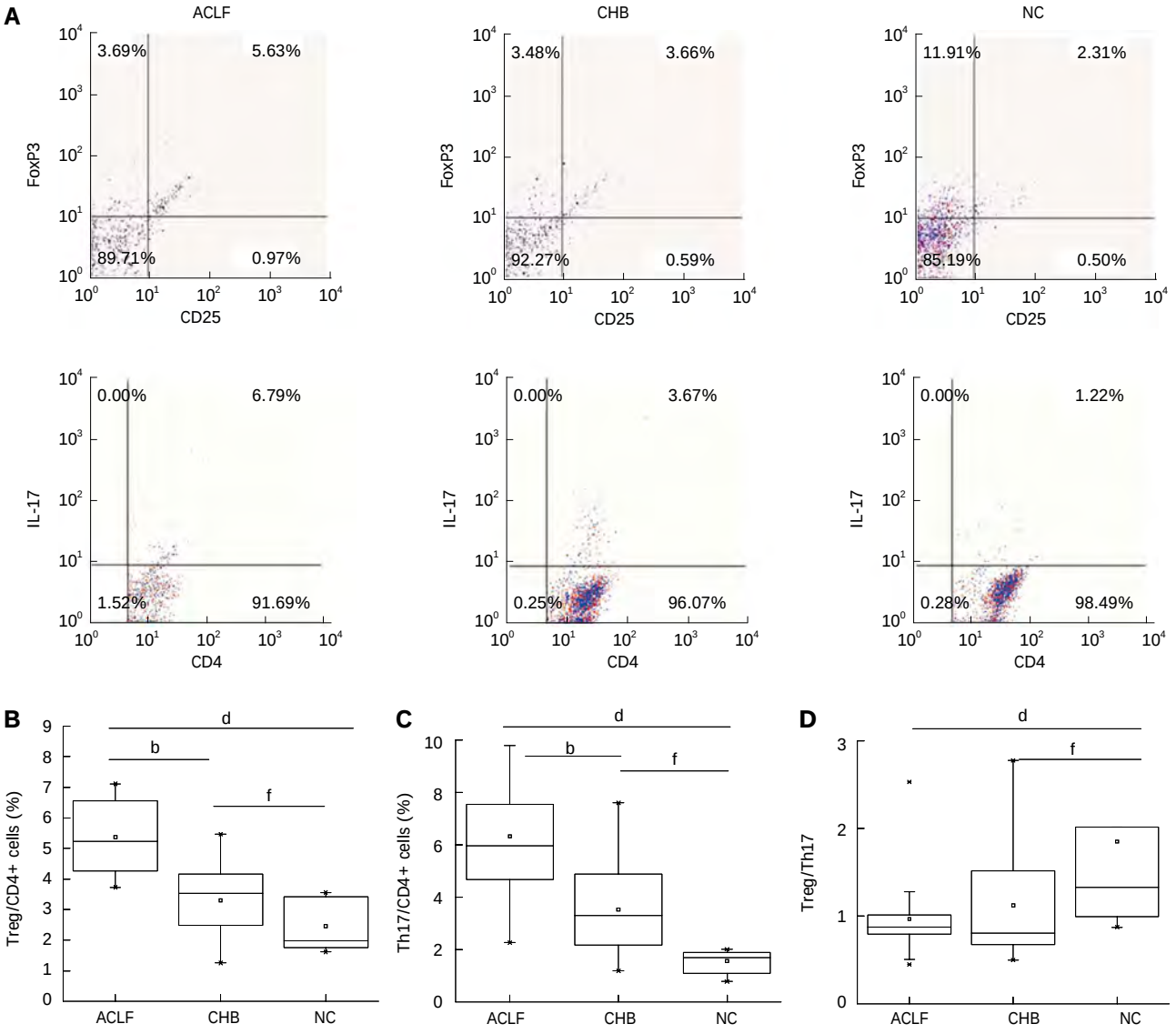

Figure 1 Percentages of T helper 17 cells and regulatory T cells, and the ratio of regulatory T cells to T helper 17 cells in hepatitis B virus-related acute-on-chronic liver failure, chronic hepatitis B, and normal control groups. Typical four-quadrant graphs of $CD25^+FoxP3^+$ cells [regulatory T (Treg)] and $IL17^+$ cells [T helper 17 (Th17)] in $CD4^+$ cells were divided into three groups (A). The proportion of Treg cells (B) and Th17 cells (C) among $CD4^+$ cells, and the Treg cell to Th17 cell ratios (D) were shown as box plot graphs. Acute-on-chronic liver failure (ACLF) *vs* chronic hepatitis B group (CHB) $^bP < 0.01$; ACLF *vs* normal control group (NC) $^dP < 0.01$; CHB *vs* NC $^fP < 0.01$.

$P = 0.008$ (*vs* CHB)]. The percentage of Treg cells in the ACLF group was also significantly higher than that in the CHB group (5.50% ± 1.15% *vs* 3.30% ± 1.13%, $P = 0.001$) (Figure 1B). Strikingly, the percentages of circulating Th17 cells in the ACLF and CHB groups were significantly higher than those in the NC group (6.32% ± 2.22% *vs* 1.56% ± 0.44%, $P = 0.000$; 3.53% ± 1.65% *vs* 1.56% ± 0.44%, $P = 0.000$) (Figure 1C). No significant difference in the Treg cell to Th17 cell ratio was observed between the ACLF group and the CHB group (0.98 ± 0.44 *vs* 1.12 ± 0.64, $P = 0.991$). However, the Treg cell to Th17 cell ratios in the two HBV infection groups were significantly lower than that in the NC group [1.85 ± 1.22; $P = 0.000$ (*vs* ACLF), $P = 0.004$ (*vs* CHB)] (Figure 1D). In summary, the percentages of Th17 and Treg cells in the peripheral blood of ACLF patients increased, whereas the ratio of Treg to Th17 cells was lower in the ACLF and CHB groups compared with that in the NC group.

Clinical correlation of Th17 and Treg expression in HBV-related ACLF

The percentage of Treg cells in the ACLF group was correlated with ALT ($r = 0.381$ $P = 0.006$), TBIL ($r = 0.378$, $P = 0.007$), and INR ($r = 0.381$, $P = 0.006$) (Figure 2A-C). The percentage of Th17 cells was positively correlated with ALT ($r = 0.360$, $P = 0.012$), TBIL($r = 0.323$, $P = 0.025$), and MELD scores (r = 0.293, $P = 0.043$) (Figure 2D-F). The percentage of Treg cells was significantly correlated with the percentage of Th17 cells ($r = 0.425$, $P = 0.003$; Figure 2G). The ratio of Treg to Th17 cells was not significantly correlated with any of the biochemical parameters. Neither the percentages of Treg

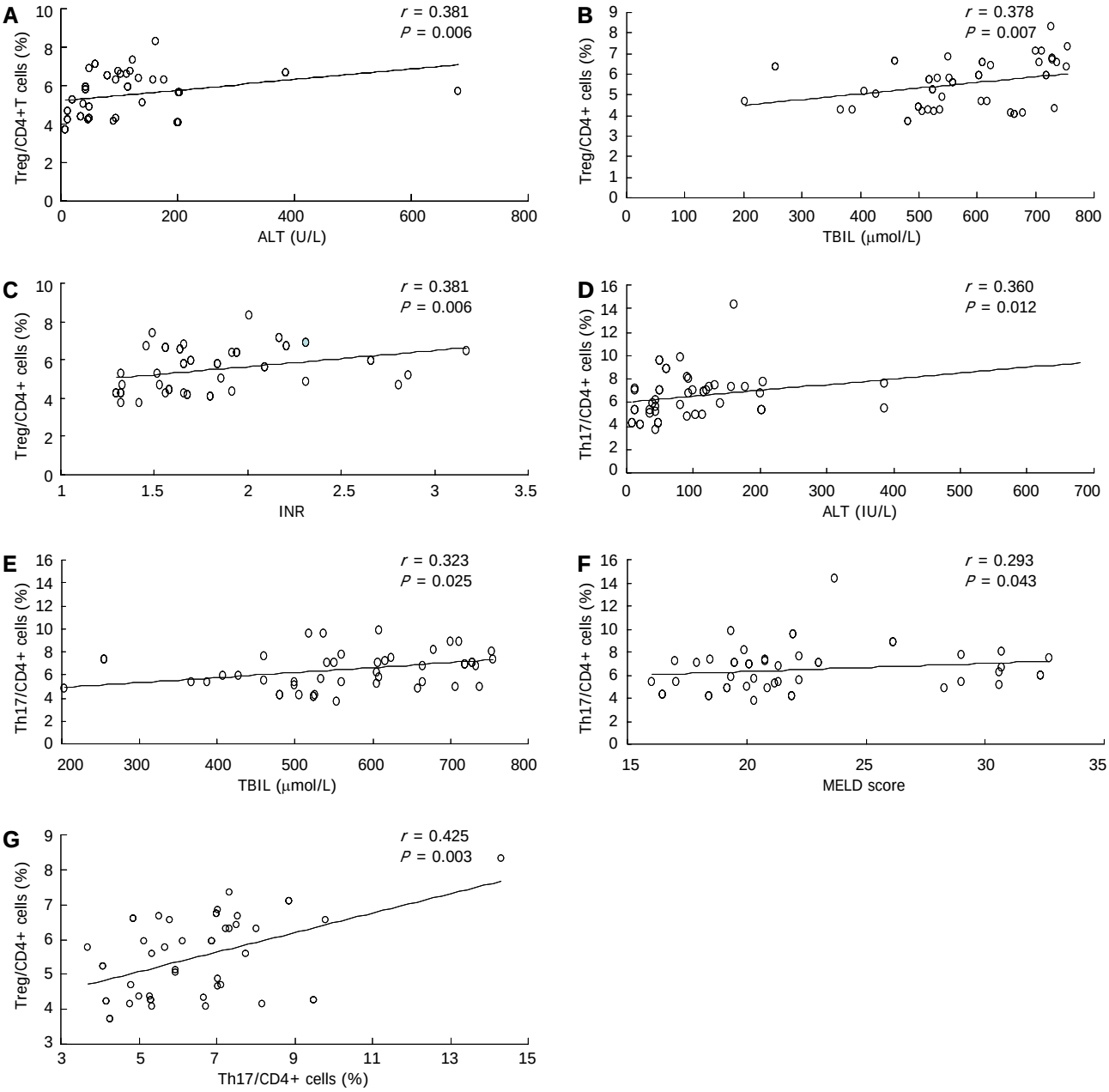

Figure 2 Correlation between T helper 17 cells and regulatory T cells counts and clinical parameters in hepatitis B virus-related acute-on-chronic liver failure group. The percentage of regulatory T (Treg) cells was significantly correlated with alanine aminotransferase (ALT) (A), total bilirubin (TBIL) (B), and international normalized ratio (INR) (C). The percentage of T helper 17 (Th17) cells was significantly correlated with ALT (D), TBIL (E), and Model for End-stage Liver Disease (MELD) scores (F).The percentage of Treg cells was significantly correlated with that of Th17 cells (G).

and Th17 cells, nor the ratio of Treg to Th17 cells, was correlated with serum HBV DNA levels.

In CHB group, the percentage of Treg cells was positively correlated with TBIL ($r = 0.431$, $P = 0.02$), whereas the percentage of Th17 cells was significantly correlated with ALT ($r = 0.367$, $P = 0.05$). Neither the percentages of Treg and Th17 cells, nor the ratio of Treg to Th17 cells, showed a relationship with serum HBV DNA levels.

Percentages of Treg and Th17 cells and the ratio of Treg to Th17 cells during follow-up phase in ACLF patients

The percentages of Treg and Th17 cells were measured serially in the ACLF group during a follow-up phase, which lasted for 8 wk. During TBIL peak, the percentage of Treg cells in non-survivors increased slightly compared with that in survivors (5.76% ± 1.21% *vs* 5.18% ± 1.02%), but the difference was not statistically significant ($P = 0.103$; Figure 3A). The percentage of Th17 cells in non-survivors was significantly higher than that in survivors (7.17% ± 2.37% *vs* 5.24% ± 1.46%; $P = 0.002$) (Figure 3B). No significant difference in Treg cell to Th17 cell ratio was observed between non-survivors and survivors (0.88 ± 0.32 *vs* 1.10 ± 0.54; $P = 0.233$; Figure 3C).

During 8th week follow-up, the percentages of Treg and Th17 cells and the Treg cell to Th17 cell ratio of the survivors were compared with those at the time of TBIL

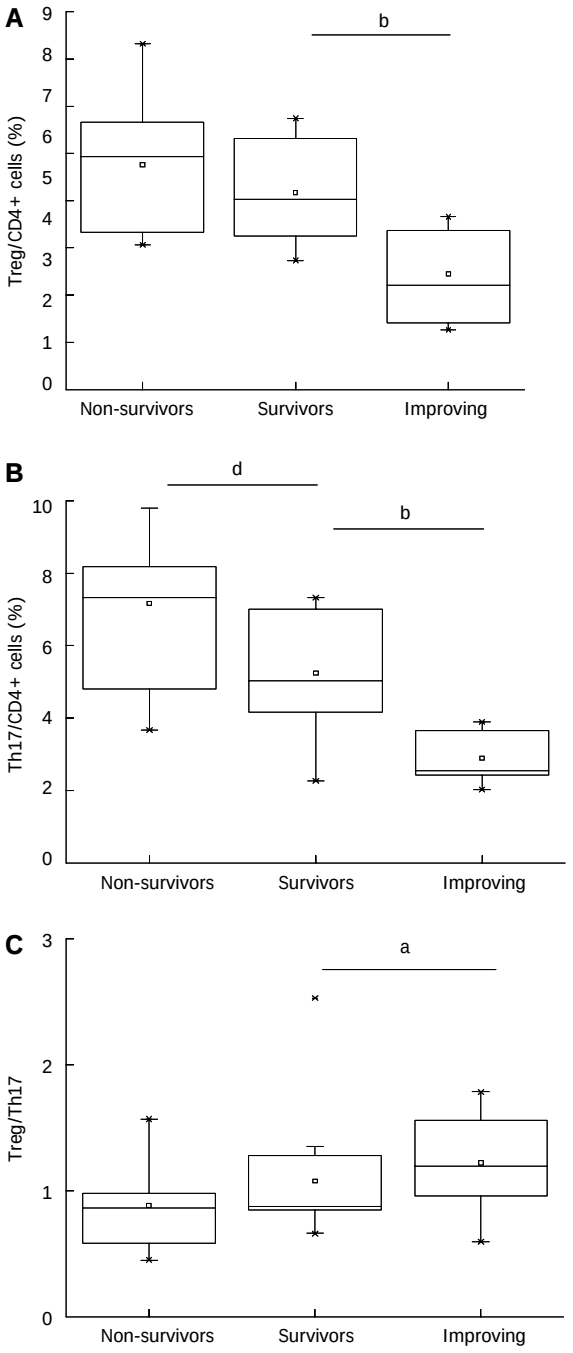

Figure 3 Comparison of regulatory T cells and T helper 17 cell counts, and the regulatory T cell to T helper 17 cell ratio between acute-on-chronic liver failure survivors and non-survivors, as well as those during the peak of acute-on-chronic liver failure severity (total bilirubin peak) and those during the 8th week of follow-up. Regulatory T (Treg) cell counts (A), T helper 17 (Th17) cell counts (B), and the Treg cell to Th17 cell ratios (C) of acute-on-chronic liver failure (ACLF) non-survivors, as well as those of ACLF survivors during the total bilirubin (TBIL) peak and during the 8th week of follow-up. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the 8th week of follow-up in ACLF survivors, $^{d}P < 0.01$ *vs* non-survivors. Non-survivors: The non-survivors in ACLF group; Survivors: The survivors in ACLF group during the TBIL peak; Improving: The survivors in ACLF group during the 8th week of follow-up.

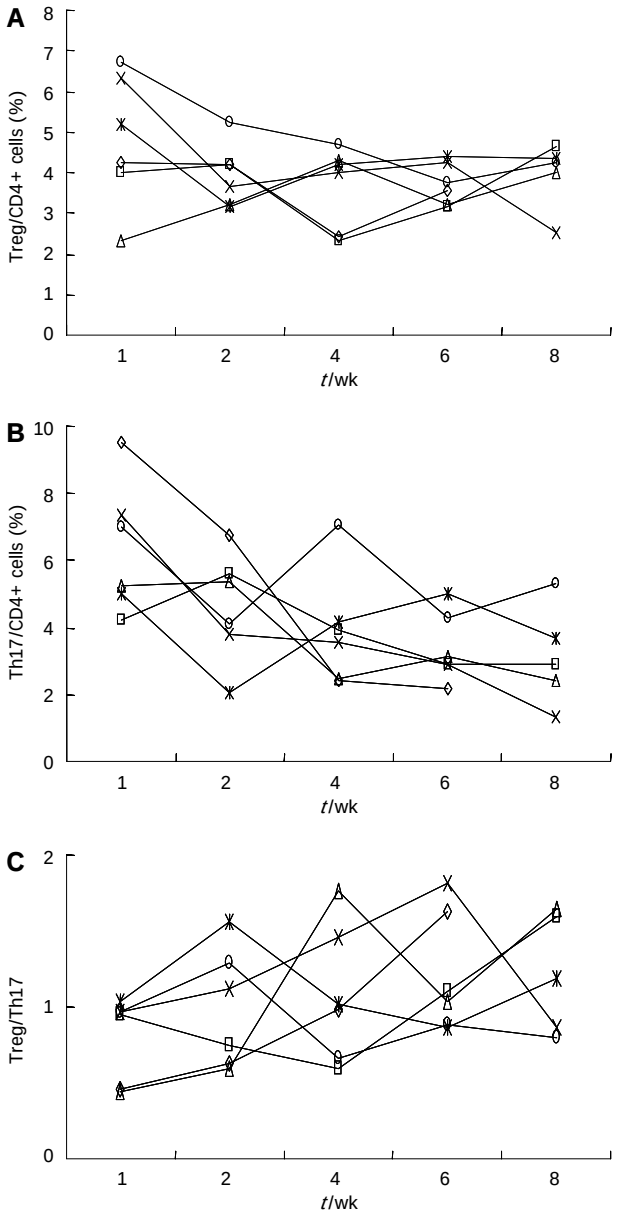

Figure 4 Changes in regulatory T cells and T helper 17 cell counts, and regulatory T cells and T helper 17 cell ratios of acute-on-chronic liver failure survivors. Changes in regulatory T (Treg) cell counts (A), T helper 17 (Th17) cell counts (B), and the Treg cell to Th17 cell ratio (C) of six acute-on-chronic liver failure survivors (5 males and 1 female).

peak. The percentage of Treg cells during the 8th week was significantly lower than that during the TBIL peak (3.45% ± 0.97% *vs* 5.18% ± 1.02%, $P = 0.000$) (Figure 3A), whereas the percentage of Th17 cells during the 8th week was significantly lower than that during the TBIL peak (2.89% ± 0.60% *vs* 5.24% ± 1.46%; $P = 0.000$) (Figure 3B). The Treg cell to Th17 cell ratio was significantly higher during the 8th week than that during the TBIL peak (1.22 ± 0.36 *vs* 1.10 ± 0.54; $P = 0.039$) (Figure 3C).

We examined changes in the percentage of Treg cells and Th17 cells, as well as the Treg cell to Th17 cell ratios, of 6 ACLF survivors (5 males and 1 female) who exhibited decreased TBIL with obvious clinical improvement. The percentage of Treg cells (Figure 4A) decreased together with the percentage of Th17 cells (Figure 4B) during the follow-up period, whereas the Treg cell to Th17

cell ratio increased gradually (Figure 4C).

DISCUSSION

Th17 and Treg cells are subsets of $CD4^+$ T helper cells with developmental pathways that contribute significantly to immune responses[22-27]. Th17 cells are implicated in host defence against a number of microorganisms[28,29]and they participate in autoimmune and chronic inflammatory diseases[30,31]. By contrast, Treg cells display suppressive and surveillance functions in immune responses and inflammatory diseases[16,17]. A study suggested that Th17 cells mediate airway inflammatory responses whereas antigen-specific Treg cells suppress Th17-mediated lung inflammation[32]. Yang *et al*[33] reported that an imbalance between Th17 and Treg cells contributes to the pathogenesis of systemic lupus erythematosus (SLE) and that regulating the balance between Treg and Th17 cells may be a promising strategy for SLE treatment. However, our aim is to determine whether an imbalance in Treg and Th17 cells contributes to the pathogenesis of acute hepatocellular injury in HBV-related ACLF and its mechanism.

Treg and Th17 cells were significantly higher in ACLF patients than in CHB patients and the normal controls. Furthermore, in ACLF patients, the percentage of Treg cells was significantly correlated with the percentage of Th17 cells, as well as with ALT and TBIL. Several recent studies demonstrated that the percentages of circulating Th17 and Treg cells increase with disease progression and are parallel to the severity of liver inflammation as CHB progresses to ACLF[14,18,19]. These results suggest that Th17 cells are a potential marker for the degree of liver injury in ACLF, whereas Treg cells may contribute to the suppression of the immune system[11].

We then analyzed the Treg and Th17 cell counts of ACLF survivors and non-survivors to determine their correlation with their clinical outcomes. The percentage of Th17 cells in ACLF non-survivors was significantly higher than that in survivors, but the percentage of Treg cells and the Treg cell to Th17 cell ratio were not significantly different between the two groups. Our findings are consistent with those of Zhai *et al*[20] who also found increased Th17 cells and Treg cells in ACLF patients. However, they found no significant difference in Th17 and Treg cell counts between ACLF survivors and non-survivors and they observed significantly lower Th17 cell to Treg cell ratios in the survivors than in non-survivors. These differences between the two studies may be attributed to differences in the severity of hepatic injury, as well as differences in the timing of sample acquisition. However, both studies found that patients with ACLF have higher Treg and Th17 cell counts and that higher Th17 cell to Treg cell ratios may predict poorer prognosis.

In China, ACLF occurs mainly in patients with CHB or HBV-related cirrhosis. Spontaneous or treatment-induced inflammatory flare ups are frequently observed in chronic hepatitis B[34]. Several researchers in Asia have demonstrated that early intervention using antiviral therapy improves the short- and long-term outcomes of HBV-related ACLF by aggressively targeting the precipitating events to prevent multi-organ failure[3,4,35]. Entecavir-induced suppression of HBV replication in nine CHB patients showed rapid increases in Th17 cells and decreases in Treg cells, which significantly reduced the Treg cell to Th17 cell ratio[36]. Thus, the effects of antiviral therapies with ACLF will affect Treg and Th17 cells.

We measured the Treg and Th17 cell counts in ACLF survivors during follow-up to determine whether antiviral therapy affects the balance between Treg and Th17 cells. The percentages of Treg and Th17 cells decreased significantly during follow-up, whereas the Treg cell to Th17 cell ratio increased significantly compared with that during the peak of illness, which coincides with the peak serum total bilirubin. Restoring the Treg cell to Th17 cell ratio could help maintain the immune system in a steady state, favouring good outcomes among patients with HBV-related ACLF.

In conclusion, Th17 cell counts may reflect the degree of liver injury, where Treg cells may regulate the protective suppression of the immune system of patients with ACLF. Treg cells and Th17 cells increased in patients with ACLF, but higher Th17 cell to Treg cell ratios may be correlated with poorer prognosis. Restoring the Treg cell to Th17 cell ratio during ACLF could help maintain the immune system in a steady state, which may improve patient prognosis.

COMMENTS

Background

Acute-on-chronic liver failure (ACLF) is an acute hepatic insult with rapid progression and high short- and medium-term mortality. In China, hepatitis B virus (HBV) infections account for 82% of all ACLF cases. Studies have demonstrated that the hepatocellular injury caused by HBV infection is predominantly immune-mediated.However, the mechanism of HBV-related ACLF is currently unclear.

Research frontiers

Several studies showed that Treg and Th17 cells may contribute to the pathologic events in the liver during HBV infection and the balance between Treg cells and Th17 cells may be disrupted during ACLF progression.

Innovations and breakthroughs

The authors found that ACLF patients have significantly increased Treg cell to Th17 cell ratios. The Treg cells and Th17 cells decreased significantly with improvement of the ACLF, but the Treg cell to Th17 cell ratio significantly increased. Thus, Th17 cells may be a marker for the degree of liver injury and poor prognosis in ACLF. Restoring the Treg cell to Th17 cell ratio during the aggravating phase of ACLF could maintain the immune system at a steady state, which favours good prognosis.

Applications

Th17 cells and Treg cells are subsets of $CD4^+$ T helper cells with related developmental pathways. The balance between Th17 cells and Treg cells is important for maintaining human immune homeostasis. The balance between Th17 cells and Treg cells should be maintained before chronic hepatitis B progresses to ACLF. Once CHB has progressed to ACLF, restoring the Treg cell to Th17 cell ratio becomes even more significant to the outcome.

Terminology

ACLF is defined as an acute hepatic insult that manifests as jaundice and coagulopathy in a patient with chronic liver disease, complicated within 4 wk by ascites and/or encephalopathy. The major aetiologic agents of ACLF are alcohol

and drugs in the West and infectious agents in the East. A characteristic feature of ACLF is its rapid progression and high short- and medium-term mortality (50% to 90%).

Peer review

The manuscript focuses on the balance between $CD4^+CD25^+FoxP3^+$ Treg cells and $CD4^+IL17^+$ Th17 cells in HBV-related ACLF. The authors examined the effects of this balance on patient responses to therapy and outcomes. This manuscript is interesting. The references used in the study are updated.

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ORIGINAL ARTICLE

Reversal of multidrug resistance in gastric cancer cells by CDX2 downregulation

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Abstract

AIM: To explore the role of CDX2 in the multi-drug resistance (MDR) process of gastric cancer *in vitro* and *in vivo*.

METHODS: A cisplatin-resistant gastric cancer cell line with stable downregulation of CDX2 was established. mRNA and protein expression levels of CDX2, survivin, cyclin D1, and c-Myc were detected by western blotting and semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR). The influence of downregulation of CDX2 on MDR was assessed by measuring IC50 of SGC7901/DDP cells to cisplatin, doxorubicin, and 5-fluorouracil, rate of doxorubicin efflux, apoptosis, and cell cycle progression detected by flow cytometry. In addition, we determined the *in vivo* effects of CDX2 small interfering RNA (siRNA) on tumor size, and apoptotic cells in tumor tissues were detected by deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling and hematoxylin and eosin staining.

RESULTS: CDX2 siRNA led to downregulation of endogenous CDX2 mRNA (0.31 ± 0.05 *vs* 1.10 ± 0.51, 0.31 ± 0.05 *vs* 1.05 ± 0.21, $P = 0.003$) and protein (0.12 ± 0.08 *vs* 0.51 ± 0.07, 0.12 ± 0.08 *vs* 0.55 ± 0.16, $P = 2.57 \times 10^{-4}$) expression. It significantly promoted the sensitivity of SGC7901/DDP cells to cisplatin (0.12 ± 0.05 *vs* 0.33 ± 0.08, 0.12 ± 0.05 *vs* 0.39 ± 0.15, $P = 0.001$), doxorubicin (0.52 ± 0.13 *vs* 4.11 ± 1.25, 0.52 ± 0.13 *vs* 4.05 ± 1.44, $P = 2.81 \times 10^{-4}$), and 5-fluorouracil (0.82 ± 0.13 *vs* 2.81 ± 0.51, 0.82 ± 0.13 *vs* 3.28 ± 1.03, $P = 1.71 \times 10^{-4}$). Flow cytometry confirmed that the percentage of apoptotic cells increased after CDX2 downregulation (32.15% ± 2.15% *vs* 17.63% ± 3.16%, 32.15% ± 2.15% *vs* 19.3% ± 2.25%, $P = 1.73 \times 10^{-6}$). This notion was further supported by the observation that downregulation of CDX2 blocked entry into the S-phase of the cell cycle (31.53% ± 3.78% *vs* 65.05% ± 7.25%, 31.53% ± 3.78% *vs* 62.27% ± 5.02%, $P = 7.55 \times 10^{-7}$). Furthermore, downregulation of CDX2 significantly increased intracellular accumulation of doxorubicin (0.21 ± 0.06 *vs* 0.41 ± 0.11, 0.21 ± 0.06 *vs* 0.40 ± 0.08, $P = 0.003$). In molecular studies, semiquantitative RT-PCR and western blotting revealed that CDX2 downregulation could inhibit expression of c-Myc, survivin and cyclin D1.

CONCLUSION: CDX2 may be involved in regulating multiple signaling pathways in reversing MDR, suggesting that CDX2 may represent a novel target for gastric cancer therapy.

Key words: Homeobox gene CDX2; RNA interference; Gastric cancer; Drug resistance; Murine model

Core tip: Modulator of multidrug resistance (*MDR*) gene is a direct transcriptional target of CDX2. However, we still speculate whether CDX2 affects MDR through other ways. Our results showed that downregulation of CDX2 significantly promoted sensitivity of SGC7901/DDP cells to anticancer drugs, and increased the percentage of apoptotic cells. Downregulation of CDX2 potentiated G1 phase arrest of the cell cycle. Furthermore, it significantly increased intracellular accumulation of doxorubicin. We conclude that downregulation of CDX2 can efficiently reverse MDR *via* inhibition of apoptosis/cell-cycle-related gene expression (c-Myc, survivin and cyclin D1).

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INTRODUCTION

The transcription factor, CDX2, is a member of the caudal-related homeobox gene family. It is expressed exclusively in the small and large intestine, playing important roles in proliferation and differentiation of intestinal epithelial cells[1]. Several investigators have reported that low levels of CDX2 are a characteristic feature of human colon and squamous esophageal cancer[2,3], but others have shown that strong and robust expression of CDX2 is found in > 80% of colorectal cancer and non-small cell lung cancer[4,5]. In addition, CDX2 enhances proliferation and has tumorigenic potential in human colon cancer cell lines LoVo and SW48[6]. These studies have suggested that CDX2 also has oncogenic activity. Together, these conflicting findings point to a complex role for CDX2 in cell regulation. In adult humans, CDX2 is associated with intestinal metaplasia in the stomach in which ectopic expression of CDX2 is speculated to cause the gastric epithelial cells to trans-differentiate and assume the intestinal phenotype[7]. In addition, CDX2 transgenic mice have been shown to have intestinal metaplasia and a high incidence of gastric carcinoma[8,9].

In a previous study[10], it has been reported that RNA interference (RNAi)-mediated inhibition of CDX2 decreases endogenous MDR1 expression. MDR1 was originally identified as an overexpressed and amplified gene in multidrug-resistant cells. Its product, P-glycoprotein (P-gp), appears to play a critical role in drug resistance, which suggests that CDX2 is associated with multidrug resistance (MDR) of gastric cancer. Previously, we have reported that CDX2 affects the cell cycle and apoptosis of gastric cancer[11], Furthermore, apoptosis is just one of the important mechanisms of reversal MDR[12]. CDX2 may play a crucial role in the control of reversal MDR.

In the present study, we constructed small interfering RNA (siRNA) sequences that targeted CDX2, transfected them into a cisplatin-resistant gastric cancer cell line SGC7901/DDP, selected stable transfectants, and explored changes in IC50, rate of doxorubicin efflux, cell cycle, and apoptosis. We also observed the effect of CDX2 siRNA on the expression of genes associated with apoptosis, including c-Myc and survivin. Moreover, we investigated the effects of CDX2 downregulation on the growth and apoptosis of SGC7901/DDP cells in nude mice.

MATERIALS AND METHODS

Reagents

5-fluorouracil, cisplatin and doxorubicin were purchased from Sigma-Aldrich (St Louis, MO, United States). Cell culture medium RPMI-1640 was purchased from Invitrogen-Gibco (Carlsbad, CA, United States). Fetal bovine serum (FBS) was from Invitrogen-Gibco. Trypsin, streptomycin and penicillin were obtained from Sunshine Biotechnology (Nanjing, China). CDX2, c-Myc, survivin, cyclin D1, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and β-actin antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, United States). All other chemicals were of the highest commercial grade available.

Cell culture

The cells were cultured in RPMI 1640 supplemented with 10% FBS (Sijiqing Biotec, Co. Ltd., Hangzhou, China), antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) in a humidified 5% CO_2 atmosphere at 37.8 ℃. For SGC7901/DDP cells, 0.6 μg/mL cisplatin was supplemented in the medium to maintain the drug-resistance phenotype.

Gene transfection

Recombinant lentiviral vector for *CDX2* gene (siRNA-CDX2) and null vector (siRNA-NC) were stored in our laboratory[13]. SGC7901/DDP cells were seeded in six-well plates with antibiotic-free medium. After 24 h incubation, cells were infected with viral supernatant at a multiplicity of infection of 150 PFU per cell (MOI = 150), and the stable-transfected cell lines were obtained by culturing transfected cells in the presence of 700 mg/mL G418 (Invitrogen, Carlsbad, CA, United States) for 3-4 wk. The cells were divided into three groups: SGC7901/DDP + siRNA-CDX2, SGC7901/DDP + siRNA-NC, and SGC7901/DDP.

Measurement of cell drug sensitivity by MTT analysis

The IC50 was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazoliumbromide] assay. Cells were plated in 96-well plates (5000 cells/well), and after

adherence, the cells were exposed to cisplatin, doxorubicin, and 5-fluorouracil. After incubation for 48 h, the cells were incubated with 20 μL MTT (at a final concentration of 0.5 mg/mL) at 37 ℃ for 4 h. The medium was removed and the precipitated formazan was dissolved in 100 μL DMSO. The absorbance at 490 nm was detected using a microplate reader (Bio-Rad, Hercules, CA, United States). The IC50 was estimated by the relative survival curve. Each assay was performed in triplicate.

Measurement of pump rate of doxorubicin by flow cytometry

The cells were inoculated into six-well plates and 4 mg/mL doxorubicin was added, and all wells were placed at 37 ℃ for 30 min. Flow cytometry was used to measure the fluorescent intensity of doxorubicin in cells with an excitation wavelength of 488 nm and emission wavelength of 575 nm. The cells were then washed twice with fresh culture medium and incubated with the new medium at 37 ℃ for 1 h to detect the retained doxorubicin. Subtraction of the fluorescence retained from the total fluorescence was the fluorescent index of doxorubicin. The procedure was repeated three times and an average value was obtained to calculate the pump rate of doxorubicin. The pump rate of the drug from the cells = (accumulated quantity of doxorubicin-retained quantity of doxorubicin)/accumulated quantity of doxorubicin.

Cell cycle analysis by flow cytometry

SGC7901/DDP cells (1×10^6) were washed twice with ice-cold PBS, treated with trypsin, and fixed in cold 70% ethanol at 4 ℃ for 30 min. The cell pellet was incubated in a solution containing 50 ng/mL propidium iodide, 0.2 mg/mL RNase, and 0.1% Triton X-100 at room temperature for 30 min. The cells were analyzed by flow cytometry using an EPICS XL-MCL FACScan (Becton-Dickinson, Mountain View, CA, United States). The data was analyzed with the MultiCycle Software for Windows (Phoenix Flow Systems, San Diego, CA, United States).

Semiquantitative reverse-transcriptase polymerase chain reaction

Total RNA was extracted from SGC7901/DDP + siRNAi-CDX2 cells, SGC7901/DDP + siRNAi-NC cells, and SGC7901/DDP cells using TRIzol Reagent (Invitrogen). All gene segments were amplified and verified by semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR). cDNAs were reverse-transcribed from 2 μg total RNA. The PCR primer sequences (CDX2 primers were sense: 5'-CGG CAG CCA AGT GAA AAC-3' and antisense: 5'-GAT GGT GAT GTA GCG ACT GTA-3'. Survivin primers were sense: 5'-AAA TGC ACT CCA GCC TCT GT-3' and antisense: 5'-TGT CGA GGA AGC TTT CAGGT-3'. Cyclin D1 primers were sense: 5'-CCC TCG GTG TCC TAC TTC AA-3' and antisense: 5'-GGG GAT GGT CTC CTT CAT CT-3. c-Myc primers were sense: 5'-TTC TCT CCG TCC TCG GAT TC-3' and antisense: 5'-GTA GTT GTG CTG ATG TGT GG-3'. GAPDH primers were sense: 5'-ACC ACA GTC CAT GCC ATC AC-3' and antisense: 5'-TCA CCA CCC TGT TGC TGT A-3'). The products of PCR were checked by agarose gel electrophoresis, and the abundance of each mRNA was detected and normalized to that of GAPDH mRNA.

Western blotting

Cell lysates were prepared in a buffer containing 100 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.6), 1 mmol/L EDTA (pH 8.0), 1 μg/mL aprotinin, 100 μg/mL phenylmethylsulfonyl fluoride, and 1% (v/v) NP-40. After protein quantitation using the Lowery protein assay, equal amounts of proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes by the semi-dry blotting method using a three-buffer system. The membranes were incubated with a dilution of primary antibody (anti-CDX2: 1:500, anti-c-Myc: 1:1000, anti-survivin: 1:1500, anti-cyclin D1:1:3000), overnight at 4 ℃. The membrane was washed with TBST and incubated with a peroxidase-conjugated secondary antibody (1:1000) (Santa Cruz Biotechnology) for 1 h. Specific antibody binding was detected using a chemiluminescence detection system (Pierce, Rockford, IL, United States), according to the manufacturer's recommendations. Western blot film was scanned, and the net intensities of the bands were quantified using Image-QuanT software (Molecular Dynamics, Sunnyvale, CA, United States). After development, the membrane was stripped and reprobed with antibody against GAPDH (1:1000) or β-actin (1:1500) to confirm equal sample loading.

Effect of CDX2 siRNA on reversing MDR of human gastric cancer in vivo

BALB/c 5-wk-old male nude mice (Guangxi Animal Center, Nanning, China) were kept under specific pathogen-free conditions and tended to in accordance with institutional guidelines. All experimental studies were approved by the Guangxi Medical University Animal Care and Use Committee. SGC7901/DDP cells were used for tumor implantation. Approximately 2×10^6 tumor cells were harvested, resuspended in 100 μL PBS, implanted subcutaneously into the flanks of the BALB/c nude mice, and resulting tumor was named as SGC7901/DDP tumor. After 7 d, when the SGC7901/DDP tumor measured 3-5 mm in diameter, these nude mice were randomly divided into the following three groups (6 mice/per group): SGC7901/DDP + siRNA-CDX2, SGC7901/DDP + siRNA-NC, and SGC7901/DDP. The animals were administered an intratumoral injection of LV-siRNA- CDX2 or LV-siRNA-NC at a titer of 5×10^6 TU in 100 μL PBS, and injection of an equal volume of PBS was used as a blank control. After the first injection, the animals were administered a similar injection every 2 d. DDP was administered by intraperitoneal injection at a dose of 25 mg/kg. After the first injection, the animals were administered a similar injection every 2 d. The tumors were monitored every day and measured every 2 d with a caliper, and the diameters were recorded. The tumor volume (TV) was calculated by the formula: $TV =$

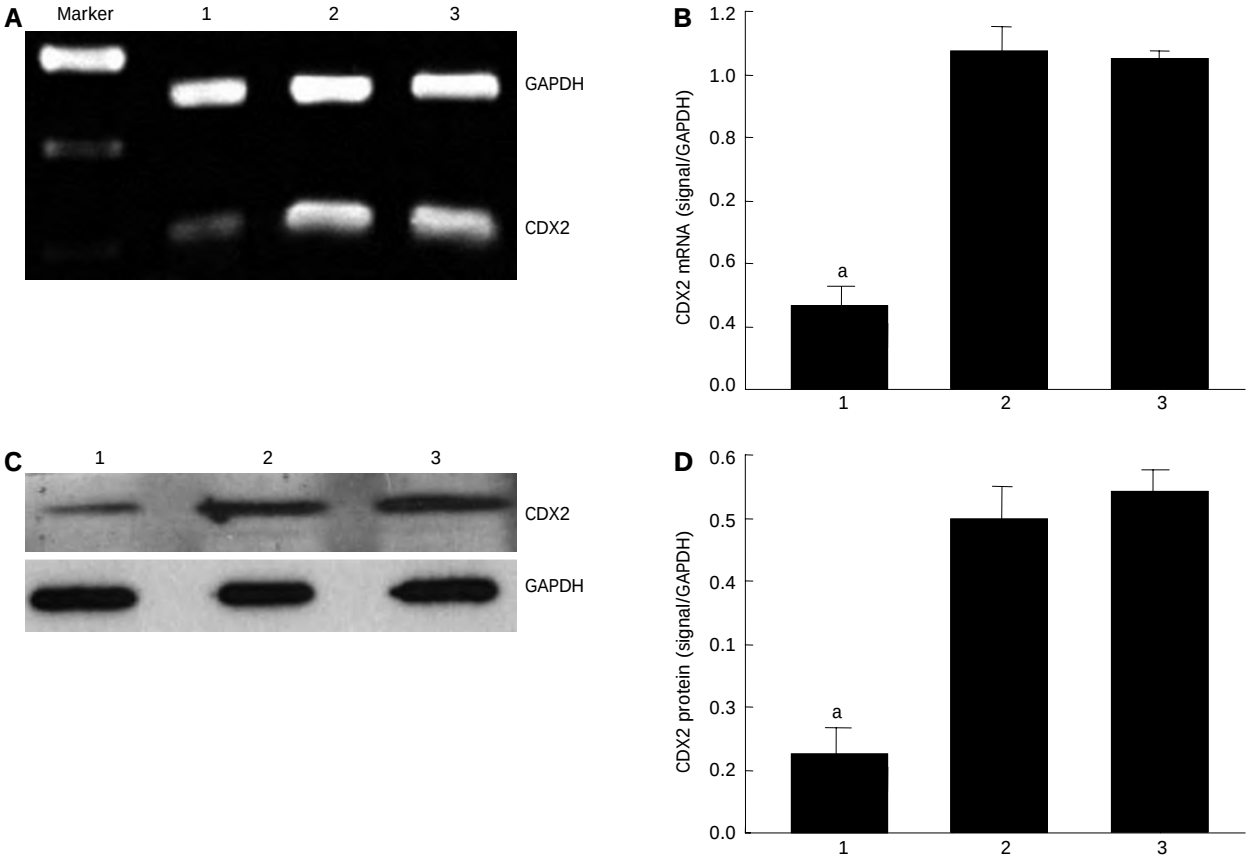


Figure 1 mRNA and protein expressions of CDX2 after RNA interference. A, B: Expression level of CDX2 mRNA was determined by semiquantitative reverse-transcriptase polymerase chain reaction; C, D: Expression level of CDX2 protein was determined by Western blotting. mRNA results were expressed as the ratio of CDX2 to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Western blotting results were expressed as the ratio of optical density of CDX2 bands to GAPDH bands. All values are mean ± SE. $^{a}P < 0.05$ for SGC7901/DDP + small interfering RNA (siRNA)-CDX2 cells *vs* SGC7901/DDP + siRNA-NC cells and SGC7901/DDP cells. Lane 1: SGC7901/DDP + siRNA-CDX2 cells; Lane 2: SGC7901/DDP + siRNA-NC cells; Lane 3: SGC7901/DDP cells.

$W^2 \times L/2$, where L is the length and W is the width of the tumor. The relative tumor volume (RTV) was calculated by the formula: $RTV = V_t/V_0$ (V_0 is the TV at the day when the chemicals were given, and V_t is the TV of subsequent measurement). The animals were sacrificed at 12 d after tumor injection and the tumors were analyzed.

Hematoxylin and eosin staining and deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay

For hematoxylin and eosin (HE) staining tumor tissues were fixed in 4% formaldehyde, dehydrated with gradient ethanol, and embedded in paraffin wax. Tissue sections were dewaxed and rehydrated according to a standard protocol. Sections were stained with HE. For the deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay, apoptotic cells in sections of mouse tumor tissue were detected using an *in situ* apoptosis detection kit (KEYGEN, Nanjing, China) as instructed by the manufacturer. Cells were visualized with a light microscope (Olympus IX70, Tokyo, Japan). The apoptotic index was calculated as follows: the apoptotic index = number of apoptotic cells/total number of cells. The *in vivo* experiments strictly obeyed the ethical principles and guidelines for scientific experiments on animals.

Statistical analysis

Data are expressed as mean ± SE. Statistical significance was determined using χ^2 test, Student's t test, or one-way analysis of variance (ANOVA). Statistical analysis were carried out using SPSS version 13.0 (Chicago, IL, United States) or Origin 7.5 software programs (OriginLab, Northampton, MA, United States). A value of $P < 0.05$ was considered as statistically significant.

RESULTS

CDX2 siRNA inhibits CDX2 mRNA and protein expression

Our previous study suggested that recombinant lentiviral vector for CDX2 gene (siRNA-CDX2) successfully inhibited CDX2 mRNA and protein expression in MGC-803 cells[13]. In the present study, we further tested the hypothesis that CDX2 siRNA downregulates CDX2 mRNA and protein expression in SGC7901/DDP cells. We treated SGC7901/DDP cells with siRNA-CDX2 and siRNA-NC (negative control). Transfection of siRNA-CDX2 into SGC7901/DDP cells led to marked inhibition of CDX2 mRNA (Figure 1A) and protein expression (Figure 1C). Densitometry analysis showed that CDX2 mRNA (Figure 1B) and protein (Figure 1D) in SGC7901/DDP

Table 1 IC50 values for anticancer drugs in SGC7901/DDP cells

	Doxorubicin (μg/mL)	5-fluorouracil (μg/mL)	Cisplatin (μg/mL)
SGC7901/DDP + siRNA-CDX2	0.12 ± 0.05[a]	0.52 ± 0.13[a]	0.82 ± 0.13[a]
SGC7901/DDP + siRNA-NC	0.33 ± 0.08	4.10 ± 1.25	2.81 ± 0.50
SGC7901/DDP	0.39 ± 0.15	4.05 ± 1.44	3.28 ± 1.03

IC50 values were evaluated by MTT assay. Each experiment was conducted in triplicate. Data are expressed as means ± SD. One-way analysis of variance followed by Dunnett's multiple comparison tests revealed statistical differences. [a]$P < 0.05$ for SGC7901/DDP + small interfering RNA (siRNA)-CDX2 cells *vs* SGC7901/DDP + siRNA-NC cells and SGC7901/DDP cells.

+ siRNA-CDX2 cells were about 3.5- and 4-fold lower, respectively, than those in SGC7901/DDP + siRNA-NC cells and SGC7901/DDP cells ($P < 0.05$). There were no differences between SGC7901/DDP + siRNA-NC cells and SGC7901/DDP cells. These results suggested that CDX2 siRNA could downregulate CDX2 mRNA and protein expression in SGC7901/DDP.

CDX2 siRNA reverses MDR

Although SGC7901/DDP cells were selected with the single anticancer drug cisplatin, they also displayed multiple resistances to other anticancer drugs. We studied the regulatory effects of CDX2 siRNA on the drug sensitivity of gastric cancer cells. MTT assay was used to detect the sensitivity of cells to one P-gp-related drug (doxorubicin) and two P-gp-non-related drugs (5-fluorouracil and cisplatin). As showed in Table 1, compared with SGC7901/DDP + siRNA-NC cells and SGC7901/DDP cells, SGC7901/DDP + siRNA-CDX2 exhibited significantly decreased IC50 values for cisplatin, doxorubicin and 5-fluorouracil ($P < 0.05$).

Effects of CDX2 siRNA on pump rate of doxorubicin

Pumping out chemotherapeutic agents is the key process in MDR[14]. We proposed that downregulation of CDX2 inhibited drug efflux in gastric cancer cells. To test this hypothesis, intracellular drug accumulation and retention were evaluated using doxorubicin as a probe. As shown in Figure 2A, compared with SGC7901/DDP + siRNA-NC cells and SGC7901/DDP cells, SGC7901/DDP + siRNA-CDX2 cells exhibited significantly increased accumulation and retention as well as a lower releasing index of doxorubicin (Figure 2B) ($P < 0.05$).

Effect of CDX2 siRNA on cell cycle control

We used flow cytometry to determine whether reversal of MDR by CDX2 siRNA in SGC7901/DDP cells was mediated, at least in part, through an effect on cell cycle progression. We found that the number of cells in G1 phase markedly increased, while those in S phase decreased in SGC7901/DDP + siRNA-CDX2 cells, compared with SGC7901/DDP + siRNA-NC cells and SGC7901/DDP cells (Figure 2C) ($P < 0.05$).

CDX2 siRNA induces apoptosis

Anti-apoptosis is an important mechanism of MDR, therefore, we investigated the effect of siRNA-CDX2 on cisplatin-induced gastric cancer cell apoptosis by calculating apoptosis index. Cells were stained with annexin V PE and 7-AAD, and then subsequently analyzed by flow cytometry. The dual parameter fluorescent dot plots showed that the viable cells were in the lower left quadrant, and the apoptotic cells were in the right quadrant. As shown in Figure 2E, compared with SGC7901/DDP + siRNA-NC cells and SGC7901/DDP cells, SGC7901/DDP + siRNA-CDX2 cells exhibited significantly increased apoptosis index (Figure 2F) ($P < 0.05$).

CDX2 siRNA influenced expression of c-Myc, survivin and cyclin D1

To investigate the mechanism by which CDX2 siRNA induces reversal of MDR in SGC7901/DDP cells, we detected expression levels of some well-known regulators of apoptosis (caspase-9, caspase-3, p53, bax, bcl-2, Survivin, and c-Myc), and an important cell cycle molecule (cyclin D1) by semiquantitative RT-PCR and Western blotting (Figure 3). The mRNA and protein expression level of c-Myc, survivin and cyclin D1 in SGC7901/DDP + siRNA-CDX2 cells was lower than that in SGC7901/DDP + siRNA-NC cells and SGC7901/DDP cells ($P < 0.05$). However, no significant difference in the expression level of caspase-9, caspase-3, p53, bax and bcl-2 was found in the cell models (data not shown).

Effect of CDX2 siRNA on reversing MDR of human gastric cancer in vivo

We examined the effect of CDX2 siRNA on growth of SGC7901/DDP cells *in vivo*, by implanting LV-siRNA-CDX2 and LV-siRNA-NC subcutaneously into the flanks of BALB/c nude mice. We detected expression levels of CDX2 *in vivo* by semi-quantitative RT-PCR and Western blotting. The mRNA (Figure 4A) and protein (Figure 4B) expression level of CDX2 in SGC7901/DDP + siRNA-CDX2 group was lower than that in SGC7901/DDP + siRNA-NC group and SGC7901/DDP group. Three weeks after implantation, TV in the SGC7901/DDP + siRNA-CDX2 group was significantly less than in the SGC7901/DDP and SGC7901/DDP + siRNA-NC groups ($P < 0.05$) (Figure 4D). The percentage of apoptotic tumor cells in SGC7901/DDP + siRNA-CDX2 cells was 7.2% ± 1.3%, which was more than the 3.1% ± 1.2% in SGC7901/DDP + siRNA-NC cells and 3.1% ± 1.4% in SGC7901/DDP cells, as determined by the HE staining and TUNEL assay (Figure 4C).

DISCUSSION

The development of MDR to cancer chemotherapy is a major obstacle to the effective treatment of gastric cancer[14]. However, the mechanism of MDR remains obscure. P-gp was the first molecule identified as a modulator of MDR. After that, various other molecules were

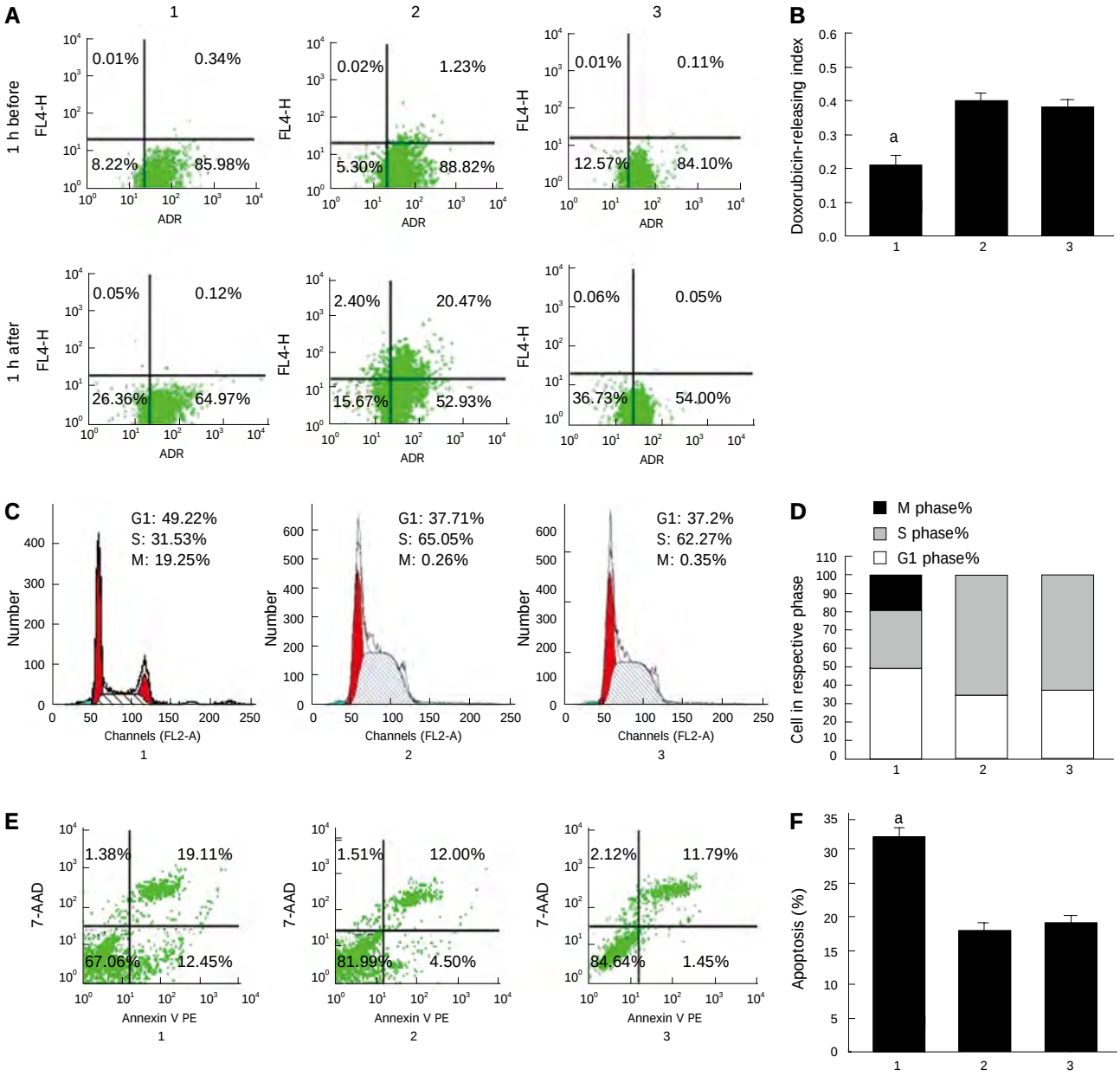
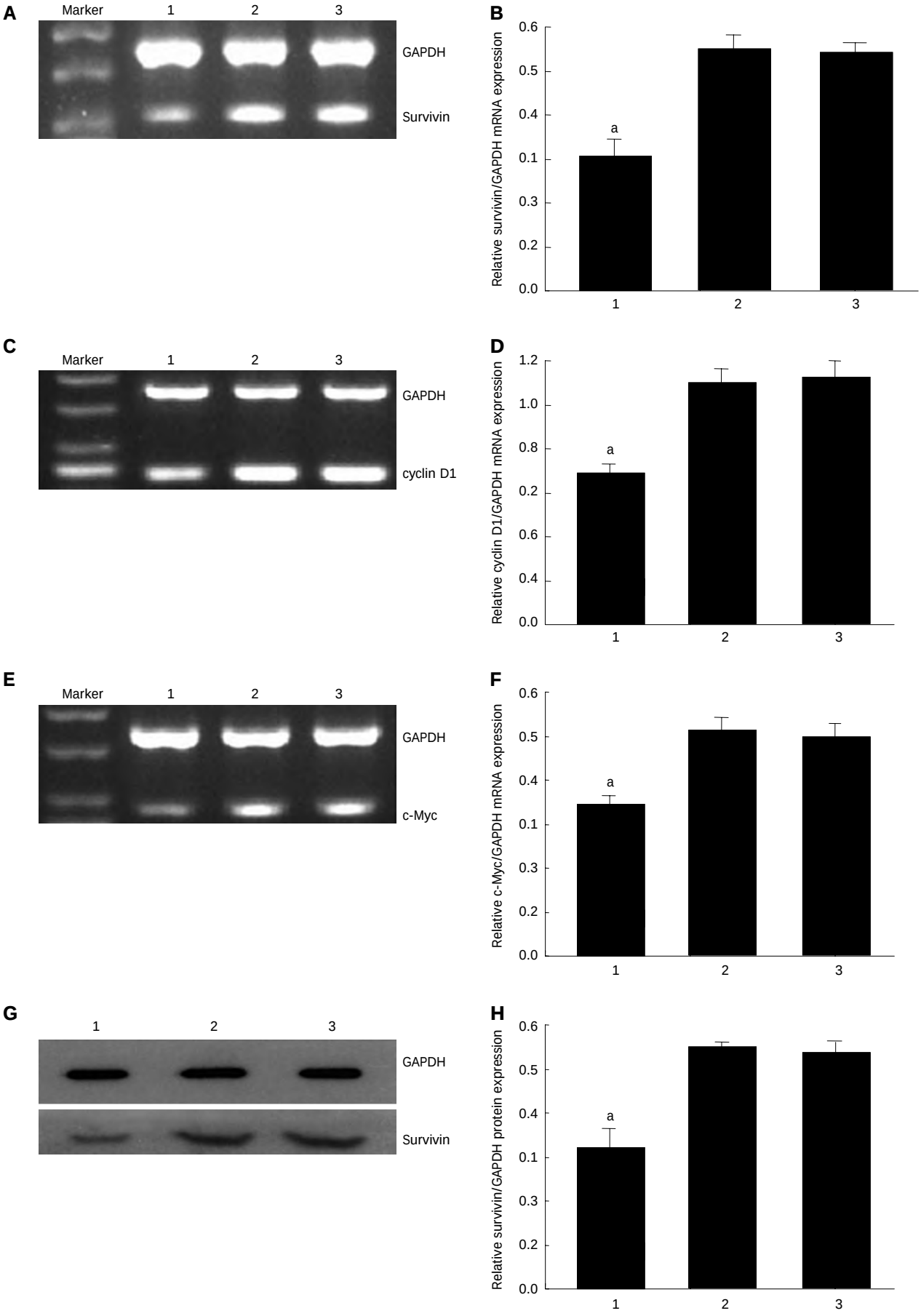


Figure 2 Effect of downregulation of CDX2 on cell pump rate of doxorubicin, cell cycle, and apoptotic rate in SGC7901/DDP cells after RNA interference. A, B: Pump rate of doxorubicin in SGC7901/DDP cells after RNAi was analyzed by flow cytometry; C, D: Cell cycle in SGC7901/DDP cells after RNAi was analyzed by flow cytometry; E, F: Apoptotic rate in SGC7901/DDP cells after RNAi was analyzed by flow cytometry. $^{a}P < 0.05$ for SGC7901/DDP + small interfering RNA (siRNA)-CDX2 cells *vs* SGC7901/DDP + siRNA-NC cells and SGC7901/DDP cells. Lane 1: SGC7901/DDP + siRNA-CDX2 cells; Lane 2: SGC7901/DDP + siRNA-NC cells; Lane 3: SGC7901/DDP cells.

shown to be involved, including transporters that eject anticancer drugs from cells, such as MDR-associated protein (MRP)[15], genes regulating apoptosis, such as p53[16], PKC[17], and Bcl-2 family[18]. Recently, the distribution of drugs in cancer cells was also considered to play a part in MDR[19]. According to our previous report, some classic molecules are involved in MDR, including caspase-3 (apoptosis-related cysteine peptidase) and caspase-9 (an initiator caspase, has been linked to the mitochondrial death pathway)[20,21], but there may be other mechanisms that control MDR of gastric cancer cells[12].

The CDX2 homeobox gene, which is homologous to the *Drosophila* gene caudal, has an essential role during early development[2], an important study by Ma *et al*[22] demonstrated that short interfering RNA-mediated knockdown of CDX2 resulted in reduced apical sodium-dependent bile acid transporter (ASBT) mRNA expression in intestinal cells. Overexpression of CDX2 in human colon cancer cells induces a less malignant phenotype, inhibiting proliferation, invasion, and migration[23]. Furthermore, CDX2 has a crucial role in the regulation of MDR1 gene expression in drug resistance[10]. However, the precise molecular mechanism of CDX2 in reversing MDR in gastric cancer cells is still poorly characterized. The present study is believed to be the first to correlate CDX2 with MDR of gastric cancer cells, and we found


A
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1
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GAPDH
Survivin
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Relative survivin/GAPDH mRNA expression
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cyclin D1
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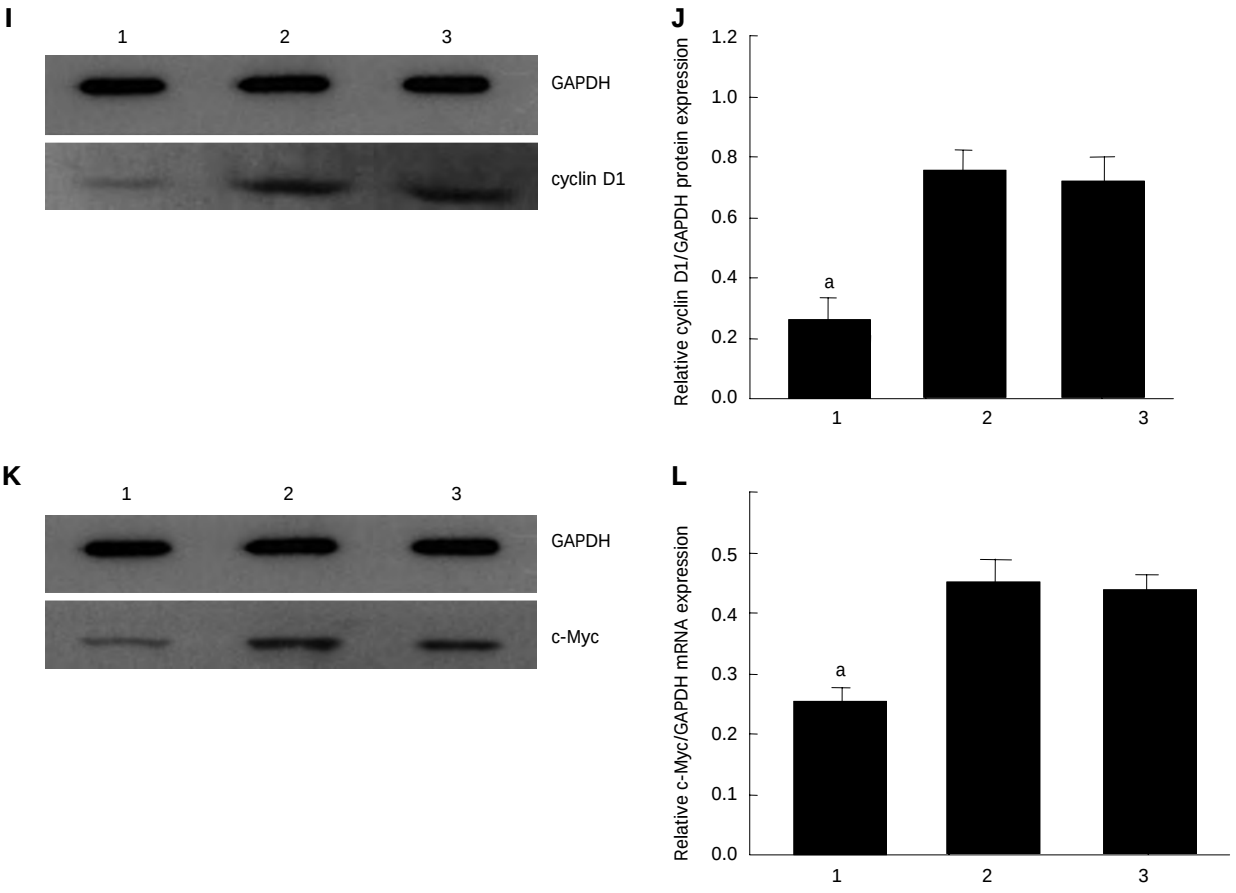

Figure 3 RNA interference-mediated inhibition of CDX2 decreased survivin, cyclin D1 and c-Myc mRNA and protein expression. mRNA expression levels of survivin (A), cyclin D1 (C), and c-Myc (E) were determined by semiquantitative reverse-transcriptase polymerase chain reaction. Protein expression levels of survivin (G), cyclin D1 (I), and c-Myc (K) were determined by western blotting. mRNA results were expressed as the ratio of survivin (B), cyclin D1 (D), and c-Myc (F) to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Western blotting results are expressed as the ratio of optical density of survivin (H), cyclin D1 (J) and c-Myc (L) bands to GAPDH bands. All values are mean ± SE. $^{a}P < 0.05$ for SGC7901/DDP + small interfering RNA (siRNA)-CDX2 cells *vs* SGC7901/DDP + siRNA-NC cells and SGC7901/DDP cells. Lane 1: SGC7901/DDP + siRNA-CDX2 cells; Lane 2: SGC7901/DDP + siRNA-NC cells; Lane 3: SGC7901/DDP cells.

that expression of CDX2 regulated drug efflux pumping, the cell cycle, and apoptosis. The multiple changes conferred by CDX2 on gastric cancer cells are not surprising, given the involvement of CDX2 in a wide range of biochemical reactions, and CDX2 is a homeobox transcription factor that contributes to reversing MDR.

Our study indicated that CDX2 siRNA led to marked downregulation of CDX2 mRNA and protein expression in SGC7901/DDP cells, caused cell cycle arrest in the G0/G1 phase, and induced apoptosis. Furthermore, downregulation of CDX2 in SGC7901/DDP cells enhanced sensitivity to cisplatin, 5-fluorouracil (P-gp-nonrelated drug), and doxorubicin (P-gp-related drug). The ability to pump doxorubicin was reduced significantly, moreover, a strong antitumor effect of CDX2 siRNA *in vivo* was observed, as tumor growth was suppressed and tumor apoptosis was increased in nude mice when CDX2 mRNA and protein were downregulated. These findings suggest that CDX2 siRNA reversed MDR of human gastric cancer cells.

Doxorubicin is a common substrate for P-gp, but SGC7901/DDP + siRNA-CDX2 cells also exhibited significantly decreased IC50 values for cisplatin and 5-fluorouracil. It should be noted that P-gp-mediated drug efflux was not the only mechanism involved in drug resistance. Previous studies have shown that the effect of P-gp on drug resistance is closely related to cell cycle distribution and apoptosis[24-26]. Cyclin D1 is a regulatory kinase of cell cycle distribution. Previously, overexpression of cyclin D1 in a human fibrosarcoma cell line has been shown to confer resistance to methotrexate[27], which suggests that cyclin D1 overexpression can contribute to the resistance of cancer cells to chemotherapeutic agents. Conversely, suppression of cyclin D1 levels has been shown to potentiate the response of human pancreatic cancer cells to cisplatinum, transfection and multidrug selection experiments have demonstrated that resistance to mitoxantrone can be associated with MDR1 and/or multidrug resistance-associated protein (MRP) overexpression[28]. Indeed, subsequent analysis of MDR1 and MRP expression has revealed that cyclin D1 suppression decreases MDR1 and MRP mRNA levels[24]. Besides regulation of cell cycle distribution, apoptosis is a common pathway that finally mediates the killing effects of anticancer drugs, which is an important cause of MDR. Mitochondria are known to play an active role in the apop-

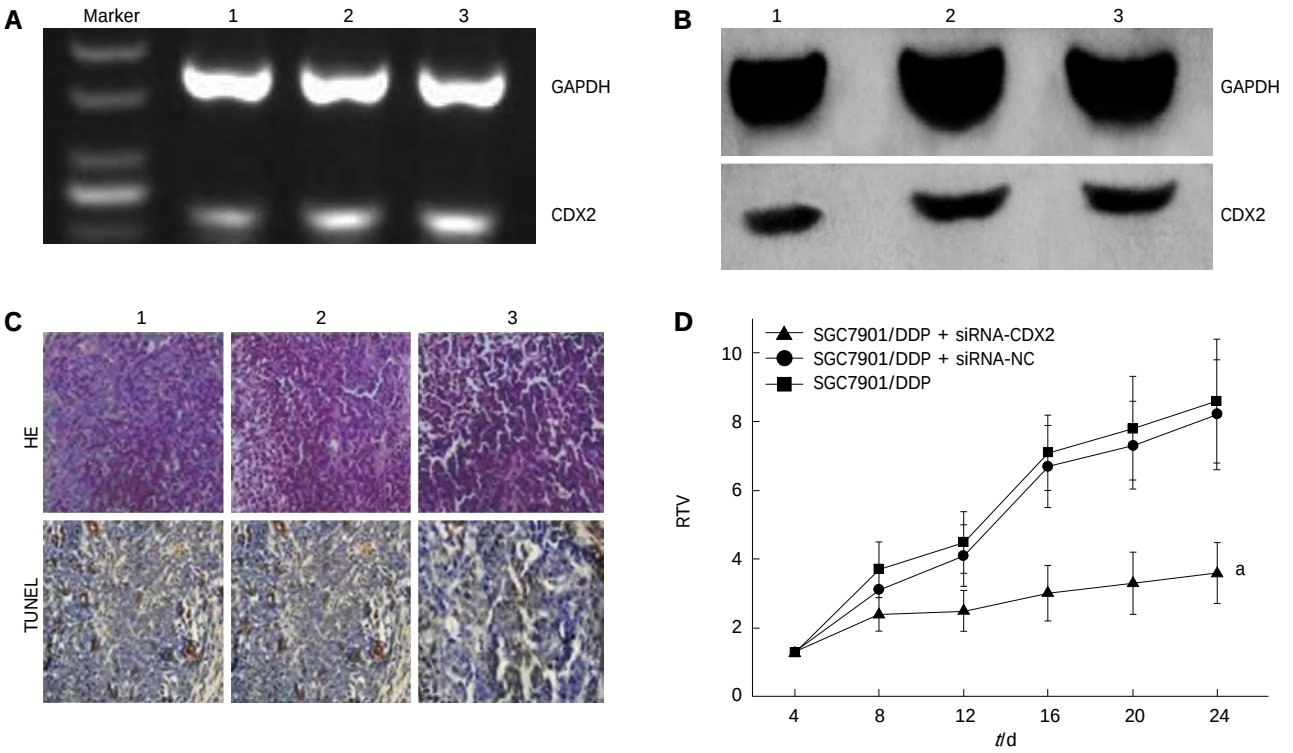

Figure 4 Effect of RNA interference-mediated inhibition of CDX2 mRNA and protein expression and downregulation of CDX2 on apoptosis *in vivo*. A: mRNA expression level of CDX2 was determined by semiquantitative reverse-transcriptase polymerase chain reaction; B: Protein expression level of CDX2 was determined by western blotting; C: Tumor cell apoptosis was assessed by HE staining and TUNEL assay. CDX2 siRNA induced more apoptosis of tumor cells (× 400); D: Relative tumor volume (RTV) of nude mice in each group is presented. Each time point represents the mean RTV for each group. RTV in the SGC7901/DDP + small interfering RNA (siRNA)-CDX2 group was smaller than that in control animals. [a]$P < 0.05$ for SGC7901/DDP + siRNA-CDX2 cells *vs* SGC7901/DDP + siRNA-NC cells and SGC7901/DDP cells. Lane 1: SGC7901/DDP + siRNA-CDX2 cells; Lane 2: SGC7901/DDP + siRNA-NC cells; Lane 3: SGC7901/DDP cells. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

totic process by various mechanisms, including release of caspase activators, disruption of electron transport and energy metabolism, and production of reactive oxygen species[29]. Survivin induces mitochondrial fragmentation and reduces mitochondrial respiration[30]. These data indicate that survivin is closely related to apoptosis. Therefore, in the present study, inhibition of CDX2 expression may have decreased cyclin D1 and survivin expression directly or indirectly, which was responsible for reversal of MDR in human gastric cancer cells *in vitro* and *in vivo*. Further studies are needed to confirm our results.

The term MDR was originally coined to define a condition enabling a disease-causing organism or cancer cells to resist distinct drugs or chemicals with a wide variety of structure and function, targeted at eradicating the organism/cancer cell. Much routine chemotherapy cannot achieve good therapeutic effects because of MDR. It is important to find a new way to reverse MDR. In this study, we showed that CDX2 plays a critical role in reversing MDR. Downregulation of CDX2 using RNAi reversed the progression of MDR in gastric cancer SGC7901/DDP cells *in vitro* and *in vivo*. In conclusion, this study lays the foundation for treatment of MDR in gastric cancer through manipulation of CDX2 expression.

COMMENTS

Background

The term multidrug resistance (MDR) was originally coined to define a condition enabling a disease-causing organism or cancer cells to resist distinct drugs or chemicals with a wide variety of structure and function, targeted at eradicating the organism/cancer cell. Much routine chemotherapy cannot achieve good therapeutic effects because of MDR. It is believed that MDR is the key factor in the failure of gastric cancer chemotherapy. It is important to find a new way to reverse MDR. The caudal-type homeobox gene, CDX2, plays an important role in intestinal metaplasia, and is a precursor of intestinal-type gastric carcinoma. However, the effect of CDX2 in reversing MDR is still not clear.

Research frontiers

CDX2 has a crucial role in the regulation of *MDR1* gene expression in drug resistance, but P-glycoprotein (P-gp)-mediated drug efflux is not the only mechanism involved in drug resistance. The CDX2 research hotspot is how it affects the reversal of MDR by other pathways.

Innovations and breakthroughs

This study is believed to be the first to demonstrate that downregulation of CDX2 causes cell cycle arrest in the G0/G1 phase, and induces apoptosis. Furthermore, downregulation of CDX2 in SGC7901/DDP cells enhances the sensitivity of SGC7901/DDP cells to cisplatin, 5-fluorouracil (P-gp-non-related drug), and doxorubicin (P-gp-related drug). The ability to pump doxorubicin was reduced significantly, moreover, a strong antitumor effect of CDX2 siRNA *in vivo* was observed, as tumor growth was suppressed and tumor apoptosis was increased in nude mice when CDX2 mRNA and protein were downregulated. CDX2 siRNA also decreased c-Myc, survivin and cyclin D1 expression as determined by semiquantitative reverse-transcriptase polymerase chain reaction and Western blotting.

Applications

This study lays the foundation for treatment of MDR in gastric cancer through manipulation of CDX2 expression.

Terminology

The transcription factor, CDX2, is a member of the caudal-related homeobox gene family, and is mainly expressed in the intestine. It is also known to be a key factor in reversing MDR by manipulation of MDR1 expression.

Peer review

This is a well-written manuscript and most of the experiments were properly controlled and clearly presented.

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BRIEF ARTICLE

Helicobacter pylori infection as a cause of iron deficiency anaemia of unknown origin

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Abstract

AIM: To assess the aetiological role of *Helicobacter pylori* (*H. pylori*) infection in adult patients with iron-refractory or iron-dependent anaemia of previously unknown origin.

METHODS: Consecutive patients with chronic iron-deficient anaemia (IDA) with *H. pylori* infection and a negative standard work-up were prospectively evaluated. All of them had either iron refractoriness or iron dependency. Response to *H. pylori* eradication was assessed at 6 and 12 mo from follow-up. *H. pylori* infection was considered to be the cause of the anaemia when a complete anaemia resolution without iron supplements was observed after eradication.

RESULTS: *H. pylori* was eradicated in 88 of the 89 patients. In the non-eradicated patient the four eradicating regimens failed. There were violations of protocol in 4 patients, for whom it was not possible to ascertain the cause of the anaemia. Thus, 84 *H. pylori* eradicated patients (10 men; 74 women) were available to assess the effect of eradication on IDA. *H. pylori* infection was considered to be the aetiology of IDA in 32 patients (38.1%; 95%CI: 28.4%-48.8%). This was more frequent in men/postmenopausal women than in premenopausal women (75% *vs* 23.3%; $P < 0.0001$) with an OR of 9.8 (95%CI: 3.3-29.6). In these patients, anaemia resolution occurred in the first follow-up visit at 6 mo, and no anaemia or iron deficiency relapse was observed after a mean follow-up of 21 ± 2 mo.

CONCLUSION: Gastric *H. pylori* infection is a frequent cause of iron-refractory or iron-dependent anaemia of previously unknown origin in adult patients.

Key words: *Helicobacter pylori*; Iron-deficiency anaemia; Iron refractoriness; Gluten-sensitive enteropathy; Menopause

Core tip: Data on the effect of *Helicobacter pylori* (*H. pylori*) eradication on adult patients with iron-refractory or iron-dependent anaemia of previously unknown origin are scarce, and thus the frequency of *H. pylori* infection as the cause of anaemia in that setting is unknown. Resolution of iron-deficient anaemia (IDA)

was observed in 32 out of the 84 *H. pylori* eradicated patients (38.1%). In all of them there was no relapse after a mean follow-up of 21 ± 2 mo. Thus, *H. pylori* infection was considered the aetiology of IDA in these cases. *H. pylori* infection as the aetiology of IDA was greater in men *plus* postmenopausal women than in premenopausal women (75.0% *vs* 23.3%, $P < 0.0001$).

Monzón H, Forné M, Esteve M, Rosinach M, Loras C, Espinós JC, Viver JM, Salas A, Fernández-Bañares F. *Helicobacter pylori* infection as a cause of iron deficiency anaemia of unknown origin. *World J Gastroenterol* 2013; 19(26): 4166-4171 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4166.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4166

INTRODUCTION

Iron-deficiency anaemia (IDA) occurs in 2%-5% of adult men and postmenopausal women in the developed world, with blood loss from the gastrointestinal tract being the most common cause[1-4]. In addition, IDA also occurs in 5%-12% of otherwise healthy premenopausal women[5]. IDA is a common cause of referral to gastroenterologists (4%-13% of referrals)[3], and for 5%-10% of patients with IDA without gastrointestinal bleeding the cause of the condition remains obscure in spite of extensive examination[6,7].

Helicobacter pylori (*H. pylori*) colonisation in gastric mucosa may impair iron uptake and increase iron loss, potentially leading to IDA. The speculative mechanisms by which *H. pylori* may produce IDA have recently been reviewed[8-10]. Four meta-analyses to assess the effect of *H. pylori* eradication combined with ferrous supplementation on the treatment of IDA have been published[11-14]. The conclusions suggest that *H. pylori* eradication therapy improves iron absorption, since *H. pylori* eradication combined with iron administration was more effective than iron administration alone for the treatment of IDA. However, there was no follow-up of patients after oral iron therapy was completed, and relapse of IDA after *H. pylori* eradication was not evaluated; thus, it was not established whether *H. pylori* infection was the cause of IDA. Most of the intervention trials have been performed in geographical areas where both IDA and *H. pylori* infection are highly prevalent, and where the aetiology of IDA may be multifactorial (malnutrition, vitamin deficiencies, chronic parasitic infections, malaria). In western countries there are only some uncontrolled intervention studies showing recovery from anaemia after *H. pylori* eradication[15,16]. In light of the above-mentioned studies, *H. pylori* infection has been considered as a risk factor for IDA. The British Society of Gastroenterology recommends eradication of *H. pylori* infection in patients with IDA and normal colonoscopy and oesophagogastroduodenoscopy (Grade of recommendation, C)[1], and the Maastricht guidelines suggest to eradicate *H. pylori* in patients with IDA (Grade of recommendation, A)[17]. However, data on the effect of *H. pylori* eradication on adult patients with iron-refractory or iron-dependent IDA of previously unknown origin are scarce, and thus the frequency of *H. pylori* infection as the cause of IDA in that setting is unknown. Therefore, the aim of the present study was to assess the aetiological role of *H. pylori* infection in such patients, in a geographical background where concomitant causes of IDA are unusual.

MATERIALS AND METHODS

Patients

Consecutive patients with unexplained chronic IDA or isolated iron deficiency (ID) referred to the Gastroenterology Department from January 2007 to December 2010 were prospectively evaluated.

Patients were included if they were older than 18 years of age with all the following: (1) chronic IDA defined as haemoglobin < 10.5 g/dL in women and < 11.5 g/dL in men, and serum ferritin < 13 μg/L or ID defined as only serum ferritin < 13 μg/L; (2) gastric *H. pylori* infection; (3) iron refractoriness or iron dependency (see below for definition); (4) negative faecal immunochemical tests for occult blood (at least three negative samples); (5) negative coeliac serology [both serum immunoglobulin A (IgA)-antiendomysial an IgA-human anti-tissue transglutaminase antibodies], although patients diagnosed with coeliac disease in whom IDA persisted in spite of being on a strict gluten-free diet with negative coeliac serology and no villous atrophy were included; (6) normal gastroscopy and full colonoscopy; (7) normal physical examination, blood analysis (including routine blood biochemistry, C reactive protein, folate and vitamin B_{12} levels), and urinalysis; and (8) normal gynaecological examination.

Patients with the following conditions were excluded from the study: (1) frequent (three times a week or more) use of non-steroidal anti-inflammatory drugs or salicylates during the previous 6 mo; (2) use of dicumarinics; (3) other conditions which cause anaemia or interfere with erythropoiesis including malignancy, haematological diseases, connective tissue disease, chronic diseases such as chronic renal failure, chronic liver disease, severe cardiac and respiratory disease, and previous gastrointestinal surgery; (4) pregnancy or lactation; (5) history of alcoholism or drug addiction; (6) heavy menstrual flow (cycles > 5 d, associated with passage of clots after the three first days) and/or metrorrhagia; (7) obvious blood loss (melena, haematochezia, haematuria, recurrent epistaxis); (8) adherence to vegetarian or iron-deficient diet; and (9) expected lack of cooperation.

Capsule endoscopy was not routinely performed since one inclusion criterion was that repeated faecal immunochemical tests for occult blood were negative. In individual cases (12 patients), it was performed by the decision of the physician at charge, yielding in all cases normal results.

All patients had received iron supplements and all of them fulfilled the criteria of either iron refractoriness or iron dependency. Iron refractoriness was defined as an inappropriate increase in haemoglobin levels (< 2 g/dL) after completion of a 5000 mg dosing cycle of ingested elemental iron over one month or longer[18]. Iron dependency was defined as the patient's requiring daily oral iron supplementation (ferrous sulphate, 100-200 mg daily of elemental iron) to maintain adequate haemoglobin levels.

Study design

The following tests were prospectively performed in all included patients: (1) two endoscopic biopsies from both gastric body and antrum, and four biopsies from distal duodenum; (2) histological examination of antral biopsies and/or ^{13}C-urea breath test to assess *H. pylori* infection; and (3) human leukocyte antigen (HLA)-DQ2 and HLA-DQ8 haplotypes of predisposition to coeliac disease.

In all patients *H. pylori* was eradicated using a standard 7-d triple regimen with omeprazole 20 mg *bid*, amoxicillin 1 g *bid*, and clarithromycin 500 mg *bid*. Two-week quadruple regimens were used as a rescue therapy for patients failing the first-line eradication therapy, and in some cases a levofloxacin-based third-line rescue therapy was used. *H. pylori* eradication was evaluated by histological examination of antral biopsies and/or ^{13}C-urea breath test. Analytical response to *H. pylori* eradication was assessed at 6 and 12 mo of follow-up. In cases with associated lymphocytic duodenitis (LD), histological follow-up was also performed at 6 and 12 mo.

Final diagnosis

H. pylori infection was considered to be the cause of IDA or ID when after eradication a complete response (*i.e.*, IDA or ID recovery, with normal serum ferritin levels), without iron supplements, was observed at 12 mo of follow-up. A diagnosis of gluten-sensitive enteropathy was performed in patients with LD and positive coeliac genetics (HLA-DQ2 and/or HLA-DQ8) without response after achieving *H. pylori* eradication, when there was a sustained (at 12 mo follow-up) complete clinical and histological response on a strict gluten-free diet[19]. The cause of either IDA or ID was considered to be unknown in patients without response to both *H. pylori* eradication and gluten-free diet (when indicated).

Histological studies

Four endoscopic biopsies from the 2nd-3rd portions of the duodenum and two biopsies from both body and antrum were obtained in the index endoscopy. *H. pylori* infection was investigated in gastric antral mucosal samples by standard histopathological assessment[20]. Duodenal samples were processed using haematoxylin/eosin staining and CD3 immunophenotyping, and these were blindly evaluated by an expert gastrointestinal pathologist (Salas A). LD was defined as 25 or more intraepithelial lymphocytes per 100 epithelial nuclei and normal villous architecture, as suggested in recent literature[21]; the cut-off value was validated in our laboratory[19]. This cut-off value was also selected to define LD due to gluten-sensitive enteropathy, which corresponds to the Marsh 1 type lesion of the coeliac disease spectrum[21].

Helicobacter pylori status

Patients were classified as having *H. pylori* infection when either histology or ^{13}C-urea breath test was positive. ^{13}C-urea breath test was performed on those patients with negative histology who either were taking proton-pump inhibitors or had antral intestinal metaplasia at the index endoscopy, as previously described[20]. In the case of proton-pump inhibitors, the breath test was performed 4 wk after discontinuation.

Ethics

The protocol was approved by the Ethics Committee of the Hospital Universitari Mútua Terrassa, and all participants provided informed consent.

Statistical analysis

Results are expressed as mean ± SE and as percentages plus their 95%CI. Chi-square statistics were used to assess significant associations between qualitative variables. The OR and 95%CI of the significant associations were computed.

RESULTS

One hundred thirty-six consecutive adult patients fulfilled the inclusion criteria during the study period. Twenty-two were excluded due to presence of exclusion criteria, 7 patients had previously unrecognised non-steroidal anti-inflammatory drug intake, and 15 had other causes of anaemia (1 infection by intestinal parasites, 2 recurrent rectal bleeding attributed to haemorrhoids, 1 chronic renal failure, 2 small bowel Crohn's disease, 1 gastrinoma, and 8 pernicious anaemia). Twenty-five additional patients (18.4%) were lost in follow-up before achieving a definite diagnosis of their anaemia. Thus, 89 patients were finally included in the study (10 men; 79 women; mean age: men, 54.0 ± 15.8 years; premenopausal women, 44.0 ± 8.6 years; postmenopausal women, 59.0 ± 9.8 years). There were no significant differences in demographic data or frequency of menopause, *H. pylori*-related chronic gastritis, associated enteropathy (LD) or coeliac genetics between included patients and those lost in follow-up (Table 1).

H. pylori was eradicated in 88 of the 89 patients. In the non-eradicated patient the four eradicating regimens failed. There were violations of protocol in 4 patients, for whom it was not possible to ascertain the cause of the anaemia. Thus, 84 *H. pylori*-eradicated patients were available to assess the effect of eradication on IDA.

Resolution of IDA or ID was observed in 32 out of the 84 *H. pylori*-eradicated patients (38.1%; 95%CI: 28.4-48.8). In all of them, IDA or ID recovery was observed at the 6-month follow-up visit after *H. pylori*-erad-

Table 1 Comparison of demographic, clinical, and biological data between included patients and those lost in follow-up

	Included patients (n = 89)	Loss of follow-up patients (n = 25)
Age (yr), mean ± SE	46.0 ± 11.8	41.0 ± 14.2
Sex (M/F)	10/79	4/21
Postmenopausal women	17.90%	16.00%
Chronic antral gastritis	81.80%	78.30%
Chronic body gastritis	83.00%	78.30%
Associated enteropathy	59.50%	60.00%
HLA-DQ2 and/or DQ8+	48.30%	40.00%

There were no significant differences in any parameter. HLA: Human leukocyte antigen; M/F: Male/female.

ication, and there was no relapse after a mean follow-up of 21 ± 2 mo. Therefore, *H. pylori* infection was considered the aetiology of IDA in these cases. Frequency of *H. pylori* infection as the aetiology of IDA was greater in men (8 of 10, 80%) *plus* postmenopausal women (10 of 14, 71.4%) than in premenopausal women (14 of 60, 23.3%) (75.0% *vs* 23.3%, $P < 0.0001$) with an OR of 9.8 (95%CI: 3.3-29.6). There were no differences in the frequency of *H. pylori* infection as the cause of IDA between patients with and those without associated enteropathy (18 of 49, 36.7%; with LD and 14 of 35, 40%, without LD).

In addition, a gluten-free diet was offered to 13 patients in whom IDA persisted after *H. pylori* eradication. Gluten-sensitive enteropathy was the aetiology of IDA in 4 (men, 1 of 10, 10%; premenopausal women, 1 of 14, 7.1%; postmenopausal women, 2 of 60, 3.3%) of the 84 *H. pylori* eradicated patients (4.8%; 95%CI: 1.8-11.6). In all of them LD was detected, and a clinical (IDA recovery without iron supplementation) and histological remission after a gluten-free diet was observed. There was no relapse of IDA or ID after the 12 mo of follow-up.

The final diagnosis of IDA in the remaining 48 patients, who were mainly premenopausal women, was unknown. Despite *H. pylori* eradication there was a need to maintain iron supplementation during follow-up, with persistent iron-dependent IDA. A relation with menstrual blood loss was observed in 30 of the premenopausal women since anaemia recovered after either entering menopause or starting hormonal contraceptive therapy. As previously mentioned, none of them had heavy menstrual blood loss at inclusion, and for all of them the gynaecologic examination had been normal.

DISCUSSION

Results of the present study suggest that *H. pylori* infection is a frequent cause of IDA in adult patients with iron refractoriness or iron dependency in whom the standard diagnostic work-up is negative. In 38% of such patients *H. pylori* eradication was associated with both IDA resolution without the need for more iron supplementation and an absence of IDA relapse after nearly two years mean follow-up. These observations argue in favour of causality of *H. pylori* infection. In addition, the efficacy of *H. pylori* eradication to recover from IDA in such patients was compared between men *plus* postmenopausal women and premenopausal women. There was IDA recovery in 75% and 23% of them, respectively, with highly significant differences. In fact, the OR of *H. pylori* infection as the cause of IDA was almost 10 times higher in the first group than the second.

We have to take into account that the *H. pylori* re-infection rate after cure in our geographical area is low, around 1% patient-year; this implies that the present results might not be extrapolated to other regions with higher re-infection rates.

Results of the present study on premenopausal women are in disagreement with previous results of Annibale *et al*[15], since they showed recovery from anaemia at 12 mo of follow-up after *H. pylori* eradication in 92% of patients, mainly premenopausal women. The discrepancies revolve around the definition of response. In our study, response to *H. pylori* eradication was defined as anaemia recovery with normalisation of serum ferritin levels. In Annibale's study, however, ferritin levels returned to normal in only 17% of the patients despite recovery from anaemia, which is a figure similar to that of the 23% obtained in our study. Taking into account the meta-analysis data mentioned in the introduction[11-14], and the results of the present study in men and postmenopausal women, *H. pylori* infection may also be a contributing factor to IDA in premenopausal women, and *H. pylori* eradication is indicated to improve iron absorption. In this sense, it would be of interest to assess whether iron requirements change after *H. pylori* eradication in these patients. Regrettably, iron requirements were not sufficiently well recorded in the present study to allow for this evaluation.

Other factors may contribute to IDA in otherwise healthy premenopausal women such as menstrual loss, increased iron demands on pregnancy and breast feeding, and dietary deficiency[22]. Hormonal contraceptive therapy may reduce menstrual blood loss by approximately 50%, even in women with average or slightly above-average blood loss[23]. In the present study, this type of therapy was effective for IDA recovery in those premenopausal women in whom increased iron requirements persisted after *H. pylori* eradication.

H. pylori infection may be a cause of LD, which may disappear after eradication of the infection[19,24]. However, whether *H. pylori*-infected patients with LD are more prone to developing IDA is unknown. The present study shows that the frequency of *H. pylori* infection as the final diagnosis of IDA is similar for patients with and without associated enteropathy. Therefore, these data argue against a pathophysiological role for this mild enteropathy in the development of IDA in *H. pylori*-infected patients.

In 4.5% of the included patients the final diagnosis was gluten-sensitive enteropathy. These results agree with

a previous study by our group showing that a subgroup of patients with IDA of previously unknown origin and positive coeliac genetics presented a gluten-sensitive mild enteropathy with negative coeliac serology[25].

In conclusion, the results of the present study show that *H. pylori* infection is a frequent cause of IDA in men and postmenopausal women with either iron refractoriness or iron dependency, in whom other causes of IDA have been previously ruled out. *H. pylori* eradication therapy produces long-term resolution of IDA in such patients. Also, *H. pylori* infection may be a contributing factor to IDA in otherwise healthy premenopausal women without heavy menstrual blood loss, and it is the aetiology of IDA in almost 25% of them.

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COMMENTS

Background

Iron deficiency anaemia (IDA) is a common cause of referral to gastroenterologists (4%-13% of referrals), and for 5%-10% of patients with IDA without gastrointestinal bleeding the cause of the condition remains obscure in spite of extensive examination. Previous data suggest that *Helicobacter pylori* (*H. pylori*) eradication therapy improves iron absorption, since *H. pylori* eradication combined with iron administration was more effective than iron administration alone for the treatment of IDA. The Maastricht guidelines suggest to eradicate *H. pylori* in all patients with IDA.

Research frontiers

H. pylori eradication may improve iron absorption, but how often is gastric *H. pylori* infection the cause of IDA? Data on the effect of *H. pylori* eradication on adult patients with iron-refractory or iron-dependent anaemia of previously unknown origin are scarce, and thus, the frequency of *H. pylori* infection as the cause of IDA in that setting is unknown.

Innovations and breakthroughs

This study is performed in a large prospective series of consecutive patients using very strict inclusion criteria, with a long follow-up after *H. pylori* cure. Resolution of anaemia was defined as both haemoglobin and iron stores normalization, which was long-term maintained without requiring iron supplements. Frequency of IDA resolution was compared between men/post-menopausal women and pre-menopausal women.

Applications

Gastric *H. pylori* infection may be a frequent cause of iron-refractory or iron-dependent anaemia of previous unknown origin in adult patients, mainly in men and post-menopausal women. In these patients, *H. pylori* infection eradication produced the cure of IDA. In addition, *H. pylori* infection may be a contributing factor to IDA in otherwise healthy premenopausal women without heavy menstrual blood loss, being the aetiology of IDA in almost a 25% of them.

Peer review

The authors in this article have focused on the possible role of *H. pylori* infection in causation of IDA. They have stated that 38% of the patients may have the anaemia duo to the infection by *H. pylori*.

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BRIEF ARTICLE

Single endoscopist-performed percutaneous endoscopic gastrostomy tube placement

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Abstract

AIM: To investigate whether single endoscopist-performed percutaneous endoscopic gastrostomy (PEG) is safe and to compare the complications of PEG with those reported in the literature.

METHODS: Patients who underwent PEG placement between June 2001 and August 2011 at the Baskent University Alanya Teaching and Research Center were evaluated retrospectively. Patients whose PEG was placed for the first time by a single endoscopist were enrolled in the study. PEG was performed using the pull method. All of the patients were evaluated for their indications for PEG, major and minor complications resulting from PEG, nutritional status, C-reactive protein (CRP) levels and the use of antibiotic treatment or antibiotic prophylaxis prior to PEG. Comorbidities, rates, time and reasons for mortality were also evaluated. The reasons for PEG removal and PEG duration were also investigated.

RESULTS: Sixty-two patients underwent the PEG procedure for the first time during this study. Eight patients who underwent PEG placement by 2 endoscopists were not enrolled in the study. A total of 54 patients were investigated. The patients' mean age was 69.9 years. The most common indication for PEG was cerebral infarct, which occurred in approximately two-thirds of the patients. The mean albumin level was 3.04 ± 0.7 g/dL, and 76.2% of the patients' albumin levels were below the normal values. The mean CRP level was high in 90.6% of patients prior to the procedure. Approximately two-thirds of the patients received antibiotics for either prophylaxis or treatment for infections prior to the PEG procedure. Mortality was not related to the procedure in any of the patients. Buried bumper syndrome was the only major complication, and it occurred in the third year. In such case, the PEG was removed and a new PEG tube was placed *via* surgery. Eight patients (15.1%) experienced minor complications, 6 (11.1%) of which were wound infections. All wound infections except one recovered with antibiotic treatment. Two patients had bleeding from the PEG site, one was resolved with primary suturing and the other with fresh frozen plasma transfusion.

CONCLUSION: The incidence of major and minor complications is in keeping with literature. This finding may be noteworthy, especially in developing countries.

Key words: Gastrostomy; Gastric feeding tube; Enteral nutrition; Enteral feeding; Endoscopy; Gastrointestinal

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INTRODUCTION

Percutaneous endoscopic gastrostomy (PEG) has been used widely for the enteral feeding of patients who have a functioning gastrointestinal tract but are unable to consume adequate nutrition orally. Patients with cerebro-

vascular diseases, Parkinson's disease, dementia and head injury and those suffering from head and neck cancer and upper digestive tract cancer are candidates for PEG[1,2]. A PEG tube can be placed using one of four methods: push (Sachs-Vine), pull (Ponsky), introducer (Russell) or versa (t-fastener). The pull and push techniques are preferred because they offer greater safety and efficacy[3,4]. Both minor and major complications may occur during PEG placement. Major complications associated with PEG include peritonitis, gastric perforation, esophageal perforation, gastrocolocutaneous fistula, gastric outlet obstruction, necrotizing fasciitis and buried bumper syndrome. Minor complications include pneumoperitoneum, temporary ileus, hematoma, hemorrhage, wound infection, aspiration, tube dislodgement, gastroesophageal erosion, and gastric ulcer. Other gastrointestinal problems include gas distension, nausea, emesis, constipation and diarrhea[5-7]. In general practice, a PEG is placed by two endoscopists[1,8]. The aim of this study is to evaluate whether single endoscopist-performed PEG is safe and to compare the major and minor complications of PEG with those reported in the literature.

MATERIALS AND METHODS

This study was approved by the Baskent University Institutional Review Board and Ethics Committee (Project No: KA12/150) and supported by the Baskent University Research Fund. Patients who underwent PEG placement between June 2001 and August 2011 at the Baskent University Alanya Teaching and Research Center were evaluated retrospectively. Patients whose PEG was placed for the first time by a single endoscopist were enrolled in the study. For all patients, the PEG was placed using the "pull method". All of the patients were evaluated for indications for PEG, major and minor complications of PEG, nutritional status (prealbumin and albumin levels), C-reactive protein (CRP) levels and antibiotic treatment or antibiotic prophylaxis prior to PEG placement. Comorbidities and the rates, time and reasons for mortality were also evaluated, as were the reasons for PEG removal and the duration of PEG placement. The patients' first-degree relatives were telephoned and interviewed about the complications associated with the PEG and the patients' outcomes.

In our medical center, the standard PEG procedure was performed by single endoscopist. Before the procedure, permission for PEG placement was obtained from the patients' first-degree relative. The procedure was performed in the intensive care unit. Lidocaine spray was administered to the throat for local anesthesia. Midazolam and/or propofol-based sedation were administered intravenously by an anesthesiologist. An upper endoscopy was performed at the beginning of the procedure to exclude severe gastric ulceration, varices and outlet obstruction. After the stomach was insufflated with air through scope, the best location for the PEG placement was determined by pressing a finger slightly against the abdominal wall. The best location was indicated by the clear indentation of the finger observed inside the stomach on the greater curvatures and the illumination of the abdominal wall (Figure 1). The nurse was then given the scope. The sterile-dressed endoscopist cleaned the abdominal wall using a povidone-iodine solution. A one-centimeter incision was made after local anesthetic was applied to the planned location. The nurse filled the patient's stomach with air, and then the endoscopist inserted the needle of the PEG set through abdominal wall into the fully insufflated stomach. After removing the trocar, the endoscopist passed the guide wire through the needle. The nurse then caught the guide wire by the snare which was inserted through the endoscope, the endoscopist then withdrew the guide wire and the endoscope from the patient's mouth. After the endoscopist redressed, attached the guide wire to the PEG tube and the wire was pulled out of the abdominal wall, moving the PEG tube down the esophagus. Control endoscopy was performed to optimally place the PEG tube tip. The PEG tube was turned to locate the appropriate position and was fixed with an external device, leaving a 5-mm distance between the external device and the abdominal wall. This site was cleaned with povidone-iodine solution and dressed with gauze. Enteral feeding began 24 h after the procedure and ensuring that no local wound infection was present. The patient was inspected for erythema, induration and discharge at the PEG site and was assessed using the scoring system developed by Jain *et al*[9] for PEG infection. The patient was also followed by the nutrition team for other complications and nutritional status until discharge. The patient's family was asked to inform the nutrition team about possible complications.

Figure 1 The best location was indicated by the clear indentation of the finger observed inside the stomach and the illumination of the abdominal wall.

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences software program (Version 11.0, SPSS Inc., Chicago, IL, United States).

RESULTS

Between June 2001 and August 2011, a total of 82 patients underwent PEG placement. Twenty patients un-

Table 1 Demographic and laboratory characteristics of the study subjects *n* (%)

Age, yr	69.9 ± 16.3
Gender, M:F	30 (55.6):24 (46.3)
Comorbidities	
Diabetes mellitus	19 (35.2)
Chronic obstructive pulmonary disease	6 (11.1)
Coronary artery disease	11 (20.4)
Cardiac arrhythmia	4 (7.4)
Hypertension	20 (37.0)
Chronic renal failure	3 (5.6)
Hyperlipidemia	2 (3.7)
Other[1]	4 (7.4)
Antimicrobial therapy prior to PEG	40 (74.1)
Laboratory findings	
Leukocytosis (> 11 kg/mm^3)	22 (40.7)
CRP elevation (> 8 mg/dL)	48 (90.6)
High CRP (> 80 mg/dL)	22 (41.5)
Low albumin levels (< 3.5 g/dL)	40 (76.9)

[1]Hydrocephaly, breast cancer, meningioma, chronic liver disease. M: Male; F: Female; PEG: Percutaneous endoscopic gastrostomy; CRP: C-reactive protein.

derwent PEG replacement and were excluded from the study. Sixty-two patients underwent the PEG procedure for the first time. Eight of these procedures were performed by 2 endoscopists and were excluded from the study. A total of 54 patients were enrolled in the study. Indications were cerebral infarct in 39 patients (72.2%), cardiac arrest and cerebral ischemia in 4 patients (7.4%), dementia in 7 patients (12.9%), head trauma in 3 patients (5.6%), and cancer in 1 patient (1.9%).

Of the patients whose PEG was placed for the first time, 24 (46.3%) were women and 30 (55.6%) were men. The mean age was 69.9 years. The comorbidities accompanying the patients' primary disease were hypertension, diabetes mellitus, cardiac arrhythmia, coronary artery disease, chronic obstructive pulmonary disease, chronic renal disease, hyperlipidemia and hydrocephaly. The mean albumin levels were 3.04 ± 0.7 g/dL, and 76.2% were below normal values. The mean CRP level was high in 90.6% of patients prior to the procedure (Table 1). In our study, 74.1% of the patients received antibiotics either for prophylaxis or for treatment for infections prior to the PEG procedure. The demographic, clinical and laboratory characteristics of the study subjects are shown in Table 1.

After hospitalization, the mean time past until PEG placement was 22 ± 15.6 d. Buried bumper syndrome was the only one major complication (1.6%), and it occurred in the third year in one patient. In that case, the PEG was removed, and a new PEG tube was placed surgically. Eight patients (15.1%) experienced minor complications, 6 (11.1%) of which were wound infections and 2 of which (3.7%) were bleeding. All wound infections except for 1, which resulted in the removal of the PEG, recovered with antibiotic treatment. Two patients experienced bleeding from the PEG site; one patient was receiving anticoagulation therapy. One case resolved with primary suture, and the other resolved with fresh frozen plasma transfusion.

The first-degree relatives of all of the patients were interviewed by phone. The family members of 6 of the 54 PEG patients could not be reached by telephone, so we do not have long-term follow-up results for these patients. In our study, 1 mo survival was 85.4%, and three-month survival was 41.7%. Twenty-nine patients died during follow-up. The PEG indications for the patients who died were as follows: 14 had cerebral infarct, 3 had head trauma, 2 had cardiac arrest and cerebral ischemia and 1 had cancer. Mortality was not related to PEG placement in any of the patients and mainly depended on the underlying medical problems. The PEG tube was withdrawn in seven patients after they regained swallowing function and in one patient with an uncontrolled local wound infection. As of this writing, eleven patients live with the PEG tube, and 6 of them underwent PEG replacement during follow-up. To date, their relatives have not mentioned any problem related to the PEG in follow-up telephone interviews.

DISCUSSION

Although PEG is usually a safe procedure, certain complications can occur that may cause mortality, especially in patients with comorbidities. In our study, no mortality was associated with the PEG procedure. Buried bumper syndrome was the only major complication, and it occurred in only one patient (1.9%) in the third year of PEG placement. Minor complications occurred in 15.1% of patients, and most of these complications were wound infections.

Survival is an important endpoint in PEG studies. One-month survival is approximately 80% to 90% in most reports[10-12]. Similar to our study, the most frequent indication for PEG insertion was a neurological condition, and several studies reported that stroke was the most common indication[12-14]. In our study, one-month survival was 85.4%, and three-month survival was 41.7%. Buried bumper syndrome is an uncommon but severe complication of the procedure. It usually occurs after four months of PEG placement; however, it has also been reported to occur as late as 7 years after placement[15-17]. Rino *et al*[5] reported this complication as early as 5 d after the procedure. Finocchiaro *et al*[10] reported that one hundred twenty-eight patients were followed long-term for more than 31 d; major complications occurred in 3% of the patients, 2 of whom had buried bumper syndrome. Other major complications included 1 case of aspiration pneumonia and 1 case of subcutaneous abscess. In our study, buried bumper syndrome was the only observed major complication, and it occurred 3 years after the procedure. In the patient with buried bumper syndrome, the PEG tube was successfully surgically removed, and a new PEG tube was placed *via* the same procedure.

As in our study, the most common complication of

PEG was infection, which sometimes results in the removal of the PEG tube[18,19]. In a prospective study in which antibiotic prophylaxis was not given, the rate of peristomal infection was 33.6%[7]. Another study reported wound infections rates of up to 18%, and antibiotic prophylaxis was shown to reduce the rate to nearly 3%[19]. In a prospective, randomized, double-blind, placebo-controlled study by Jain *et al*[9], antibiotic prophylaxis with cefazolin was associated with decreased local PEG site infection. In a meta-analysis by Jafri *et al*[20], antibiotic prophylaxis before the PEG procedure was effective in reducing postprocedure local infection rates. In our study, 74.1% of the patients received antibiotics for either prophylaxis or the treatment of infections prior to the PEG procedure. The local wound infection rate was 11.1%, which is comparable to the rates reported by other studies in the literature. Only one patient developed a PEG site infection that did not resolve with antibiotic therapy; in this case, the PEG was removed. The other minor complication in our study was bleeding from the PEG puncture site, which occurred in two patients. One patient was treated with fresh frozen plasma, and the other was treated *via* primary suture of the abdominal wall vessel. Bleeding from the puncture site occurs as a result of a puncture of the abdominal wall vessel soon after the procedure. It can also be treated by tightening the outside apparatus of the PEG tube. Singh *et al*[21] reported that gastrointestinal bleeding after PEG placement occurred in 3.3% of patients, and bleeding directly attributed to PEG was noted in 0.4%.

Many factors contribute to PEG complications. The PEG tube placement team's experience, the PEG tube size, underlying malignancy and the institution in which the PEG procedure is performed are risk factors for wound infection. Low albumin levels and high CRP levels, age over 65 years and low BMI have also been associated with increased mortality risk[7,8,22-26]. In our study, the mean age was 69.9 years. High CRP levels were found in 41.5% of the patients, and low albumin levels were found in 76.9%. Although these unfavorable parameters existed prior to the procedure, there was no evidence of mortality related to PEG.

The nonrandomized and retrospective nature of our study are its restrictions. A prospective and randomized study might better define the safety and appropriateness of the single endoscopist-performed procedure.

In conclusion, the major and minor complications of single-endoscopist PEG are consistent with those reported in the literature for PEG procedures performed by two endoscopists. This finding may be noteworthy, especially in developing countries.

COMMENTS

Background

Percutaneous endoscopic gastrostomy (PEG) is lifesaving for patients who cannot feed orally for certain reasons. PEG is routinely placed by two endoscopists and carried inherent complications, some of which are life-threatening. It is not known whether PEG placement performed by a single endoscopist is safe or appropriate.

Research frontiers

The complications of PEG are significant. No study has reported the single-endoscopist PEG procedure or its related complications.

Innovations and breakthroughs

Although this study is retrospective and lacks the advantages of prospective and randomized trials, it provides important information indicating that PEG procedures can be applied by a single endoscopist, and the complications encountered are similar to those reported in other studies of PEG performed by two endoscopists.

Applications

Single endoscopist-performed PEG may be an appropriate and safe method for performing the procedure, especially in developing countries.

Peer review

This is a well-written retrospective study about the PEG procedure, which is performed here by a single endoscopist. The results show that it may be safe and appropriate for a single endoscopist to perform PEG. This study may lead to prospective and randomized trials in this field.

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BRIEF ARTICLE

Comparison of double pants with single pants on satisfaction with colonoscopy

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Abstract

AIM: To increase satisfaction and diminish anxiety and shame during colonoscopy, we developed novel double pants (NDP) which consist of doubled fabrics with an inner hole. The aim of study was to compare satisfaction, anxiety and shame between NDP and conventional single pants (CSP).

METHODS: Total 160 consecutive examinees were randomly divided into NDP and CSP group. Before colonoscopy, questionnaires identifying state and trait anxiety were completed. After colonoscopy, questionnaires for overall satisfaction (Group Health Association of America 9) and pants-specific satisfaction (5-20), state anxiety (20-80), and shame (6-24) were interviewed.

RESULTS: Pants-specific satisfaction scores regarding willingness to repeat colonoscopy using same pants (3.3 ± 0.8 *vs* 2.1 ± 0.9, $P < 0.001$) and recommendation of same pants to other people (3.3 ± 0.7 *vs* 2.0 ± 1.0, $P < 0.001$) were significantly higher in NDP than CSP groups. State anxiety (33.0 ± 7.0 *vs* 35.4 ± 6.9, $P = 0.028$) and shame (6.6 ± 1.5 *vs* 8.1 ± 3.2, $P = 0.001$) after colonoscopy was lower in NDP group compared with CSP group.

CONCLUSION: The NDP contribute to increase satisfaction and decrease anxiety and shame after colonoscopy.

Key words: Pants; Colonoscopy; Satisfaction; Shame; Anxiety

Core tip: We developed novel double pants (NDP) those are consisted of double fabrics with an inner hole. We compared the satisfaction, anxiety and shame between NDP and conventional single pants (CSP). The examinees in NDP group responded higher pants specific satisfaction, lower state anxiety after colonoscopy and lower shame score compared to those in CSP group. NDP developed in our institute may contribute to increase satisfaction and decrease anxiety and shame after colonoscopy.

Chung SH, Park SJ, Hong JS, Hwang JY, Lee SA, Kim KR, Lee HS, Hong SP, Cheon JH, Kim TI, Kim WH. Comparison of double pants with single pants on satisfaction with colonoscopy. *World J Gastroenterol* 2013; 19(26): 4177-4184 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4177.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4177

INTRODUCTION

Colonoscopy has recently increased in importance worldwide due to its use in screening for colon polyps and colorectal cancer[1]. Even in institutions where conscious sedated colonoscopy is available, colonoscopy without sedation is still performed due to patient comorbidities, the economic burden of sedation, and examinee preference. There is considerable global variation in the prevalence of sedative endoscopy[2]. Most colonoscopies in the United States are performed as sedated procedures, but some countries rarely use sedation in colonoscopy[3,4].

Consideration of the factors affecting satisfaction during colonoscopy has also recently increased because satisfaction may represent an important quality indicator for colonoscopy[5,6]. Moreover, the satisfaction of examinees may be reduced by anxiety, shame, discomfort, and embarrassment[7-11]. Many factors have been identified which determine satisfaction, anxiety, and shame, including the circumstance of the endoscopy room, the clothing for the procedure, endoscopist's skill, and the unfamiliarity of the medical staff with whom examinees interact during the procedure.

During colonoscopy at our center, examinees previously wear conventional single pants (CSP), where there is no hole for insertion of the scope. Examinees remove the CSP to below the level of the buttocks and expose their buttocks area during colonoscopy. Exposing the buttocks during colonoscopy can make examinees feel shameful and anxious, which can diminish their satisfaction with the colonoscopy. To decrease the shame and anxiety induced by exposing the buttocks of examinees wearing CSP during colonoscopy, we developed novel double pants (NDP). Examinees wearing NDP can undergo colonoscopy without taking off the inner pants. The smaller area of the buttock exposed by using NDP could minimize shame and anxiety during colonoscopy. Therefore we hypothesized that NDP could decrease shame and anxiety and increase satisfaction. We aimed to assess satisfaction, anxiety, and shame of patients wearing NDP compared with patients wearing CSP during colonoscopy through a prospective randomized single-center study. We also investigated the factors associated with patient satisfaction, anxiety, and shame during colonoscopy.

MATERIALS AND METHODS

Participants and study design

The study included examinees over 20 years old who agreed to undergo colonoscopy without sedation in the endoscopy unit of Severance Hospital, Yonsei University College of Medicine, Seoul, South Korea from January 2012 and July 2012. All of included people were the patients who visited the clinic for routine health check-up or for evaluating their mild gastrointestinal symptoms. Because we tried to minimize the selection bias of tertiary referral medical center, we excluded the patients who were referred by the physicians of primary or secondary medical center and needed more special care due to their objective medical problems. Before they signed at written informed consent, the nurse in outpatient clinic explained the purpose of the study. They got the information about not only the purpose of the study, but also the study design, the types of pants, the possible adverse events of procedure, and the contents of interview. Examinees under 20 years of age, those who had undergone colonoscopy within the past three years, those who were treated with an emergent colonoscopic procedure, those with a history of bowel resection surgery, inflammatory bowel disease (IBD), cancer, colostomy, ileostomy, psychotic disease including depression, anxiety disorder, or obsessive compulsive disorder, women who were pregnant or lactating, illiterate patients, foreigners, or examinees with American Society of Anesthesiologists (ASA) Scores of ⩾ 3 were excluded. We used our preliminary survey data to perform a power calculation due to the lack of the prior published reports to guide this analysis. We calculated that a sample size of 80 participants was sufficient to detect an effect value of 0.5 (mean difference/common SD) at a significance level of 0.05% (two-sided) with 80% power and 20% drop-out rate. A total of 185 colonoscopy examinees were enrolled in this study between February 2012 and July 2012. Eight examinees were excluded because of colon cancer found during the colonoscopy, ten examinees were excluded because of psychologic disorder, three examinees were excluded because of a history of bowel resection, and four examinees were excluded because of ASA scores ⩾ 3, as shown in Figure 1. Finally a total of 160 consecutive examinees were randomly divided into NDP and CSP groups. Participants in this study were randomly assigned to a "CSP group" or a "NDP group" using a permuted four-block randomization method. Biostatistician made permuted four-block randomization table and calculated the numbers of participants. All random code was contained in a closed box. The nurse in outpatient clinic enrolled the examinees. Before colonoscopy, participants, outcome assessors, and care providers (endoscopists and nurses) were blinded to assignment to

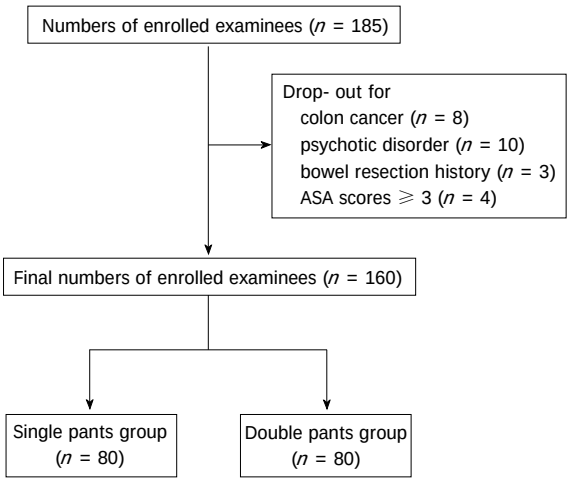

Figure 1 Consort diagram. ASA: American Society of Anesthesiologists.

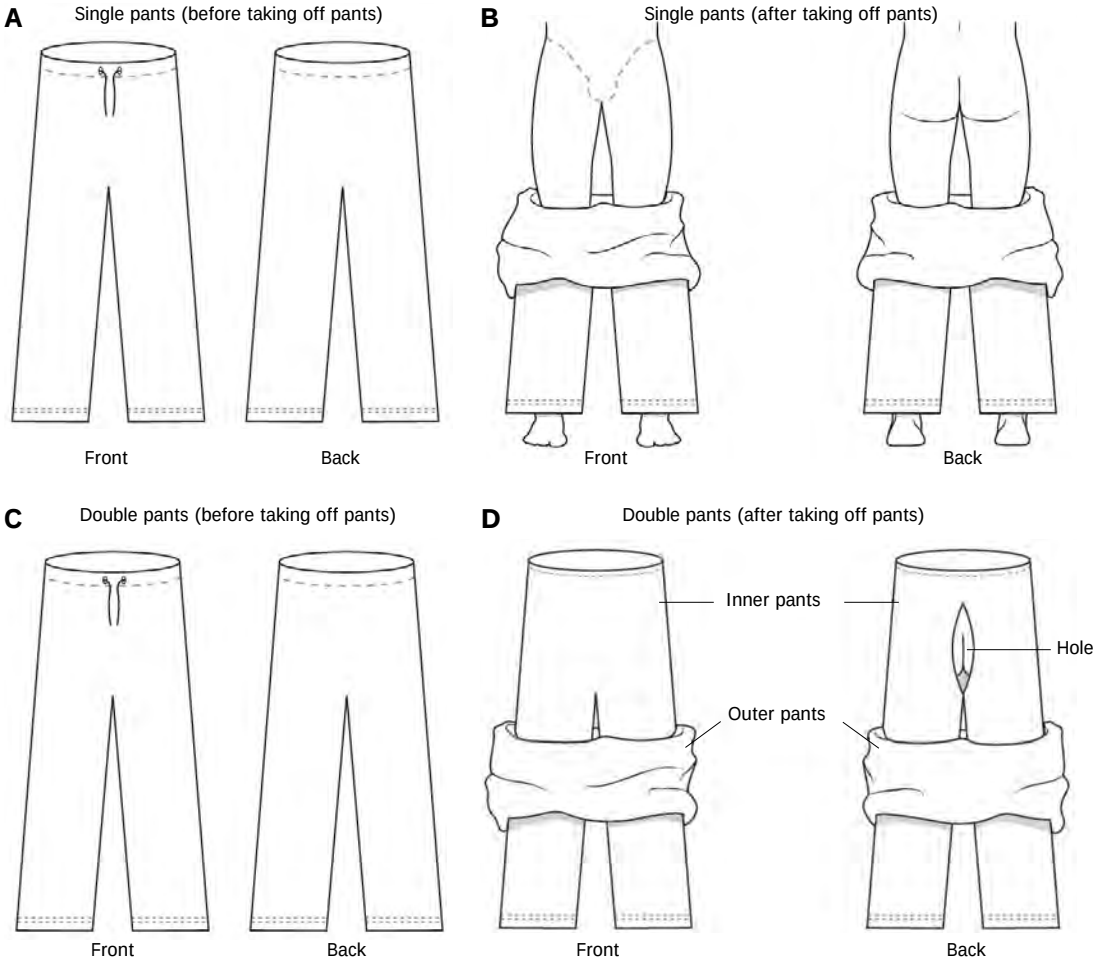

Figure 2 Drawings of the conventional single pants and novel double pants. A: The conventional single pants were composed of a single layer of fabric; B: If the examinees wearing conventional single pants dropped the outer pants below hips, the total area of the hip is exposed; C: The novel double pants were composed of a double layer of fabric from the hip to the thigh and a single layer of fabric below the thigh; D: In the inner layer of the double pants, there is a hole for insertion of scope in the back. If the examinees wearing novel double pants dropped the outer pants below hips, the only buttock area is exposed through the hole (25 cm × 15 cm) for insertion of scope.

the allocation because the third party examiner allocated the enrolled subjects regarding to the prepared permuted-block randomization table. Written informed consent was provided by all participants in the study.

Pants

In CSP, there is no hole for insertion of the scope. Examinees remove the CSP to below the level of the buttocks and expose their buttocks area during colonoscopy, as shown in Figure 2A and B. NDP consist of single fabric only below the thigh, and doubled fabric from the hip to the thigh with a hole in the inner pants at the level of the buttocks. The hole is 25 cm wide and 15 cm long. Examinees wearing NDP can undergo colonoscopy without taking off the inner pants, as shown in Figure 2C and D. We supplied the 80 NDP (Bobo trading, Seoul, South Korea) to NDP group and 80 CSP (Seodaemun uniform, Seoul, South Korea) to CSP group. All patients got the pants in the hospital just before the procedure according to the randomized allocation by the nurse in outpatient clinic. The pants had been prepared with marking "A type" (CSP) and "B type" (NDP).

Interview

In this study the only one nurse, as the third party examiner, interviewed the patients before and after the procedure to exclude interviewer's influence on the answers in this study. Before colonoscopy, examinees underwent a one-on-one interview in a quiet, separated room with a third-party examiner in which they completed questionnaires identifying state and trait anxiety, marital status, education, and residence. After colonoscopy, examinees had a similar one-on-one interview to complete the questionnaires, which included the Group Health Association of America 9 (GHAA 9) scales and questions regarding pant-specific satisfaction, state anxiety, and shame. After colonoscopy, examinees' pain during colonoscopy was assessed after the procedure and scored from 0 to 10 using a face pain scale (0-very happy, no pain, 2-hurts just a little bit, 4-hurts a little more, 6-hurts even more, 8-hurts a lot, 10-hurts as much as you can imagine; don't have to be crying to feel this much pain)[12].

Colonoscopy

Professional endoscopists who had performed more than 1000 colonoscopies performed all of the study colonoscopy procedures with a standard colonoscope (CF Q240L, CF Q240I, CF H260AI, CF Q260AI; Olympus Optical Co, Ltd, Tokyo, Japan). The indication for colonoscopy, ASA status, Ottawa quality scale, procedural time, number of polyps, number of examinees with polyps, method of polyp removal, rates of adverse events after polyp removal, rates of successful cecal approach, and gender difference between examinees and endoscopists in the NDP and CSP groups were investigated.

Outcome measurements

Satisfaction: The questionnaires measuring satisfaction included 14 questions regarding the examinees' colonoscopy experience. Nine of the questions regarding overall satisfaction were derived from a previously validated GHAA 9 satisfaction survey[13]. These questions used a 5-point scale to grade satisfaction (1: poor, 2: fair, 3: good, 4: very good, 5: excellent). A score of more than 3 was considered a favorable response. Five questions were asked to elicit pants-specific satisfaction as related to the following: difficulty of defecation wearing the pants; difficulty in position change during the colonoscopy; worriness about the exposed buttock area; willingness to try the same pants for the next exam; and recommendation of the same pants to other people. The five pants-specific satisfaction questions used a 4-point scale to grade satisfaction (1, not at all; 2, somewhat; 3, moderately so; 4, very much so). Three of the five questions were negative questions about satisfaction and thus scored in reverse. Scores on pants-specific satisfaction ranged between 5 and 20.

Anxiety: The trait anxiety questionnaire has been shown to reflect the general disposition of patients or stable tendency for anxiety, while the state questionnaire reflects a patient's anxiety related to a particular set of circumstances[9,14]. The trait and state questionnaires each consists of 20 statements, and all answers were graded using a 4-point scale (1, not at all; 2, somewhat; 3, moderately so; 4, very much so). Seven of the 20 questions from the STAI−Trait anxiety and 10 of the 20 questions from the STAI-State anxiety were negative questions and scored in reverse. The scores of the trait and state questionnaire range between 20 and 80, and higher scores reflected higher anxiety.

Shame: Among the experienced shame scale, 4 questions were used to measure body shame during colonoscopy[15]. We designed another 2 questions to measure shame following the exposure of the body during position change for the colonoscopy and exposure of the buttock area while walking to the colonoscopy room. In total, 6 different questions were used to measure shame. All answers were graded using a 4-point scale (1, not at all; 2, somewhat; 3, moderately so; 4, very much so). Scores on the body shame questionnaire ranged between 6 and 24, and higher scores reflected higher level of shame.

Ethical considerations

Informed consent was obtained from each patient included in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008) as reflected in a prior approval by the institution's human research committee. The study protocol was approved by the ethical committee of Yonsei University College of Medicine. The study protocol was also approved by clinicaltrial.gov (NCT 01524042).

Statistical analysis

All differences in CSP and NDP were examined using SPSS Statistics (version 18.0.0, IBM Corp., Armonk, NY, United States). Continuous variables were compared using Student's t test, while categorical data were analyzed using the χ^2 test (Fisher's exact test) between two groups. Univariate and multivariate linear regression analysis were used to assess independent predictive factors associated with satisfaction, state anxiety after colonoscopy, and shame. For multivariate analysis, variables with $P < 0.1$ by univariate analysis were included. All statistical tests were two-tailed and considered statistically significant with a P value < 0.05.

RESULTS

Baseline characteristics and endoscopic characteristics of the study population

There was no significant difference in baseline characteristics including age, gender, marital status, education, and residence in the study population and in the NDP and CSP groups in Table 1. There were no significant differences in the endoscopic characteristics of study population including indication for colonoscopy, Ottawa quality scale, procedural time, number of polyps, number of examinees with polyps, method of polyp removal, rates of adverse events after polyp removal, rates of successful cecal approach, pain scale, and gender difference between examinees and endoscopists in the NDP and CSP groups, as shown in Table 2.

Outcomes of satisfaction, state anxiety, trait anxiety and shame

There was no significant difference in GHAA9 score (Likert scale, 0-5) between the NDP and CSP groups. There were high favorable response rates (FRRs) which were greater than 90% for waiting time, waiting on procedure day, personal manner of physician and support staff, technical skills, adequacy of explanation, overall rating of the visit, and willingness to have the procedure repeated by the same physician and at the same facility in Table 3. In terms of pants-specific satisfaction in Table 3, the CSP group worried more about exposing the buttock area during the colonoscopy than did the NDP group (3.3 ± 0.7 *vs* 2.9 ± 0.7, $P < 0.001$). The NDP group was more willing to wear same pants when they undergo their

Table 1 Baseline characteristics of study population *n* (%)

	CSP (*n* = 80)	NDP (*n* = 80)	*P* value
Age (yr)	59.1 ± 11.4	59.4 ± 11.7	0.847
20 ⩽ age < 40	3 (3.8)	5 (6.2)	
40 ⩽ age < 60	38 (47.5)	43 (53.8)	
age ⩾ 60	39 (48.7)	32 (40)	
Gender			0.210
Male	41 (51.3)	45 (56.3)	
Female	39 (48.7)	35 (43.7)	
Marital status			> 0.999
Married	75 (93.8)	76 (96.0)	
Single or divorced	5 (6.2)	4 (4.0)	
Education			0.320
Middle school	20 (25.0)	13 (16.2)	
High school	31 (38.8)	28 (35.0)	
University	22 (27.5)	32 (40.0)	
Graduate school	7 (8.7)	7 (8.8)	
Residence			0.609
Urban	73 (91.3)	70 (87.5)	
Rural	7 (8.7)	10 (12.5)	
ASA status			0.896
Class Ⅰ	32 (40)	36 (45.0)	
Class Ⅱ	48 (60)	44 (55.0)	
Class Ⅲ	0 (0.0)	0 (0.0)	
Class Ⅳ	0 (0.0)	0 (0.0)	
Class Ⅴ	0 (0.0)	0 (0.0)	

CSP: Conventional single pants; NDP: Novel double pants; ASA: American Society of Anesthesiologists.

Table 2 Endoscopic characteristics of study population *n* (%)

	CSP (*n* = 80)	NDP (*n* = 80)	*P* value
Indication of colonoscopy			0.260
Abnormality on other study	2 (2.5)	4 (5.0)	
Stool occult blood positive	9 (11.2)	13 (16.2)	
Screening	47 (58.8)	42 (52.5)	
Anemia	0.0 (0)	1.0 (1.2)	
Diarrhea	3(3.8)	4 (5.0)	
Surveillance after polyp removal	6 (7.5)	11 (13.8)	
Abdominal pain	13 (16.2)	5 (6.3)	
Ottawa Quality Scale	3.9 ± 2.5	4.3 ± 2.8	0.382
Procedural time (min)			
Insertion	10.7 ± 8.5	8.6 ± 5.5	0.059
Withdrawal	12.8 ± 6.2	11.8 ± 5.5	0.309
Number of polyp	1.1 ± 1.8	0.8 ± 1.5	0.266
Number of examinees with polyps	47 (58.8)	42 (52.5)	0.525
Method of polyp removal			0.521
Biopsy	25 (52.1)	22 (56.4)	
Snaring polypectomy	15 (31.3)	8 (20.5)	
Endoscopic mucosal resection	8 (16.6)	9 (23.1)	
Adverse events			
Bleeding	0 (0.0)	0 (0.0)	-
Perforation	0 (0.0)	0 (0.0)	-
Cecal approach	80 (100)	80 (100)	-
Pain scale[1]	3.5 ± 2.7	3.7 ± 2.4	0.719
Genders between examinee and endoscopist			0.525
Same gender	42 (52.5)	47 (58.8)	
Different gender	38 (47.5)	33 (41.2)	

Data are expressed as absolute numbers (percentage) or mean ± SD. [1]Examinees' pain during colonoscopy was assessed after the procedure and scored from 0 to 10 using a face pain scale (0-very happy, no pain, 2-hurts just a little bit, 4-hurts a little more, 6-hurts even more, 8-hurts a lot, 10-hurts as much as you can imagine; don't have to be crying to feel this much pain). CSP: Conventional single pants; NDP: Novel double pants.

next colonoscopy than the CSP group (3.3 ± 0.8 *vs* 2.1 ± 0.9, $P < 0.001$). The NDP group was also more willing to recommend other people wear the same pants when they undergo their own colonoscopies than the CSP group (3.3 ± 0.7 *vs* 2.0 ± 1.0, $P < 0.001$). A significantly lower shame score was estimated in the NDP group compared with CSP group (6.6 ± 1.5 *vs* 8.1 ± 3.2, $P < 0.001$), which is shown in Table 3.

Predictive factors for satisfaction, state anxiety, and shame after colonoscopy

To investigate the predictive factors related to satisfaction, state anxiety, and shame after colonoscopy, univariate and multivariate regression analysis was performed (Table 4). In the multivariate analysis of pants-specific satisfaction, unmarried examinees had less pants-specific satisfaction than married examinees [B (SE) = -1.82 (0.61), $P = 0.004$], and the NDP group had higher pants-specific satisfaction than the CSP group [B (SE) = 2.72 (0.28), $P < 0.001$]. In multivariate analysis of state anxiety after the colonoscopy, female participants had higher state anxiety score after the procedure than males [B (SE) = 3.52 (1.14), $P = 0.002$]. Unmarried examinees had higher state anxiety score after colonoscopy than married examinees [B (SE) = 4.45 (2.22), $P = 0.047$]. Urban examinees had higher state anxiety score after colonoscopy than rural examinees [B (SE) = 4.68 (1.70), $P = 0.007$]. The NDP group had a tendency of lower state anxiety score after colonoscopy than the CSP group [B (SE) = -1.80 (1.04), $P = 0.086$]. In the multivariate analysis of shame, female examinees had higher shame score than male examinees [B (SE) = 1.25 (0.37), $P = 0.001$]. Unmarried examinees also had higher shame score than married examinees [B (SE) = 2.78 (0.81), $P = 0.001$]. The NDP group had lower shame score than the CSP group [B (SE) = -1.37 (0.37), $P = 0.001$].

DISCUSSION

In this study, we compared the satisfaction, anxiety and shame between NDP and CSP with prospective randomized control trial. The examinees in NDP group responded with higher pants-specific satisfaction, lower state anxiety after colonoscopy, and lower shame scores compared to those in CSP group. Thus, the NDP developed at our institution may contribute to increased satisfaction and decreased anxiety and shame after colonoscopy.

Although there have been a wide range of studies regarding satisfaction[1,16-20] and anxiety[9,21-23] during colonoscopy, our study was unique because it specifically addressed colonoscopic pants and investigated the differences in emotional change by the type of colonoscopic pants participants wore. Colonoscopic pants were designed considering the maximization of the efficiency of colonoscopies and hygiene aspects. Various types of colonoscopic pants have been used at different institutions. In some centers, examinees wear CSP or pants with

Table 3 Comparison of satisfaction rated by Group Health Association of America 9 survey by favorable response rate and likert scale, pants specific satisfaction, state anxiety and shame after colonoscopy between novel double pants and conventional single pants

	FRR, *n* (%)			Likert scale (mean ± SD)		
	CSP	NDP	*P* value	CSP	NDP	*P* value
GHAA9						
Appointment wait time	73 (91.3)	72 (90.0)	> 0.999	3.7 ± 0.7	3.6 ± 0.9	0.351
Waiting on procedure day	74 (92.5)	76 (95.0)	0.746	3.8 ± 0.8	3.9 ± 0.8	0.649
Personal manner of physician	78 (97.5)	79 (98.8)	> 0.999	4.4 ± 0.6	4.4 ± 0.6	0.712
Technical skills of physician	74 (92.5)	76 (95.0)	0.746	4.1 ± 0.8	4.1 ± 0.7	0.564
Personal manner of support staff	77 (96.3)	78 (97.5)	> 0.999	4.3 ± 0.7	4.0 ± 0.5	0.546
Adequacy of explanation of what was done	74 (92.5)	78 (97.5)	0.276	4.0 ± 0.6	4.0 ± 0.5	0.902
Overall rating of visit	75 (93.8)	74 (92.5)	> 0.999	3.9 ± 0.6	3.9 ± 0.6	0.806
Yes/No questions						
Would have procedure by same physician: Yes	78 (97.5)	77 (96.3)	> 0.999			
Would have procedure at same facility: Yes	79 (98.8)	79 (98.8)	> 0.999			
Pants specific satisfaction						
Difficulty in defecation				3.7 ± 0.5	3.8 ± 0.4	0.106
Difficulty in position change				3.7 ± 0.5	3.8 ± 0.3	0.175
Worriness about exposing buttock area in procedure				3.3 ± 0.7	2.9 ± 0.7	< 0.001
Will to wear same pants at next colonoscopy				2.1 ± 0.9	3.3 ± 0.8	< 0.001
Will to recommend to other people to wear same pants				2.0 ± 1.0	3.3 ± 0.7	< 0.001
Before colonoscopy						
Total scores of trait anxiety				39.2 ± 8.8	39.4 ± 8.2	0.904
Total scores of state anxiety				37.9 ± 8.2	39.9 ± 8.8	0.144
After colonoscopy						
Total scores of state anxiety				35.4 ± 6.9	33.0 ± 7.0	0.028
Total scores of shame				8.1 ± 3.2	6.6 ± 1.5	< 0.001
Change of state anxiety before and after the procedure				-2.4 ± 7.6	-6.9 ± 8.4	0.001

The scores on pants-specific satisfaction ranged between 5 and 20, and the scores of the trait and state anxiety questionnaire range between 20 and 80. The scores on the body shame questionnaire ranged between 6 and 24. A score of more than 3 of Likert scale was considered a FRR (Excellent: 5; Very Good: 4; Good: 3; Fair: 2; Poor: 1). Likert scale: Excellent: 5; Very Good: 4; Good: 3; Fair: 2; Poor: 1. Scores of pants specific satisfaction, Not at all: 1; A little: 2; Moderately: 3; Very much: 4. CSP: Conventional single pants; NDP: Novel double pants; GHAA9: Group Health Association of America 9; FRR: Favorable response rate.

Table 4 Univariate and multivariate analysis of pants specific satisfaction after colonoscopy

Predictive factors	Outcome (pants specific satisfaction)				Outcome (state anxiety after colonoscopy)				Outcome (shame after colonoscopy)			
	Univariate		Multivariate		Univariate		Multivariate		Univariate		Multivariate	
	B (SE)	*P* value	B (SE)	*P* value	B (SE)	*P* value	B (SE)	*P* value	B (SE)	*P* value	B (SE)	*P* value
Sex												
Male	Ref.				Ref.		Ref.		Ref.		Ref.	
Female	0.04 (0.36)	0.913			4.29 (1.06)	0.913	3.52 (1.14)	0.002	1.31 (0.40)	0.001	1.25 (0.37)	0.001
Age	-0.02 (0.01)	0.104			0.01 (0.04)	0.104			-0.01 (0.01)	0.928		
Marital status												
Married	Ref.		Ref.		Ref.		Ref.		Ref.		Ref.	
Unmarried	-1.6 (0.7)	0.033	-1.82 (0.61)	0.004	4.48 (2.40)	0.033	4.45 (2.22)	0.047	2.79 (0.87)	0.002	2.78 (0.81)	0.001
Education												
Middle school	Ref.				Ref.				Ref.			
High school	-0.04 (0.49)	0.931			-1.81 (1.53)	0.931			-0.01 (0.57)	0.998		
University	0.27 (0.50)	0.506			-2.64 (1.55)	0.506			0.22 (0.58)	0.697		
Graduate school	0.57 (0.73)	0.430			-2.50 (2.24)	0.430			0.15 (0.84)	0.853		
Type of pants												
CSP	Ref.		Ref.		Ref.		Ref.		Ref.		Ref.	
NDP	2.70 (0.29)	< 0.001	2.72 (0.28)	< 0.001	-2.43 (1.10)	< 0.001	-1.80 (1.04)	0.086	-1.47 (0.39)	< 0.001	-1.37 (0.37)	0.001
Residence												
Rural area	Ref.				Ref.		Ref.		Ref.			
Urban area	-0.46 (0.58)	0.430			4.2 (1.7)	0.430	4.68 (1.70)	0.007	0.93 (0.67)	0.165		
Genders between examinee and endoscopist												
Same gender	Ref.				Ref.		Ref.		Ref.			
Different gender	-0.36 (0.36)	0.308			3.90 (1.07)	< 0.001	1.45 (1.15)	0.209	0.65 (0.41)	0.115		

CSP: Conventional single pants; NDP: Novel double pants.

a hole in the buttock area with a movable flap, which can potentially expose buttock area during walking. In other centers, examinees wear single pants with hole in the buttocks and outer gown to hide the hole, which is likely inferior to the NDP we describe in terms of repair and maintenance expenses.

In our study there was no significant difference in GHAA 9 patient satisfaction between NDP and CSP groups as the contents of GHAA 9 were not closely connected with satisfaction regarding colonoscopic pants. However, in the survey of pants-specific satisfaction, the NDP group showed increased satisfaction than the CSP group in terms of their willingness to wear the same pants in the next time and to recommend the same pants to other people. High patient satisfaction during colonoscopy may result in a higher rate of compliance with screening and clinical surveillance programs[1]. The American Society for Gastrointestinal Endoscopy and the American College of Gastroenterology recommends assessment of patient satisfaction during colonoscopy to evaluate the quality of colonoscopy[5,6].

The sensitivity related to the psychological aspects including anxiety and shame might partly depends on the different characteristics including age, gender, behavior, social environment and sensitivity of the society. As example lower anxiety scores was reported to be associated with older age, male sex, lower income, experience of previous colonoscopy and lower education[9]. During gastrointestinal endoscopic procedures, female examinees have been reported to have higher state and trait anxiety levels than male examinees[10,22]. In our study, female, unmarried, urban examinees and CSP group had more state anxiety after colonoscopy than that of their male, married, rural examinees and NDP group counterparts. The differences of shame and state of anxiety after colonoscopy between the two groups were moderate in most of the items. But, the difference in change of state of anxiety before and after the procedure might be clinically significant. Because all participants are healthy persons in psychological aspect at baseline, even moderate change of anxiety level after colonoscopy could result in decreased satisfaction of the procedure and reduced compliance to next examinations in clinical practice.

There were some limitations to our study. Endoscopists could not be completely blinded to the types of colonoscopic pants worn because they ultimately saw which pants they were wearing during the colonoscopic procedure. However, the outcome assessors were blinded during the total period of study. In addition, all the endoscopists who performed the colonoscopy were excluded from the outcome assessors. And there was no questionnaire for endoscopists in this study. Examinees with cancer or IBD were suspected to have higher anxiety levels during colonoscopy, and thus were excluded. Therefore understanding the satisfaction, anxiety, and satisfaction of these particular examinees would require further investigation. Most of examinees in this study were over 40 years old and more than half of examinees underwent colonoscopy for screening purposes; the number of young unmarried examinees was very small in this study, and the level of anxiety, shame and satisfaction in this group may be significantly different. For a more ideal comparision of anxiety, shame and satisfaction during colonoscopy between the NDP and CSP group, it would be better for one examinee to wear single pants and double pants during colonoscopy, but this was not logistically feasible. To compensate for this limitation in state anxiety we measured the change in state anxiety before and after colonoscopy in the same examinees.

In conclusion, the examinees in the NDP group had higher pants-specific satisfaction and lower state anxiety and lower shame after colonoscopy compared to CSP group. Therefore NDP could help to increase satisfaction and decrease anxiety and shame after colonoscopy. Future studies should continue to investigate factors for anxiety, shame and satisfaction.

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COMMENTS

Background

Exposing buttocks during colonoscopy can make examinees feel unsatisfied, anxious, and shameful. To increase satisfaction and diminish anxiety and shame during colonoscopy, the authors developed novel double pants (NDP) which consist of doubled fabrics with an inner hole.

Research frontiers

Pants-specific satisfaction scores regarding willingness to repeat colonoscopy using same pants and recommendation of same pants to other people were significantly higher in NDP than conventional single pants (CSP) groups. State anxiety and shame after colonoscopy was lower in NDP group compared with CSP group.

Innovations and breakthroughs

Although there have been a wide range of studies regarding satisfaction and anxiety during colonoscopy, this study was unique because it specifically addressed colonoscopic pants. The authors developed NDP, which consist of single fabric only below the thigh, and doubled fabric from the hip to the thigh with a hole in the inner pants at the level of the buttocks. Examinees wearing NDP can undergo colonoscopy without taking off the inner pants.

Applications

Through these findings, the NDP developed at our institution may contribute to increased satisfaction and decrease anxiety and shame after colonoscopy.

Terminology

NDP are single fabric only below the thigh, and doubled fabric from the hip to the thigh with a hole in the inner pants at the level of the buttocks. The hole is 25 cm wide and 15 cm long.

Peer review

Satisfaction studies are important in relationship with the compliance of colorectal cancer screening programs and less so in the group of patients with specific suspicion of diseases affecting the anus, rectum or colon. This complex study is well designed and analyzed. The topic of the manuscript is interesting and the work performed is ambitious.

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BRIEF ARTICLE

Cap polyposis: A rare cause of rectal bleeding in children

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Abstract

AIM: To evaluate the clinicopathological features and treatment outcomes of cap polyposis in the pediatric population.

METHODS: All pediatric patients with histologically proven diagnosis of cap polyposis were identified from our endoscopy and histology database over a 12 year period from 2000-2012 at our tertiary pediatric center, KK Women's and Children's Hospital in Singapore. The case records of these patients were retrospectively reviewed. The demographics, clinical course, laboratory results, endoscopic and histopathological features, treatments, and outcomes were analyzed. The study protocol was approved by the hospital institutional review board. The histological slides were reviewed by a pediatric histopathologist to confirm the diagnosis of cap polyposis.

RESULTS: Eleven patients were diagnosed with cap polyposis. The median patient age was 13 years (range 5-17 years); the sample included 7 males and 4 females. All of the patients presented with bloody stools. Seven patients (63%) had constipation, while 4 patients (36%) had diarrhea. All of the patients underwent colonoscopy and polypectomies (excluding 1 patient who refused polypectomy). The macroscopic findings were of polypoid lesions covered by fibrinopurulent exudates with normal intervening mucosa. The rectum was the most common involvement site (n = 9, 82%), followed by the rectosigmoid colon (n = 3, 18%). Five (45%) patients had fewer than 5 polyps, and 6 patients (65%) had multiple polyps. Histological examination of these polyps showed surface ulcerations with a cap of fibrin inflammatory exudate. Four (80%) patients with fewer than 5 polyps had complete resolution of symptoms following the polypectomy. One patient who did not consent to the polypectomy had resolution of symptoms after being treated with sulphasalazine. All 6 patients with multiple polyps experienced recurrence of bloody stools on follow-up (mean = 28 mo).

CONCLUSION: Cap polyposis is a rare and under-recognised cause of rectal bleeding in children. Our study has characterized the disease phenotype and treatment outcomes in a pediatric cohort.

Key words: Cap polyposis; Polyps; Rectal bleeding; Pediatrics; Inflammatory bowel disease

Core tip: Cap polyposis is a rare and under-recognized condition with distinct clinical, endoscopic and histopathological features. All children with cap polyposis invariably present with rectal bleeding. Awareness of this diagnosis is important as its clinical and endoscopic features can mimic inflammatory bowel disease result-

ing in prolonged and inappropriate treatment. This article evaluates the clinicopathological features and treatment outcomes in a series of children with cap polyposis. Complete polypectomy should be performed where possible in combination with medical therapy. Prognosis is good for children with few polyps although recurrence rate is high in those with multiple polyps at diagnosis requiring further surgical intervention.

Li JH, Leong MY, Phua KB, Low Y, Kader A, Logarajah V, Ong LY, Chua JHY, Ong C. Cap polyposis: A rare cause of rectal bleeding in children. *World J Gastroenterol* 2013; 19(26): 4185-4191 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4185.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4185

INTRODUCTION

Cap polyposis (CP) is a rare and under-recognized condition with distinct clinical, endoscopic and histopathological features. It was first described by Williams *et al*[1] in 1985. CP is characterized by inflammatory polyps that are usually located from the rectum to the distal descending colon. Histologically, these polyps consist of elongated, tortuous, and often distended crypts covered by a "cap" of inflammatory granulation tissues. Macroscopic findings include dark red, sessile polyps that are commonly situated on the apices of transverse mucosal folds, with normal intervening mucosa.

Characteristic symptoms in adults include mucous diarrhea, tenesmus and rectal bleeding[2]. CP may be confused with other inflammatory conditions of the large intestine, in particular inflammatory bowel disease (IBD), due to their similarities in clinical and endoscopic features. The pathogenesis of CP is unknown, and no specific treatment has yet been established.

CP has been rarely described in the pediatric population. We report a case series of 11 pediatric patients diagnosed with CP and characterized their clinical, endoscopic, and histological features.

MATERIALS AND METHODS

All pediatric patients with histologically proven diagnosis of CP were identified from our endoscopy (total number = 1905) and histology database over a 12 year period from 2000-2012 at our tertiary pediatric center, KK Women's and Children's Hospital in Singapore. The case records of these patients were retrospectively reviewed. The demographics, clinical course, laboratory results, endoscopic and histopathological features, treatments, and outcomes were analyzed. The study protocol was approved by the hospital institutional review board (Singhealth Centralised Institutional Review Board). The histological slides were reviewed by a pediatric histopathologist to confirm the diagnosis of CP.

RESULTS

Patients

There were 11 pediatric patients diagnosed with cap polyposis from 2000 and 2012. The clinical features of these patients are summarized in Table 1. There were 7 males and 4 females, with a median age of 13 years (range 5-17 years). The racial distributions included 5 Malays, 4 Chinese, and 2 Indian patients.

Clinical features

Common presenting features of these patients included per-rectal bleeding, constipation and straining, diarrhea, and abdominal pain (Table 1). All 11 patients presented with blood in the stools. Seven patients (63%) had constipation and/or straining, and 4 patients (36%) had diarrhea. Abdominal pain was a presenting complaint in 6 (54%) patients. Digital rectal examinations revealed rectal polypoid masses in 7 (63%) patients and 1 patient had perianal fissure.

The median hematological values at diagnosis were hemoglobin 10.9 (IQR 12.6-14) g/dL, white cell counts 6.7 $\times$ 10^9/L (IQR 4-11)10^9/L and platelet counts of 342 (IQR 150-400) $\times$ 1000/μL. All of the patients had normal coagulation profiles. Eight of the 11 patients had normal serum albumin measured with a median value of 38 g/L (IQR 35-45). Only 2 patients (IDs 8 and 10) had hypoalbuminemia. Inflammatory markers C-Reactive protein/Erythrocyte Sedimentation Rate were measured in 6 patients (ID 2, 5, 7, 8, 9, and 10), and they were normal.

Endoscopic features and histology

All patients underwent colonoscopy with macroscopic findings of polyps or polypoid lesions. These were mainly small, red and sessile polyps covered by a thick layer of fibrinopurulent exudates predominantly found on the apices of the mucosa folds. The intervening mucosa was normal both macroscopically and on histological examination (Figure 1A and B). The polyps were most commonly located in the rectum only (n = 9, 82%). Two patients (18%) had polyps in the rectum and sigmoid colon. The number of polyps ranged from 1 to more than 10. Five (45%) patients had fewer than 5 polyps, and 6 (65%) patients had multiple (> 5) polyps on initial colonoscopy.

On histological examination, these colonic polyps showed a variable degree of surface ulceration associated with a cap of fibrin inflammatory exudates and granulation tissue. Focally, the surface epithelium is preserved but attenuated. The crypts within the polyps showed crypt elongation and luminal epithelial serration. In some cases, the crypts were mildly distended towards the surface. The lamina propria contained a variably increased number of acute and chronic inflammatory cells (Figure 2). These histological features were consistent with inflammatory cap polyps. The mucosa surrounding these polyps was normal.

Three patients presented with abdominal pain and

Table 1 Clinical features and treatment outcomes of cap polyposis patients

ID	Age (yr)	Sex	Diarrhea	C + S	Abdo pain	PR bleeding	No. of polyps	Site	Antibiotics	Stool softeners	Recur	Follow up (mo)
1	5	M	No	No	No	Yes	1	R	No	Yes	No	24
2	13	M	No	Yes	No	Yes	4	R	No	Yes	No	4
3	8	M	NA	Yes	No	Yes	1	R	No	Yes	No	3
4	8	M	No	Yes	No	Yes	1	R	Metro	No	No	3
5	15	F	Yes	No	No	Yes	3	R	No	No	No	24
6	15	M	Yes	Yes	Yes	Yes	$n > 5$	R	Metro	Yes	Yes	36
7	10	M	Yes	No	Yes	Yes	$n > 5$	R	Metro	Yes	Yes	5
8	11	F	No	Yes	Yes	Yes	$n > 5$	R	Metro	Yes	Yes	72
9	13	F	No	Yes	Yes	Yes	$n > 5$	R	No	Yes	Yes	72
10	14	F	No	Yes	Yes	Yes	$n > 5$	R + S	No	Yes	Yes	36
11	17	M	Yes	No	No	Yes	$n > 5$	R + S	No	Yes	NA	Lost
												Mean = 28

C + S: Constipation or straining; R: Rectum; S: Sigmoid; Metro: Metronidazole; M: Male; F: Female.

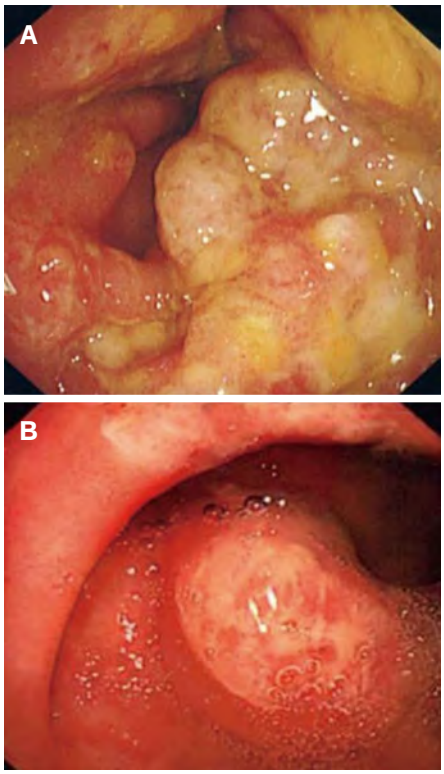

Figure 1 Colonoscopy images of 2 patients with cap polyposis. A: Colonoscopy image of a patient showing multiple small sessile polypoid lesions with mucous exudates of cap polyposis (CP) in the rectum; B: Colonoscopy image of a patient showing a single sessile red polypoid lesion located on the transverse folds with normal intervening mucosa.

underwent simultaneous upper gastrointestinal (GI) endoscopies; the histological findings showed mild gastritis but no evidence of *Helicobacter pylori* (*H. pylori*). There were no polypoid lesions noted in the stomach of these 3 patients.

Management and outcomes

All but one patient underwent polypectomies. Patients 1-5 had fewer than 5 polyps detected by the initial colonoscopy. Patients 1-4 had polypectomy performed and were subsequently treated with stool softeners. Patient 4 also received a course of metronidazole. All 4 of these patients had complete resolution of their symptoms with no further rectal bleeding at mean follow-up period of 28 mo. Patient 5 had 3 small sessile polyps and did not consent for polypectomy but had complete resolution of symptoms at 18 mo after being treated with sulphasalazine.

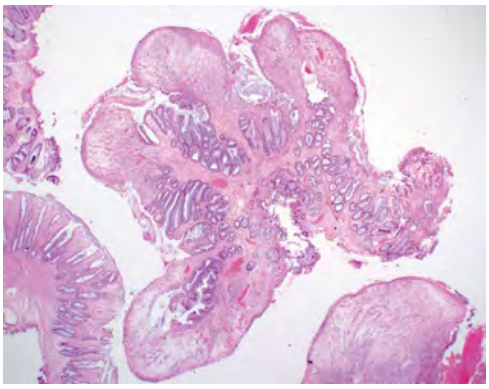

Figure 2 Histology of a sessile colonic polyp from a patient with cap polyposis. The polyp was comprised of granulation tissue and focally distended crypts with a slightly serrated luminal surface. Surface ulceration with fibrinomucoid exudates was also present.

Six patients with multiple polyps (Patients 6-11) underwent colonoscopy and polypectomies. One patient was lost to follow-up (Patient 11). The other 5 patients were given stool softeners and 3 patients were given metronidazole. All of the patients experienced a recurrence of symptoms, mainly blood in the stools at subsequent follow-up (mean follow-up period of 28 mo). These patients required repeated colonoscopies with polypectomies and continued to have intermittent per rectal bleeding. Patient 8 eventually had a resolution of his symptoms after 6 colonoscopies with multiple polypectomies at follow-up of 6 years. Patient 11, whose polyps were confined to the recto-sigmoid area, had persistent rectal bleeding that required recurrent blood transfusions and will require more extensive surgical resection in the future. All of the 5 patients with multiple polyps were otherwise well-thrived and had normal inflammatory markers at subsequent follow-up.

DISCUSSION

CP is a rare but distinct disorder with characteristic endoscopic and histological features first described by Williams[1]. Although CP was first described more than 20 years ago, this disease is still not well recognized by physicians. Only approximately 60 cases have been reported in the English language medical literature, mainly as case series or case reports. Due to its rarity, CP is often underrecognized and misdiagnosed as inflammatory bowel disease IBD, leading to prolonged and inappropriate treatment.

To our knowledge, this report is the first and largest case series describing CP in the pediatric population. Shimuzu *et al*[3] have previously described CP in a 12 year old girl. Previous adult studies have found that CP occurs more frequently in females than males[3]. In contrast, there were more boys than girls (7 males and 4 females) in our study, and their median age was 13 years old. We also noted a slightly higher proportion of Malay patients (approximately 40%) in our cohort.

Common presenting symptoms of previously described cases of CP include mucous and/or bloody diarrhea, habitual straining with defecation, chronic constipation and abdominal pain[4]. Rectal bleeding occurred universally in all 11 patients in our study. In total, 65% of our patients had chronic straining and/or constipation, while 35% of the patients had diarrhea. Almost half of our patients presented with abdominal pain, particularly those with multiple polyps. Anemia was a predominant feature in our cohort, with median Hb of 10.9 g/dL.

Protein-losing enteropathy has been reported to be associated with CP[5-9]. Shiomi *et al*[9] reported a case of CP in which protein loss from the lesions was confirmed *via* technetium 99m-labeled diethylenetriaminepentaacetic acid complexed to human serum albumin. There have also been reports of lower limb edema resulting from protein-losing enteropathy from CP[5,7-10]. In addition, laboratory investigations often reveal low total protein and serum albumin levels in CP patients. Symptoms of pre-tibial edema and low protein levels have been shown to normalize with resolution of cap polyposis[5,6-9]. None of the patients in our study presented with lower limb edema. Moreover, hypoalbuminemia was not a predominant feature in our cohort, with only 2 patients having documented low albumin levels.

Endoscopic and histological features

CP is characterized by polyps covered with fibrinopurulent exudates on the surface. The polyps range in size from several millimeters to as large as 7 cm[11,12]. The number of polyps varies from 1 to a few hundred, and the polyps are typically located at the apices of mucosal folds. These polyps have varying morphologies, including polypoid, ulcerative, and flat types[13]. The intervening mucosa has been described as normal or covered with white specks[13], although the significance of this morphology has yet to be ascertained. Initial edematous, flushed mucosa with subsequent development of polyps at the same area, has been reported with serial endoscopic studies[7,14]. The rectum and rectosigmoid colon are the most commonly affected sites, although pan-colonic and gastric involvement has been described[4,6,15]. In agreement with previous findings, all patients in our series had polyps localized to the rectum or rectosigmoid region with normal intervening mucosa.

Histologically, cap polyps consist of elongated, tortuous and hyperplastic crypts that are attenuated towards the mucosal surface. The surfaces of these polyps are ulcerated and covered by a thick layer of fibrinopurulent exudates, hence the term "cap polyps". The lamina propria also contain inflammatory cells[5]. These histological features were reported in our patients.

Role of abnormal colonic motility

The exact etiology and pathogenesis of inflammatory cap polyps are unclear. Various possible causes, including infection[6,14], mucosal ischemia[2], inflammation[16], abnormal bowel motility, and repeated trauma to the colonic mucosa caused by straining[8] have been proposed. Campbell *et al*[2] proposed that abnormal colonic motility may lead to prolapse of redundant mucosa at the apices of transverse mucosal folds. The resultant local ischemia produces characteristic histological appearances of fibromuscular obliteration of the lamina propria; superficial erosion associated with granulation tissue; and elongated, tortuous, hyperplastic glands. These histological features are also present in other conditions, such as prolapsing mucosal polyps, solitary rectal ulcers, inflammatory cloacogenic polyps, and gastric antral vascular ectasia. These findings, along with the fact that CP predominantly affects the rectosigmoid and has a circumferential involvement of the colonic mucosa, have led many researchers to attribute CP to abnormal colonic motility and repeated trauma to the colonic mucosa caused by straining during defecation. Hence, CP has been considered part of a spectrum of "mucosal prolapse syndromes"[5,17,18].

However, aberrant motility of the distal large bowel may only partially contribute to the development of cap polyposis. Although many CP patients present with a history of straining at defecation, these individuals usually lack a typical mucosal prolapse that involves the anterior wall of the lower rectum[18]. There have also been reports of CP developing in patients without evidence or history of abnormal colonic motility. Géhénot *et al*[14] and Konishi *et al*[16] reported the differentiating features between and mucosal prolapse syndrome, where there was significant thickening of the second layer on endoscopic ultrasound in cap polyposis, as opposed to smooth, diffuse thickening of the third layer and minimal thickening of the second layer in mucosal prolapse. Furthermore, avoidance of straining alone has not been reported as an effective treatment modality. In our cohort of patients, stool softeners were prescribed in 8 of 11 patients to avoid straining on defaecation but the symptoms still recurred in those with multiple polyps.

The role of inflammation and infection

In recent years, the role of infectious organisms, such as *H. pylori*[5,6,7,19,20], and inflammation[14] has been proposed. Three of our patients underwent an upper GI endoscopy, but none of them showed any evidence of *H. pylori* on in their gastric mucosa biopsies.

Konishi *et al*[21] described a patient in whom CP was noted to progress along the surgical anastomotic line after a laparoscopic sigmoid colectomy was performed, leading to the hypothesis that local inflammation plays a role in the development of cap polyposis. The effectiveness of infliximab in 2 case reports[22,23] also supports the role of inflammation in the pathogenesis of CP. However, molecular studies of the abnormal mucus in CP have proven inconclusive. Buisine *et al*[24] found a predominance of non-sulphated mucins in cap polyps, compared to both non-sulphated and sulphated mucins expressed in normal colonic mucosa. This finding has been associated with a wide range of pathological conditions, including colorectal carcinomas, ulcerative colitis, and familial polyposis, and there has been a lack of data to show a direct involvement of mucins in the initial pathogenesis of cap polyposis.

Treatment of cap polyposis

Different treatment modalities have been trialed including steroids, aminosalicylates, infliximab, *H. pylori* eradication, endoscopic and surgical resection with variable clinical outcomes. The clinical course of CP has been reported to range from spontaneous remission[19,22] to a disease course requiring surgical resection of the affected bowel segments.

Metronidazole has been used widely in the treatment of CP, often in combination with other modalities[5,7,8,25-27]. Shimizu *et al*[3] reported improvement of symptoms and stromal infiltration on colonic biopsies in a patient treated with metronidazole after failed treatment with mesalamine and levofloxacin, leading to the hypothesis that the role of metronidazole may be related to its anti-inflammatory effects rather than antibiotic action against specific pathogens. It has been postulated that by acting as a radical scavenger, metronidazole can inhibit leukocyte emigration and adherence[5]. In our series, 4 patients received a course of metronidazole together with polypectomy. Of these 4 patients, 3 patients had a recurrence of symptoms. This result suggests that metronidazole may have a limited role in the treatment of patients who have multiple cap polyps.

Limited reports of treatment with infliximab have yielded varying results[22,23,28]. Kim *et al*[26] reported a resolution of symptoms and endoscopic lesions after a single infusion of infliximab, but Maunory *et al*[27] reported a case of recurrence despite 2 infusions of infliximab.

The effective eradication of *H. pylori* has been reported to play a role in the treatment of CP. A review of the English language medical literature has identified 6 reports[6,7,15,19,20,25] in which 9 CP patients were treated with *H. pylori* eradication treatment, 8 of whom showed a complete resolution of symptoms and cap polyps (88.9%) and 1 patient experienced a partial improvement of symptoms. Interestingly, all 9 patients tested positive for *H. pylori*, and the patient who showed only a partial improvement in symptoms had persistent *H. pylori* infection despite eradication therapy.

The possible role of *H. pylori* in extragastric diseases, including idiopathic thrombocytopenic purpura, iron deficiency anemia, chronic urticaria and ischemic heart disease, has been suggested[28-30]. Various mechanisms, including the release of inflammatory mediators, molecular mimicry and a systemic immune response, have been postulated[20]. Although *H. pylori* has not been detected in the colonic mucosae of CP patients, these results suggest that *H. pylori* infection may indirectly play a part in the etiology of CP. Similar histological features between Menetrier's disease (in which *H. pylori* has been postulated to play a role) and CP, such as elongated tortuous crypts, have been highlighted by Akamatsu *et al*[7]. Testing for *H. pylori* in all CP cases and subsequent eradication therapy, if necessary, has been recommended by various authors[7,20]. In our series of pediatric patients, 3 patients underwent upper endoscopy, but none of these patients had *H. pylori* detected in their gastric mucosal biopsies. One patient received triple therapy but continued to have recurrence of symptoms. The role of *H. pylori* eradication therapy needs to be further evaluated in the pediatric population.

Steroids and aminosalicylates have been used with varying results[8,25]. Chang *et al*[25] reported a series of 7 patients, 2 of whom maintained clinical response after 2 courses of systemic steroids: Symptoms persisted in 1 patient despite 2 courses of steroids and aminosalicylates; 3 patients experienced spontaneous remission, and 1 patient showed partial improvement after *H. pylori* eradication therapy. None of our patients received steroid therapy, although one patient was successfully treated with aminosalicylic acid. All of our patients for whom inflammatory markers were measured had normal values for these markers.

Polypectomy and surgical removal of the affected colon have produced inconsistent results, with Ng *et al*[4] reporting recurrence in 2 of 5 patients receiving polypectomy and recurrence in 2 of 4 patients who underwent surgical resection of the affected colon[7,31]. In our series, 10 of 11 patients underwent polypectomies. However, only half of these patients achieved complete remission at mean follow-up of 28 mo. These were patients with fewer than 5 polyps at presentation. Those patients with persistent symptoms despite polypectomies were more likely to have multiple polyps at presentation.

Clinical course

The clinical course and long-term prognosis of CP remain largely unknown. A self-limiting course has been reported, despite whether polypectomy or surgery has been performed[32,33]. A polypectomy will, however, provide a definitive histological diagnosis and may also be war-

ranted when the patient presents with significant lower gastrointestinal bleeding. A complete polypectomy was effective in several studies[4,5], and this approach is recommended whenever possible. Our findings suggest that patients with multiple polyps at diagnosis are more likely to experience symptom recurrence.

In conclusion, CP is a rare cause of rectal bleeding in children. Awareness of this diagnosis is important as the clinical and endoscopic features of CP can mimic Inflammatory Bowel Disease[32] and a misdiagnosis can result in prolonged and inappropriate treatment. CP polyps are distinctive inflammatory polyps covered by a cap of fibrinopurulent exudates normally located at the apices of the mucosal folds with normal intervening mucosa both macroscopically and on histological examination. Although the pseudopolyps in IBD have granulation tissues, the intervening mucosa is usually associated with inflammatory changes, such as superficial ulcerations, granularity and/or friability with crypt abscesses[34]. CP is mainly localized to the rectum and sigmoid, whereas the pseudopolyps in IBD may involve the entire colon. Clinically, CP patients are also more likely to have normal inflammatory markers with no extraintestinal manifestations, such as weight loss, oral ulcers, joint pain *etc*.

The clinical course of CP has not been well described. CP may in some instances, be a self-limiting condition. A complete colonoscopy should be performed as polyps have been described throughout the colon[35,36] when possible, a total polypectomy is recommended. Patients with predominant straining/constipation symptoms can be treated with laxatives and advised to avoid straining. Medical treatment including antibiotics (*e.g.*, metronidazole) and eradication therapy for *H. pylori* has been shown to be effective in some reports. There is currently no good evidence for using aminosalicylic acid or immunosuppressive therapy for treatment of CP. Surgical resection may be indicated if symptoms persist despite medical therapy, although recurrence has been described post-operatively.

In summary, CP is a rare cause of rectal bleeding in children. Awareness of this diagnosis is important as its clinical and endoscopic features can mimic inflammatory bowel disease and a misdiagnosis can result in prolonged and inappropriate treatment. Response to medical treatment has been shown to be inconsistent and unsatisfactory. Endoscopic or surgical excision can be curative, but the recurrence rates are high, particularly if numerous polyps are present. Longer term studies are necessary to understand the natural course of this condition.

COMMENTS

Background

Cap polyposis (CP) is a rare and under-recognized condition with distinct clinical, endoscopic and histopathological features first described by Williams *et al*. Little is known of CP in the pediatric population.

Research frontiers

The pathogenesis of CP is unknown and no specific treatment has been established. CP has been rarely described in the paediatric population. The research hotspot is to better define the clinical features and course of CP in children, and identify effective treatment modalities.

Innovations and breakthroughs

The study is the first case series in available literature to characterise the disease phenotypes and treatment outcomes in a group of paediatric patients.

Applications

Cap polyposis is a rare condition especially in children. It is commonly misdiagnosed as inflammatory bowel disease subjecting patients to unnecessary immunosuppressive therapy. The clinical course and long-term prognosis of cap polyposis remain largely unknown. A case series describing the treatment modalities and clinical course of CP in children will raise the awareness of this rare condition amongst paediatricians and gastroenterologists, as well as improve treatment outcomes.

Terminology

Cap polyposis is characterised by inflammatory polyps located in the rectum to distal descending colon. Histologically, these polyps consist of elongated, tortuous crypts covered by a "cap" of inflammatory granulation tissue.

Peer review

The manuscript is interesting and the main importance of the research is represented by the help offered to clinicians and endoscopists in recognizing and treating a rare pathology that could be misdiagnosed as Inflammatory Bowel Disease. These series of children demonstrate the importance of cap polyposis in young patients. Manuscript is well presented and well written.

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BRIEF ARTICLE

Transcatheter arterial chemoembolization followed by immediate radiofrequency ablation for large solitary hepatocellular carcinomas

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Abstract

AIM: To assess the technical safety and efficacy of transcatheter arterial chemoembolization (TACE) combined with immediate radiofrequency ablation (RFA) for large hepatocellular carcinomas (HCC) (maximum diameter ⩾ 5 cm).

METHODS: Individual lesions in 18 patients with HCCs (mean maximum diameter: 7.5 cm; range: 5.1-15.5 cm) were treated by TACE combined with percutaneous RFA between January 2010 and June 2012. All of the patients had previously undergone one to four cycles of TACE treatment. Regular imaging and laboratory tests were performed to evaluate the rate of technical success, technique-related complications, local-regional tumor responses, recurrence-free survival time and survival rate after treatment.

RESULTS: Technical success was achieved for all 18 visible HCCs. Complete response (CR) was observed in 17 cases, and partial response was observed in 1 case 1 mo after intervention. The CR rate was 94.4%. Local tumors were mainly characterized by coagulative necrosis. During follow-up (2-29 mo), the mean recurrence-free survival time was 16.8 ± 4.0 mo in 17 cases of CR. The estimated overall survival rate at 6, 12, and 18 mo was 100%. No major complications were observed. Levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the blood of 17 patients transiently increased on the third day after treatment (ALT 200.4 ± 63.4 U/L *vs* 24.7 ± 9.3 U/L, $P < 0.05$; AST 228.1 ± 25.4 U/L *vs* 32.7 ± 6.8 U/L, $P < 0.05$). Severe pain occurred in three patients, which was controlled with morphine and fentanyl.

CONCLUSION: TACE combined with immediate RFA is a safe and effective treatment for large solitary HCCs. Severe pain is a major side effect, but can be controlled by morphine.

Key words: Large hepatocellular carcinoma; Transcatheter arterial chemoembolisation; Radiofrequency ablation; Combination therapy; Synchronism

Core tip: Transcatheter arterial chemoembolization (TACE) immediately followed by radiofrequency ablation (RFA) under digital subtraction angiography-computed tomography is used to treat large hepatocellular carcinomas. This technology can improve the synergistic treatment effects of TACE and RFA, as well as reduce the need for repeated treatments and amount of radiation exposure. Furthermore, different treatment technologies are fused into one machine, thereby simplify-

ing the operational process. TACE immediately followed by RFA enhances tumor inactivation ability, decreases recurrence rates, prolongs patient survival time and improves prognosis.

Wang ZJ, Wang MQ, Duan F, Song P, Liu FY, Chang ZF, Wang Y, Yan JY, Li K. Transcatheter arterial chemoembolization followed by immediate radiofrequency ablation for large solitary hepatocellular carcinomas. *World J Gastroenterol* 2013; 19(26): 4192-4199 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4192.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4192

INTRODUCTION

Hepatocellular carcinoma (HCC), the sixth most malignant tumor worldwide, is the third most common tumor leading to death; unfortunately, only 10%-54% of all patients with HCC are suitable for surgery[1-3]. Transcatheter arterial chemoembolisation (TACE) is one of the modalities used to treat unresectable HCC; however, its low complete tumor necrosis rate results in tumor recurrence and metastasis and influences long-term efficacy[1,3-6]. In addition, the effect of TACE is influenced by tumor size which decreases inactivation ability, especially for HCCs with diameters larger than 5 cm[7,8]. Compared with TACE, the combination of TACE with radiofrequency ablation (RFA) shows enhanced efficacy against HCC and prolonged survival in patients[7-11]. RFA is usually performed 1-4 wk after TACE[3,11-13]. However, the combination of TACE with immediate synchronous RFA for unresectable and large HCCs has not yet been reported. We retrospectively summarized 18 patients treated between January 2010 and June 2012 to assess the technical safety and efficacy of TACE combined with immediate synchronous RFA as a treatment modality for HCC.

MATERIALS AND METHODS

Patients

This retrospective study was approved by the Ethics Committee of the People's Liberation Army (PLA) General Hospital, and all patients signed informed consent forms. A total of 18 patients were admitted to the Department of Interventional Radiology of the PLA General Hospital between January 2010 and June 2012 and were diagnosed with HCC by ultrasound, computed tomography (CT), magnetic resonance imaging (MRI) and α-fetoprotein (AFP) blood test or pathological examination according to the diagnostic criteria for HCC established by the National Association for the Study of Liver Cancer. All patients, including 16 males and 2 females, with an average age of 55 ± 8.0 years (47-63 years) underwent TACE followed immediately by synchronous RFA. The AFP level was higher than 20 ng/mL in 10 cases. On the basis of the Child-Pugh score, 13 cases were classified as Grade A and 5 cases were classified as Grade B.

Patients were allowed to receive the combination therapy if: (1) various imaging examinations (ultrasound, CT, MRI and intra-procedure imaging in TACE) showed one lesion in the liver with a maximum diameter larger than 5 cm and the patients had no surgical indications or refusal to surgery, and (2) the Child-Pugh score was Grade A or B. Patients were excluded from the treatment if they had: (1) cancer embolus in the main portal vein and its left and right portal veins, arteriovenous fistula formation, biliary invasion and extrahepatic metastases and (2) severe coagulation disorders, such as prothrombin activity < 40% and platelet concentration < 30×10^9/L. All the RFA lesions were located 1 cm away from the gall bladder, intestinal canal, bile duct and major blood vessels. All patients underwent regular physical examination and tests (routine blood test, hepatorenal function, electrolytes, blood coagulation and tumor markers), as well as other relevant examinations (liver CT, ultrasound or MRI, lung and brain CT and bone ECT). The maximum diameter of the lesion was determined on enhanced CT or MRI. TACE was performed in these 18 patients one to three times prior to the procedure.

Methods

TACE: All the interventional procedures were performed *via* INNOVA4100 IQ digital subtraction angiography (DSA) (GE Company, United States) by an interventional radiologist with 8-10 years of experience at the Department of Interventional Radiology. After the right femoral artery was punctured by Seldinger's technique, a 4F catheter (RH, Terumo Corporation, Japan) was used for celiac artery and superior mesenteric artery angiography, as well as selective hepatic arteriography if necessary. Chemoembolisation was then conducted with a 3F microcatheter (Progreat, Terumo Corporation, Japan) on the feeding arteries of the tumor. Three to four of the following drugs were administered during the procedure: epirubicin (30-50 mg), cisplatin (40-60 mg) or oxaliplatin (100-150 mg), mitomycin (10-14 mg), 5-FU (500-750 mg), calcium folinate (200-300 mg) and hydroxycamptothecine (10-14 mg). Each drug powder was mixed with lipiodol to form an emulsion and liquid chemotherapy drugs for target vessel perfusion through a microcatheter. When the branch of the portal vein had developed or blood flow had obviously slowed down after chemoembolisation induced by the lipiodol emulsion, gelatine sponge particles were used to perform embolisation. Patients with large lesions or a large number of blood vessels around the tumor were administered the same drugs, but with the addition of 500-700 μmol/L polyvinyl alcohol (Cook Medical, Bloomington, IN, United States). Collateral artery embolisation was conducted if branches such as the phrenic artery and internal thoracic artery were involved in the tumor blood supply.

Immediate synchronous RFA: Percutaneous RFA was immediately performed under general anaesthesia after TACE with the guidance of a C-arm cone beam CT

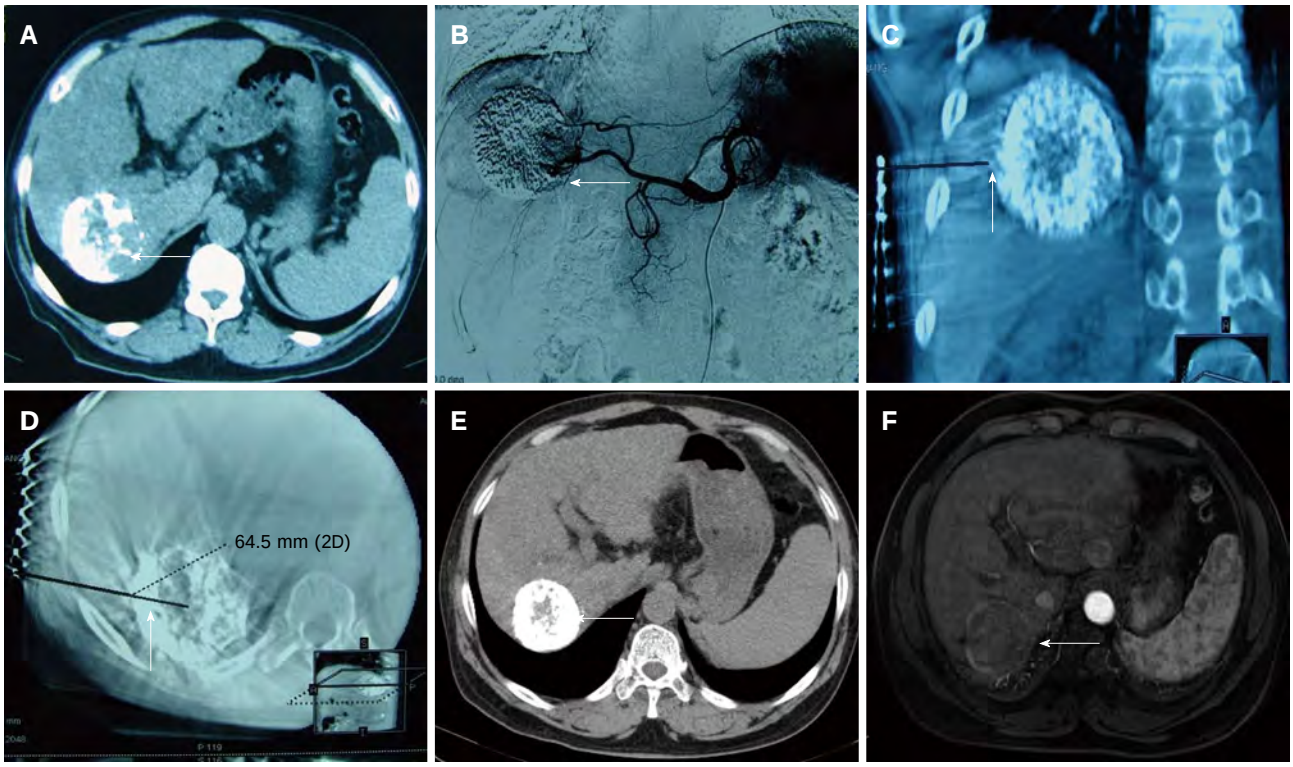

Figure 1 A male patient aged 60 years with hepatocellular carcinoma. Computed tomography (CT) scan showed a residual lesion in the liver after the first interventional therapy. Thus, transcatheter arterial chemoembolisation (TACE) immediately followed by radiofrequency ablation (RFA) was performed under the guidance of digital subtraction angiography (DSA)-CT. A: Liver CT scan showed a partial lipiodol deposit (arrow) after first interventional therapy; B: Angiogram performed before RFA showed a lipiodol deposit in the liver and staining of the delay phase around the lipiodol (arrow); C, D: INNOVA4100 IQ DSA-CT was used to obtain the coronal section and cross section of the reconstructed image to design the puncture path and angle (arrow); E, F: Liver CT scan 23 mo after combination therapy showed good lipiodol deposits without enhancement (arrow).

and DSA. Three-dimensional (3D) CT navigation with INNOVA4100 IQ DSA was used. To establish 3D CT images, the radiofrequency (RF) puncture path and its parameters, 6 × 6 square metal grid lines (diameter: 1 cm) were placed in parallel on the right side of the 8^{th}-10^{th} costal margin or below the xiphoid. Then, a 3D CT scan of the target lesion and image reconstruction (Figure 1) were conducted. The corresponding site was located on the body surface instead of the ribs, and the puncture path was identified through the surface point. The target lesion was determined to avoid important organs, such as the intestinal canal, gallbladder and lung. The puncture path was calibrated to one (*i.e.*, bull's eye configuration), and various parameters, such as the angle of the head, lateral position of the tube ball and needle depth, were determined. The device was then switched on to automatically set the system, which adjusted the tube ball to the correct position. To puncture and localize the target lesion under the perspective, the RF needle was inserted toward the target, and the bull's eye graphic converged on the target. When the puncture needle was in the correct position, the multipolar RF needle was switched on and the tube ball was rotated 70°-90° toward the right and left lateral positions to verify whether the RFA needle was located in the target lesion. For the RFA parameter setting, different RFA needles and RFA parameters were selected according to the tumor position, size and shape. A multipolar RF needle (RITA Company, Cristal Lake, IL, United States) with a maximum ablation diameter of 5 cm and needle length of 15 to 25 cm was used in all cases due to the presence of large HCCs in some patients. The following settings were used: power, 150 to 200 W; ablation time, 6 min (3 cm), 8 min (4 cm) and 15 min (5 cm) and target temperature, 105 ℃. RFA was performed twice as routine and three times if necessary. After ablation, final solidification of the puncture path and inactivation of tumor tissue were conducted to avoid bleeding and tumor implantation metastasis.

Post-procedure treatment and follow-up: Local pressure (RF puncture site and puncture site in right femoral artery) was applied after RFA. To alleviate pain, an analgesic pump was used continuously for 3 d which injected 0.2 mg/kg fentanyl with 10 mg of tropisetron hydrochloride and normal saline at a total volume of 80 mL. Electrocardiographic monitoring was performed for 24 h. The following procedures were also conducted: anti-infection, improvement of damaged liver function, nutritional support and defaecation. About 3 d to 1 wk after the procedure, routine blood examination, hepatorenal function and electrolytes were examined. Exactly 1 mo after the procedure, the patients underwent CT, ultrasound or enhanced MRI scan, and examinations for hepatorenal function and tumor markers (AFP, CA199

and carcinoembryonic antigen). If the tumor was well controlled, subsequent reviews were arranged at 2-3 mo intervals. All the images were analysed by a radiologist with over 8 years of experience. The efficacy of the combination therapy on local tumors was assessed according to the evaluation method recommended by the European Association for the Study of the Liver. Complete response (CR) was defined as the absence of signs of intensified lesions in and around the tumor. Partial response (PR) referred to a minimum of 50% reduction in size of the enhancing tumor. Progressive disease (PD) described the presence of new lesions or at least one lesion with 25% reduction in the size of the enhancing tumor. Stable disease (SD) referred to the presence of a stable lesion between PR and SD. During follow-up, the complete inactivation rate, duration and necrosis characteristics of the local tumor and survival condition of the patients were assessed. The appropriate treatment (combination therapy, close observation or single RFA or TACE) of the patients was determined on the basis of clinical conditions, such as the characteristics of new or residual lesions. Major complications were evaluated on the basis of bleeding and injuries of the intestinal canal, bile duct and gallbladder. Minor complications were assessed using several indicators, including changes in hepatorenal function after combination therapy, and changes in syndrome symptoms after embolisation, such as pain.

Statistical analysis

Quantitative data were analysed using CHISS2004 software. The *t* test was employed to compare liver function (Child-Pugh Grade) before and after intervention.

RESULTS

Technical evaluation and clinical efficacy

All patients were tolerant of the concurrent combination therapy. A total of 18 lesions were confirmed by previous images and successfully labelled with lipiodol deposit in TACE. One-off RFA puncture was successfully conducted with two to three RFA needles per lesion (Figures 2, 3). The success rate of the combination therapy was 100%. For 9 cases with lesions near the diaphragm, the puncture avoided normal lung tissue under the perspective and entered the lesions for RFA treatment.

One month after the intervention, all 18 patients underwent routine imaging (ultrasound, enhanced CT or MRI of liver) and AFP examination. Local lesions were mainly characterized by coagulative necrosis without liquefactive necrosis. Increased AFP was discovered in 10 patients before intervention, which significantly decreased 1 mo after surgery. CR was observed in 17 cases, and PR was observed in 1 case. During follow-up, the mean recurrence-free survival time of the 18 cases was 16.8 ± 4.0 mo (2-29 mo). The estimated overall survival rate at 6, 12 and 18 mo was 100%.

Complications after intervention

No TACE and percutaneous RFA-related complications, such as bleeding or necrosis of the gallbladder, bile duct, intestinal canal, pneumothorax or hepatapostema or liver/kidney failure, occurred after the intervention. Pain was completely alleviated by injection of the fentanyl mixture in 16 patients using the analgesic pump, and by 3-4 mg of morphine once every 24 h in 3 patients in addition to the analgesic pump. All patients received oral central analgesics (such as oxycodone hydrochloride) 4 d after treatment to relieve pain. Different degrees of constipation, low fever (37.3-38.4 ℃) and nausea and vomiting were observed in 5, 7 and 5 cases, respectively, and relieved with medication. The ALT (200.4 ± 63.4 U/L) and AST (228.1 U/L ± 25.4 U/L) levels in all patients transiently increased ($P = 0.00 < 0.05$) on the third day after treatment relative to levels before surgery (24.7 ± 9.3 and 32.7 ± 6.8 U/L, respectively). ALT and AST levels decreased to 29.8 ± 11.5 U/L and 36.8 ± 10.2 U/L ($P = 0.15$, $P = 0.16 > 0.05$), respectively, on the 7th day after treatment. No statistical differences in bilirubin and albumin were found before and after treatment.

DISCUSSION

Large solitary HCCs are a special type of liver cancer, the prognosis of which is better than that of the multinodular type after complete inactivation by chemoembolisation or resection[14-17]. Although surgery is still the primary treatment mode for HCC, it has several disadvantages resulting from the large size or location of the carcinoma, such as difficulty in complete resection, heavy bleeding, high incidence of complications and high recurrence rates after surgery[16,17]. Aside from surgery, many other non-surgical treatments such as TACE and RFA are used to cure solitary HCCs. However, TACE or RAF can only inactivate local carcinomas with diameters smaller than a specific value. For instance, TACE treatment requires repetition and a large dose of lipiodol, involves a high likelihood of collateral formation after multiple embolisations and has low inactivation rates after the procedure and adverse effects on long-term liver function and prognosis[17]. In our study, all the patients underwent one to three cycles of TACE before the combination therapy and were reviewed for the presence of residual or new tumors. The results indicate that a single technique cannot completely inactivate HCC and that recurrence rate of the cancer was high after the procedure.

The combination of TACE and RFA is one of the major treatments used to enhance the inactivation rate of local tumors, decreasing short- or long-term recurrence rates and extending patient survival[3-12,17-20]. However, the current combination therapy is mainly performed separately or several times and the local recurrence rate increases as tumor diameter increases[21,22]. The interval between TACE and RFA is longer than 1 or 4 wk, during which recanalization after embolisation, collateral formation and elimination of lipiodol-chemotherapeutant may occur. Therefore, this method is not strictly concurrent, and the effects of TACE and RFA are not fully synergistic.

To increase local tumor inactivation rates, prelimi-

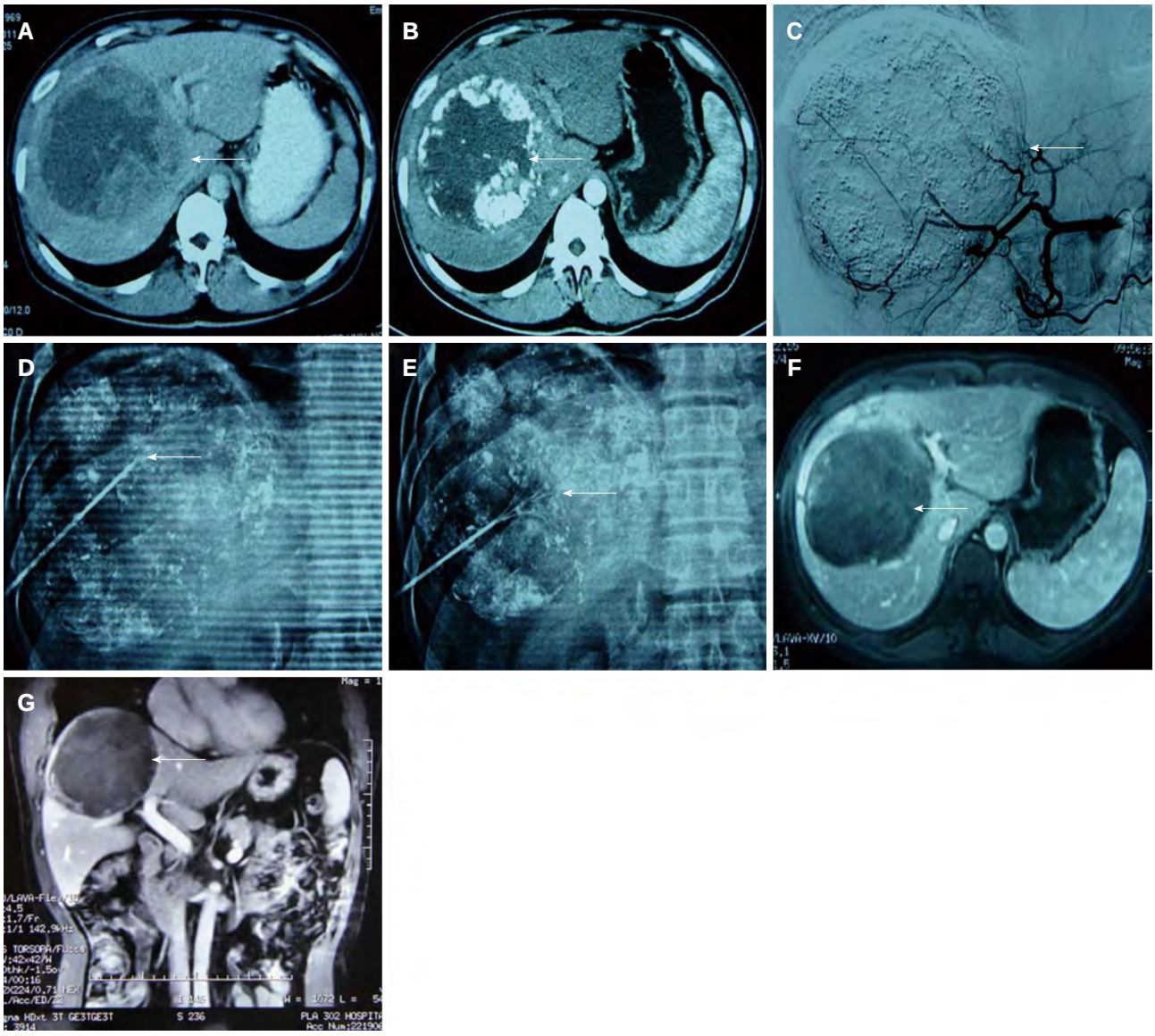

Figure 2 A male patient aged 42 years with poorly differentiated hepatocellular carcinoma. Computed tomography (CT) scan showed a residual lesion in the liver after first interventional therapy with α-fetoprotein (345 μg/mL). Thus, transcatheter arterial chemoembolisation combined with radiofrequency ablation (RFA) was performed. A: CT scan showed a large solitary tumor in the right hepatic lobe before interventional therapy (arrow); B: A lipiodol deposit appeared around the liver tumour after the first procedure. CT revealed an enhanced residual lesion in the artery phase (arrow); C: Hepatic artery angiogram before RFA showed staining of the residual lesion around the lipiodol in the liver (arrow); D, E: A multipolar probe with a maximum extended diameter of 5 cm, which could cover the residual lesion, was designed to perform RFA in the region labelled by lipiodol and the original lesion (lipiodol deposition area). A diaphragmatic dome was involved, and the puncture tunnel detoured the lung tissue under the fluoroscope (arrow); F, G: The hepatic lesion was well controlled, and no recurrence was found after 15 mo follow-up (arrow).

nary studies on the safety and effectiveness of TACE combined with immediate RFA in the treatment of liver tumors (diameter, ≤ 5 cm) have been conducted. Gadaleta *et al*[23] used immediate combination therapy to treat HCC and liver metastatic tumors of different sizes and achieved a success rate of 100% with an 88% CR. Kang *et al*[18] treated HCC (diameter, ≤ 5 cm) by TACE combined with immediate RFA. Approximately 1 mo later, the complete necrosis rate of tumors successfully labelled by TACE was 100%, and the cumulative incidence of local tumor progression at 1 year and 3 years was 1.8% and 9.4%. These results demonstrate the positive effects of immediate combination therapy. We studied the application of immediate combination therapy for large solitary HCCs (diameter, > 5 cm) and showed the favorable clinical efficacy of this method. The clinical efficiency was 100% in 18 patients, with 17 cases of CR and 1 case of PR. Compared with single TACE, combination therapy increased the local inactivation rate and prolonged recurrence-free survival. During follow-up, the estimated overall survival rate at 6, 12 and 18 mo was 100%. Based on the literature and clinical practice, as well as comparisons with conventional sequential therapy, the advantages of immediate combination therapy are as follows: (1) Single TACE or RFA cannot completely inactivate the tumor, especially the tissue on the tumor border, resulting in recurrence. In immediate combination therapy, lipiodol precipitation in the lesion wraps around

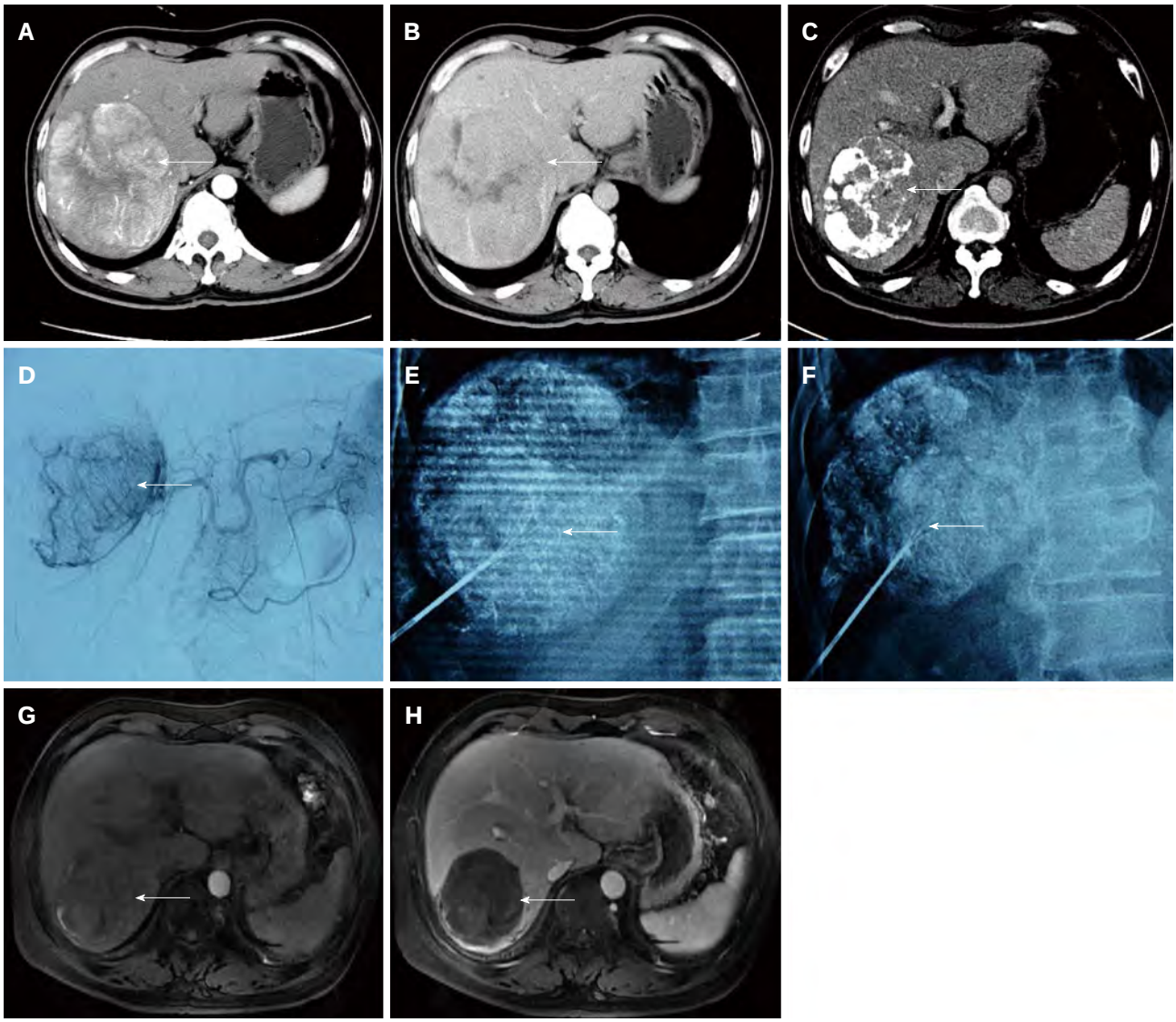

Figure 3 A male patient aged 53 years with a large lesion in the right lobe of the liver. A residual lesion was found after three cycles of transcatheter arterial chemoembolisation (TACE). TACE combined with radiofrequency ablation (RFA) was performed under the guidance of digital subtraction angiography-computed tomography. A, B: A large hypervascular hepatocellular carcinomas was observed in the right lobe (arrow); C, D: A residual lesion around the lipiodol deposit was still visible after three cycles of TACE (arrow); E, F: The lesion was successfully labelled after TACE. A multipolar needle was opened and rotated 70° to the left (E) and right (F) sides to verify whether the RF needle was in the center of the residual lesion (arrow); G, H: The large lesion was well controlled, and no recurrence was observed by magnetic resonance imaging during 13 mo follow-up after combination therapy (arrow).

and inactivates the surrounding tissue of the tumor, thereby preventing recurrence from residual tumors[3,24]; (2) Immediate combination therapy also fully enhances the synergistic effects of chemotherapeutics and thermal ablation. In TACE, lipiodol, which is the carrier of chemotherapeutics, is uncontrolled and unstable. Lipiodol cannot be released slowly with sustained high concentrations. Chemotherapeutics in lipiodol are eliminated over time if no other embolisation agents (such as sponge) are added to the drug. However, in TACE with immediate RFA, chemotherapeutics, such as adriamycin, can inhibit tumors due to their high accumulative concentration in and around the lesions[25,26]; and (3) TACE accurately locates and labels new and residual lesions, as well as lesions that cannot be observed by conventional CT and B ultrasound, through lipiodol precipitation. Thus, RFA targets are more specific. The labelling of lesions by lipiodol precipitation is more effective, especially in lesions that have become relatively complicated after several cycles of TACE because previous necrotic and new or residual lesions are labelled[27,28].

TACE combined with RFA is a safe treatment with 4%-6% incidence of various complications; severe complications are rare[3,18,23,28]. Kang *et al*[18] reported two cases of serious liver damage and one case of hemorrhage from a ruptured hepatic artery in combination therapy. Only one case of hepatic function damage without other complications, such as severe liver failure, was found in our series; this complication may have resulted from the application of a super-selective embolisation technique and bypassing of normal liver tissue by the RFA needle. Most patients only experienced post-embolisation syn-

drome (pain, low fever, *etc.*), transient liver dysfunction and constipation after surgery, all of which were relieved with medication. Constipation may be associated with the continuous application of analgesics, and low fever may be related to absorption after tumor necrosis. Transient liver dysfunction is related to combination therapy. Given that concurrent TACE with RFA increases pain in patients, RFA was performed immediately after TACE under general anaesthesia and tracheal intubation. An analgesic pump was utilized for 3 d to relieve pain. However, three patients still experienced intense local pain, which was alleviated by the addition of analgesics. Therefore, instead of the conventional combination of TACE and RFA, we recommend the application of an analgesic pump for 3 d after immediate combination therapy. Compared with the liquefactive necrosis induced by single TACE or RFA, the tumors were characterized by coagulative necrosis, which has a lower risk for local secondary hepatapostema[5].

Our study has several limitations: (1) The small sample size in this retrospective research may have influenced the results to some extent; and (2) the follow-up period was too short to accurately evaluate long-term efficacy. More patients should be included in comparative studies and further assessment of the advantages of combination therapy.

In conclusion, TACE immediately followed by RFA is a safe and effective treatment for large HCCs. To prevent pain after the procedure, an analgesic pump should be used for 3 d. The long-term efficacy of this combination therapy requires further assessment.

COMMENTS

Background

Transcatheter arterial chemoembolisation (TACE) is the main treatment method for unresectable primary hepatocellular carcinomas (HCC). However, simple TACE has low tumor inactivation and high recurrence rates. In addition, with increasing tumor size, the tumor inactivation rate significantly decreases.

Research frontiers

Combination therapy [mainly TACE combined with radiofrequency ablation (RFA)] is one of the main modalities for treating unresectable HCC. However, RFA is often performed 1 to 2 wk after TACE, which considerably reduces the synergistic effects of the combination therapy, especially for large HCCs.

Innovations and breakthroughs

The combination of TACE with immediate RFA under digital subtraction angiography-computed tomography (CT) guidance is applied to treat large single HCCs. With this technique, different treatment technologies are fused into one angiographic machine, which improves their synergistic effects (*e.g.*, heat treatment with epirubicin and embolisation with thermal ablation), thereby enhancing the inactivation rate of large HCCs and reducing the radiation dose applied to patients and the risk of repeated treatment. Preliminary results show that combination therapy has obvious advantages and can help improve the long-term survival of patients. This technique also has significance in the treatment of other metastatic and hypervascular HCCs.

Applications

TACE immediately followed by RFA is a safe and effective treatment for large HCCs. This technology can improve the synergistic treatment effects of TACE and RFA, as well as reduce the need for repeated treatments and amount of radiation exposure. Furthermore, different treatment technologies are fused into one machine, thereby simplifying the operational process. TACE immediately followed by RFA enhances tumor inactivation ability, decreases recurrence rates, prolongs patient survival time and improves prognosis.

Terminology

TACE: Transcatheter arterial chemoembolisation with lipiodol and chemical drugs. Immediate RFA: RFA procedure is performed immediately after TACE. A three-dimensional CT image, radiofrequency puncture path and parameters are first established to target the lesion and avoid non-target lesions. This combination therapeutic modality requires general anesthesia. Post-procedure pain management is required for 2 to 3 d. The combined therapeutic modality can be used for HCCs and single hypervascular metastatic tumors.

Peer review

In this manuscript, the authors summarised 18 patients treated between January 2010 and June 2012 to assess the technical safety and efficacy of TACE combined with immediate synchronous RFA as a means of treating HCC. The manuscript is very interesting. It is a very good study about the technical safety and efficacy of the combined therapy for large hepatocellular carcinomas.

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BRIEF ARTICLE

Metabonomic studies of pancreatic cancer response to radiotherapy in a mouse xenograft model using magnetic resonance spectroscopy and principal components analysis

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Abstract

AIM: To investigate the metabolic profiles of xenograft pancreatic cancer before and after radiotherapy by high-resolution magic angle spinning proton magnetic resonance spectroscopy (HRMAS ^{1}H NMR) combined with principal components analysis (PCA) and evaluate the radiotherapeutic effect.

METHODS: The nude mouse xenograft model of human pancreatic cancer was established by injecting human pancreatic cancer cell SW1990 subcutaneously into the nude mice. When the tumors volume reached 800 mm^3, the mice received various radiation doses. Two weeks later, tumor tissue sections were prepared for running the NMR measurements. ^{1}H NMR and PCA were used to determine the changes in the metabolic profiles of tumor tissues after radiotherapy. Metabolic profiles of normal pancreas, pancreatic tumor tissues, and radiation- treated pancreatic tumor tissues were compared.

RESULTS: Compared with ^{1}H NMR spectra of the normal nude mouse pancreas, the levels of choline, taurine, alanine, isoleucine, leucine, valine, lactate, and glutamic acid of the pancreatic cancer group were increased, whereas an opposite trend for phosphocholine, glycerophosphocholine, and betaine was observed. The ratio of phosphocholine to creatine, and glycerophosphocholine to creatine showed noticeable decrease in the pancreatic cancer group. After further evaluation of the tissue metabolic profile after treatment with three different radiation doses, no significant change in metabolites was observed in the ^{1}H NMR spectra, while the inhibition of tumor growth was in proportion to the radiation doses. However, PCA results showed that the levels of choline and betaine were decreased with the increased radiation dose, and conversely, the level of acetic acid was dramatically increased.

CONCLUSION: The combined methods were demonstrated to have the potential for allowing early diagnosis and assessment of pancreatic cancer response to radiotherapy.

Key words: High-resolution magic angle spinning proton magnetic resonance spectroscopy; Principal components analysis; Pancreatic cancer; Radiotherapy

Core tip: In the present study, for the first time to our knowledge, high-resolution magic angle spinning proton magnetic resonance spectroscopy and principal components analysis were combined to highlight metabolite profiles of pancreatic cancer after radiotherapy,

by analyzing the correlation between radiotherapy effect and metabolic change, and optimizing the therapeutic scheme. The results showed that metabolic profile changes of pancreatic cancer after radiotherapy were closely correlated with therapeutic effect. The outcome of the study is both interesting and beneficial to pathological research, early diagnosis, and therapy evaluation of pancreatic diseases.

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INTRODUCTION

Pancreatic cancer is a malignant tumor with very poor prognosis, and surgery has been considered as the only radical therapy. However, about 85% of newly diagnosed cases have developed distant metastasis, and only 5%-25% of pancreatic head cancer and less than 10% of pancreatic body cancer can be treated with surgical excision, and the postoperative recurrence rate is high. Therefore, radiation therapy has become the predominant treatment method for locally advanced pancreatic cancer[1-3]. Therapeutic evaluations of radiotherapy are mainly: remission from the symptoms of pain and jaundice, solid tumor size and its survival time, and the lack of a specific targeted method. During the last three decades, there has been ongoing magnetic resonance spectroscopy (MRS) research in malignant diseases. These studies provided valuable data on the biochemistry and metabolism of tumors, and on the effects of nutrients, hormones, and growth factors[4,5]. The mechanisms of action of anticancer drugs and the acquired resistance to these agents were delineated[6,7]. MRS was also used for monitoring the response to therapy[8,9].

High-resolution magic angle spinning proton magnetic resonance spectroscopy (HRMAS ^{1}H NMR) is a well-recognized technique in metabonomics studies *in vitro*, by which biopsy or postmortem samples of intact tissues are spun at the magic angle, resulting in a significant improvement in the resolution of the spectrum obtained for some of the line-broadening factors, such as dipole–dipole interactions and chemical shift anisotropy, and magnetic field inhomogeneities are averaged out[10,11]. This approach requires minimal sample preparation and, unlike convenient ^{1}H NMR spectroscopy of tissue extracts, both aqueous and lipid-soluble metabolites can be observed simultaneously *in situ*. In addition, information about the metabolic environment of the tumor can also be obtained. Therefore, HRMAS ^{1}H NMR has proved to be an efficient method for studying a wide variety of cancers, including breast cancer[12], cervical cancer[13], kidney cancer[14], prostate cancer[15], malignant lymph nodes[16], and liposarcoma[17] of animals and humans. However, so far, there are very few metabonomic studies in cancer therapeutics by the application of HRMAS ^{1}H NMR.

HRMAS ^{1}H NMR spectra obtained from tissues reflect the dynamic biological systems and processes that contribute to the overall metabolic status of an organism. It is not possible to isolate the effects of any single metabolite signal in a spectrum and, furthermore, the manual analysis of even a small number of such spectra is a laborious and complex task. Therefore, metabonomists utilize data reduction and multivariate analysis techniques, such as principal components analysis (PCA), to facilitate automated NMR pattern recognition[18,19]. Moreover, our previous study demonstrated that using ^{1}H NMR and PCA could discriminate pancreatic cancer from chronic pancreatitis accurately[20]. In the present study, HRMAS ^{1}H NMR and PCA were combined to highlight metabolite profiles of pancreatic cancer after radiotherapy, in order to analyze the correlation between radiotherapy effects and metabolic changes, and to optimize the therapeutic scheme. The study has an important implication for reference guides on therapeutic evaluation by nuclear magnetic resonance spectroscopy on pancreatic cancer *in vivo*.

MATERIALS AND METHODS

Animals and experiment schedule

Six- to eight-week-old female nude mice were obtained from the Planned Parenthood Research Institute, Shanghai, People's Republic of China. All animals in this study were housed under pathogen-free conditions and maintained in accordance with the guidelines of the Committee on Animals of the Second Military Medical University, Shanghai, China. Human pancreatic cancer cell line SW1990 in mid-log-growth phase was harvested by trypsinization. Single-cell suspensions (5×10^{6} cells in 0.1 mL HBSS) were injected subcutaneously into the nude mice. The tumors were measured every 4 d with a caliper, and the diameters were recorded. Tumor volume was calculated by the formula: $a^{2}b/2$, where a and b are the two maximum diameters. When tumors reached 2.0 cm × 2.0 cm, the duration of survival was recorded and the mouse euthanized.

For the radiotherapy experiment, when the tumor volume reached 800 mm^{3}, the mice were divided into four groups. Group A mice were used as untreated controls. Groups B, C, and D received 10, 20, and 30 Gy radiation doses, respectively. Tumor size was measured as described above. Two weeks later, tumor tissue sections were prepared for histological tests or for running the NMR measurements.

NMR spectroscopy

HRMAS ^{1}H NMR experiments were carried out using a DRX-500 spectrometer (1H frequency at 500.13 MHz;

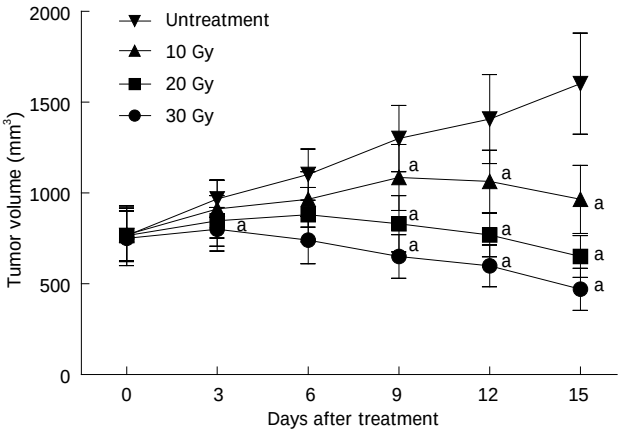

Figure 1 Effect of radiotherapy on the growth of human pancreatic tumor in nude mouse. Mice received a subcutaneous injection of SW1990 cells. When the tumor volume reached about 800 mm^3, the mice were divided into four groups. Group A mice were used as untreated controls. Groups B, C, and D received 10, 20, and 30 Gy radiation doses, respectively. Tumor size was measured for two weeks. $^{a}P < 0.05$ *vs* the untreated group.

Bruker Biospin, Rheinstetten, Germany). Tissue samples were rinsed three times with D_2O and placed into a 4-mm zirconium oxide MAS rotor with drops of D_2O (deuterium lock reference). Spectra were acquired at 300.0 K using single-pulse and CPMG pulse sequences, both with water presaturation during the relaxation delay of 2 s. CPMG pulse sequence was applied as a T2 filter to suppress signals from the molecules with short T2 values (such as macromolecules and lipids) using a total TE of 320 ms. The main parameters used for ^{1}H NMR spectra were: SW = 15 kHz; TD = 64 k; NS = 256; and MAS rate = 5 kHz. Spectral assignments were confirmed by 2-dimensional ^{1}H-^{1}H TOCSY and ^{1}H-^{1}H COSY (data not shown), together with values obtained from the literature[10,21].

The stability of tissue samples was evaluated by repeating a 1-dimensional NMR experiment after overall acquisition. No biochemical degradation was observed for any of the tissue samples.

Principal components analysis

Spectral data were phased and baseline-corrected using XWINNMR (Bruker Biospin). All FID were multiplied by an exponential function equivalent to a 0.3-Hz line broadening factor prior to Fourier transformation. Each HRMAS ^{1}H NMR spectrum was segmented into 211 regions of equal width (0.04 ppm) over the region 0.00-10.00, and the signal intensity in each region was integrated using AMIX version 3.6 (Bruker, Biospin). The region 4.50-5.00 was removed to eliminate baseline effects of imperfect water saturation. Prior to PCA, each integral region was normalized by dividing by the sum of all integral regions for each spectrum[12,14]. In order to exclude the effects of lipids and concentrate on the impacts of LMW metabolites in the CCM region, PCA was again done for ^{1}H CPMG NMR spectra over the range 0.7-4.70, each 0.04 ppm wide. PCA was used to calculate a new, smaller set of orthogonal variables from linear combinations of the intensity variables, while retaining the maximum variability present within the data. These new variables are the derived principal components, and the distribution of their values (scores) permits the simple visualization of separation or clustering between samples. The weightings (loadings) given to each integral region in calculating the principal components allows for the identification of those spectral regions of greatest influence to the separation and clustering and, hence, the deduction of biomarkers of pancreatic cancer.

Statistical analysis

Continuous variables are expressed as mean ± SD. Statistical analysis of data was done by Student's *t* test using SigmaPlot software. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Radiotherapy of human pancreatic tumor-bearing nude mouse

One week after SW1990 tumor cell inoculation, tumor size was measured and tumor volume recorded weekly. All 32 nude mouse models generated tumor tissues, and the success rate of model construction was 100% (32/32). Tumor volume in the control group (untreated), and the three groups which were given 10, 20, and 30 Gy radiation are shown in Figure 1. The transplanted tumor volume before treatment was 0.8 cm^3 on average, increasing with breeding period in the control group. Compared with the control group, the tumor volume of the treatment groups reduced significantly, with the most obvious being the 30 Gy dose treatment group. These data showed that radiotherapy could effectively suppress the growth of pancreatic cancer in the nude mice. The changes in the morphological levels are expected to be accompanied with observable changes in the tissue biochemical composition which can be accessed with HRMAS ^{1}H NMR spectroscopy *ex vivo*.

Metabolic profiles of normal pancreas and pancreatic tumor tissues

Using ^{1}H NMR spectroscopy, components such as Cho, taurine (Tau), betaine (Bet), glutamic acid (Glu), glycerophosphocholine, and choline phosphate (GPC + PC), acetic acid (Ace), alanine (Ala), and lactic acid (Lac) were detected and identified in the normal pancreas and isolated transplanted tumor tissues in the nude mouse by their spectrum peaks. The literature was referred to before (18-20) and 2-D spectrum estimation (J-res, COSY, TOCSY) (Figure 2A). Score plots of PCA based on ^{1}H NMR spectra were performed on 8 normal and 8 tumor samples, in which the spectra region was δ = 0.70-4.70, and the minimal region δ = 0.04 (Figure 2B). As shown in the loading plots, the main factors that differentiated the samples were δ 0.90-0.86, δ 1.34-1.26, δ 1.50-1.46, δ 3.30-3.18, δ 3.46-3.38, and δ 3.70-3.66, which were con-

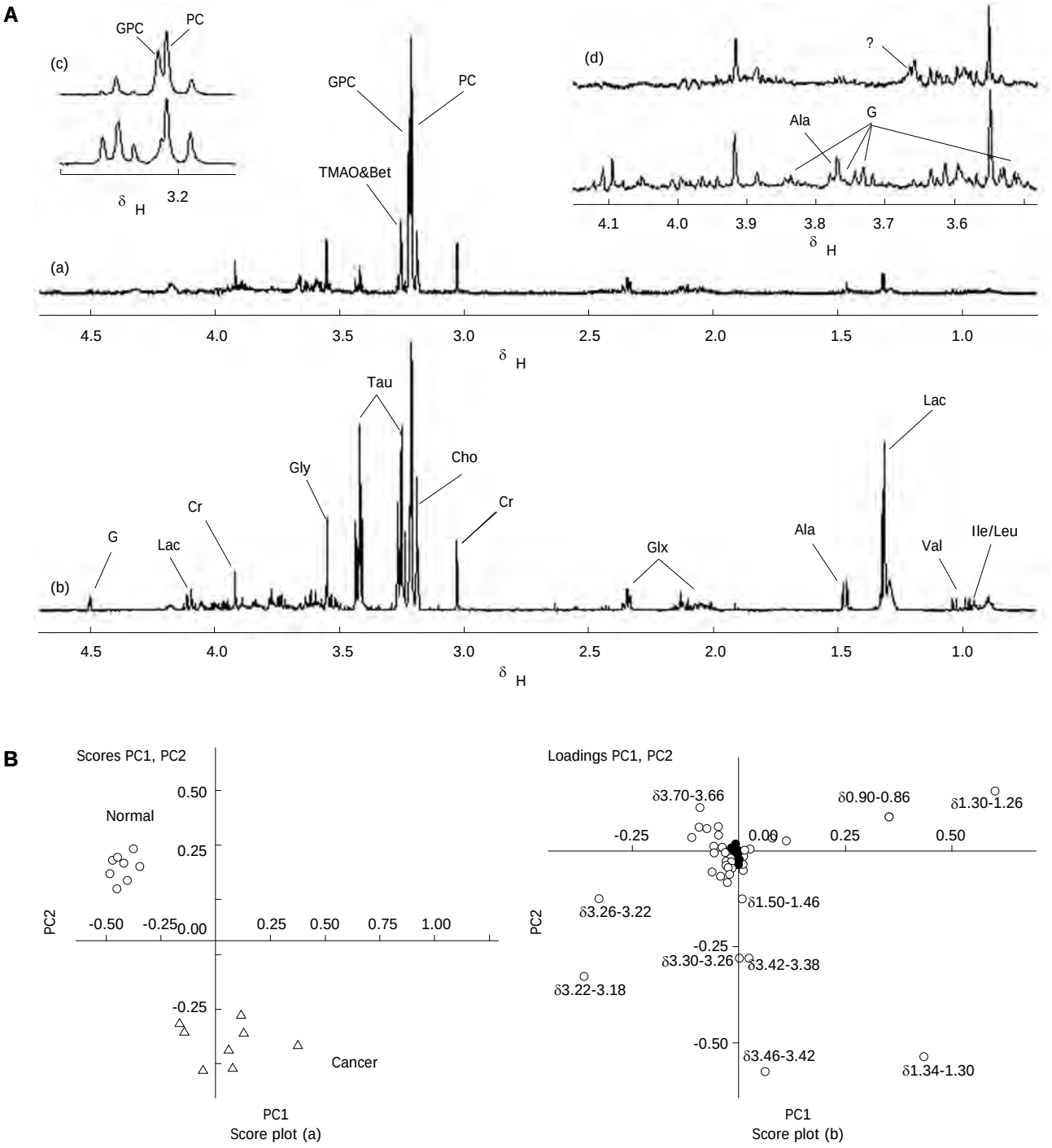

Figure 2 High-resolution magic angle spinning proton magnetic resonance spectroscopy spectra of normal pancreas and transplanted pancreatic tumor (500 mHz). A: Normal pancreas (a); Transplanted pancreatic tumor (b); Amplified data from spectra region δ3.30-3.15 (c); Amplified data from spectra region δ3.15-3.48 (d). For peak assignments, see list of abbreviations used; B: Principal Component Analysis to compare the metabolic profiles between normal pancreas and pancreatic cancer based on the high-resolution magic angle spinning proton magnetic resonance spectroscopy spectra. Panels (a) and (b) are score and loading plots. ❍: Normal pancreas; △: Pancreatic cancer.

sistent with what was observed in Figure 2A, corresponding to the residual lipid, Lac, Ala, Cho compound, Tau, and unknown chemicals.

As is well-known, absolute concentration quantification for metabolites is difficult in HRMAS spectroscopy, and the metabolite ratios are commonly used for statistical analysis. Table 1 shows the relative signal integrals and signal ratios for some metabolites that contributed to the classification of normal pancreas and pancreatic tumor tissues discussed in the above sections. Compared to the normal pancreas, concentrations of Ileu, Leu, Val, Lac, Ala, Glu, Tau, Cho, and some carbohydrates (G, contained galactose β-H possibly due to characteristic twin peak at δ 4.52) increased relatively in the pancreatic tumor samples, while GPC + PC, Bet, GPC/Cre, and unknown chemicals at δ 3.66 decreased relatively. The level

Table 1 Relative integrals and their ratios from some selected metabolites contributing to the classification of normal pancreas and pancreatic tumor tissues

		Normal pancreas	Pancreatic tumor	*P*-value
Metabolites	Choline	2.75 ± 1.37	3.99 ± 0.35	0.0376
	Taurine	1.99 ± 0.55	13.63 ± 2.92	0.0001
	Betaine	2.91 ± 0.57	1.58 ± 0.47	0.0002
	Glutamic acid	0.29 ± 0.11	0.46 ± 0.13	0.0260
	Alanine	0.60 ± 0.14	1.93 ± 0.16	0.0001
	Lactate	1.93 ± 0.86	8.30 ± 1.02	0.0001
	Acetic acid	0.06 ± 0.10	0.06 ± 0.03	0.7942
	Glycerophosphocholine+ phosphocholine	19.47 ± 1.36	16.61 ± 1.31	0.0007
Metabolites ratio	Glycerophosphocholine/ Creatine	3.51 ± 0.76	2.35 ± 0.58	0.0042
	Phosphocholine/ Creatine	5.19 ± 0.96	6.22 ± 1.52	0.1284

of Ace and PC/Cre showed no significant change.

Metabolic profiles of pancreatic tumor tissues after radiotherapy

The metabolic profiles of tumor tissues after radiotherapy were also detected by ^{1}H NMR. As shown in Figure 3A, no significant metabolic changes were observed in the ^{1}H NMR spectrum. PCA analysis was further conducted on samples in each group, with the spectrum integration region δ = 0.70-4.70, and the minimal region δ = 0.04 (Figure 3B). In score plots, most of samples in the control group concentrate in the upper left, but overlap partly with samples in the 10 Gy radiation dose group. A partial overlap is shown between the 10, 20, and 30 Gy radiation dose groups, but overall it seems that the three groups have a left, upper, and lower distribution trend in terms of scores. Loading plots showed the changes of Cho-containing compounds, along with Ace and Bet content among the three dose groups.

Table 2 shows the relative signal integrals and signal ratios for some metabolites that contributed to the evaluation of pancreatic tumor tissues response radiotherapy. Cho content showed a significant difference between the control and 30 Gy dose groups, as well as the 10 and 30 Gy dose groups. The Cho content decreased with an increase of radiation dosage. Bet content also decreased with an increase of radiation dosage. In contrast, Ace content showed a positive relationship with the radiation dosage.

DISCUSSION

Although HRMAS ^{1}H NMR combined with PCA has been demonstrated as an efficient method for studying a wide variety of animal and human cancers[12-17], this combined method has not been reported to analyze the metabolic features of cancer response to therapy. Here, for the first time to our knowledge, our findings demonstrate that applying this combined method has the potential for clinical assessment of the pancreatic cancer radiotherapeutic response.

Kaplan *et al*[22] conducted ^{1}H NMR analysis on perchlorate extract (water-soluble) of heterotopic transplanted pancreatic cancer tissue in nude mice. Compared with the normal pancreas of nude mice, Tau and Lac content in transplanted tumors increased, GPC content decreased, and there was little change in Cho and PC. However, in previous studies, some important information may be missed, and human factors introduced as a destructive process in extraction will lead to a negative impact on the results, along with poor experimental repeatability results from different pH values. Therefore, in this study, ^{1}H NMR combined with PCA was applied to the metabolic study on transplanted tumor tissues in a human pancreatic tumor-bearing nude mouse model. This has avoided the error factor involved in complex processes such as tissue extraction. Moreover, due to the application of the 500 mHz high-field strength NMR instrument, the spectrum resolution obtained is significantly higher than that reported in the literature, with more metabolites being found and variation characteristics of metabolites embodied more clearly. Consequently, not only did the accuracy of spectrum peak identification improve, but statistical analysis errors were also reduced. In this study using ^{1}H NMR combined with PCA, pancreatic cancer was shown to have higher Tau, Ileu, Leu, Val, Lac, Ala, Glu, and Cho levels relative to normal pancreas, while GPC + PC, and Bet and GPC/Cre levels decreased relatively. Compared to the other metabolites, Tau, Lac, and Ala had the most noticeable differences between normal pancreas and pancreatic cancer. Ace and PC/Cre showed no significant difference between normal and pathological conditions. The results suggest that these changes in the metabolite profile might be used as metabolic markers for the early diagnosis of pancreatic cancer.

Radiotherapy is a local treatment, and its ultimate goal is to eradicate tumor cells thoroughly, while protecting normal tissues and vital organs as much as possible[23]. The application of computer tomography simulations and the three-dimensional conformal technique in radiotherapy has boosted the pancreatic target dosage and offered better protection for the gastrointestinal tract. Currently, therapeutic evaluations of radiotherapy are mainly: remission from the symptoms of pain and jaundice, solid tumor size and its survival time, and the lack of a specific targeted method[23]. By imaging examination, tumor size and contrast agent enhancement were observed to determine tumor activity, and indirectly determine therapy efficacy, although lacking strong direct evidence[24-27]. In this study, we use ^{1}H NMR and PCA to compare pancreatic cancer metabolic variation characteristics before and after radiotherapy. Although no significant metabolic changes were observed in the ^{1}H NMR spectra, PCA results showed a trend of certain changes among different dosage groups. We found that the Ace level was increased, which positively correlated with the radiation dose. In contrast, Cho and Bet levels were decreased, which in-

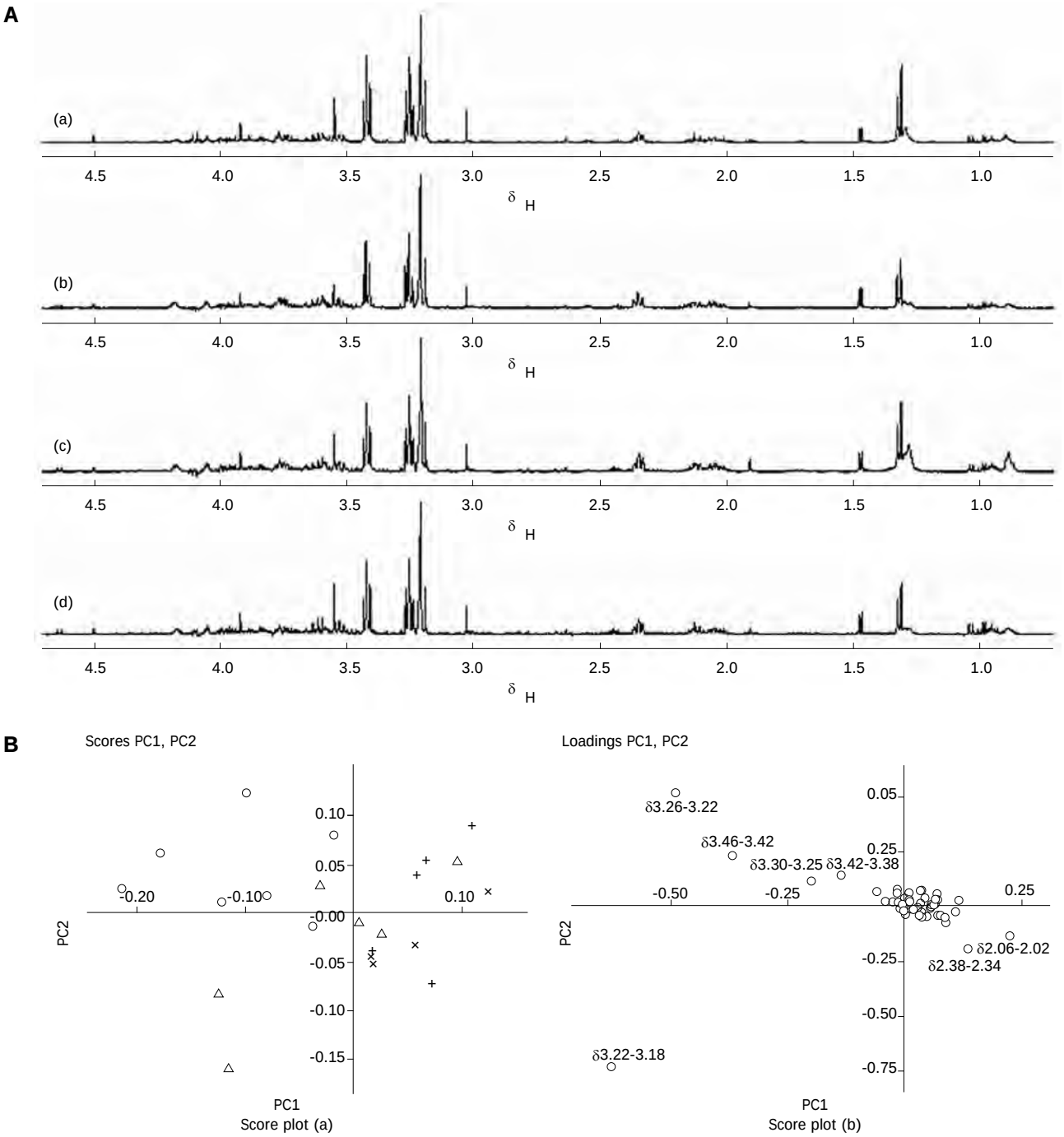

Figure 3 High-resolution magic angle spinning proton magnetic resonance spectroscopy spectra of transplanted pancreatic tumor after radiotherapy (500 mHz). A: Untreated group(a); 10 Gy treatment group (b); 20 Gy treatment group (c); 30 Gy treatment group (d); B: Principal component analysis to compare the metabolic profiles of the pancreatic tumor after radiotherapy based on the high-resolution magic angle spinning proton magnetic resonance spectroscopy spectra. Panels (a) and (b) are scores and loadings plots. ❍: Untreated group; Δ: 10 Gy treatment group; ×: 20 Gy treatment group; +: 30 Gy treatment group.

versely correlated with the radiation dose. Additionally, other metabolites, including Tau, Ileu, Leu, Val, Lac, Ala, Glu, and GPC + PC showed no significant change after radiotherapy. Thus, these data suggest that the changes in these metabolite profiles might provide a reference guide on therapeutic evaluation by NMR on pancreatic cancer *in vivo*.

Choline-containing metabolites (CCM) have already been chosen as biomarkers in various carcinoma studies[28,29]; however, they have not been mentioned in cancer treatment so far. CCM levels were shown to increase in most cancer tissues, which were explained as a result of high membrane concentration during the proliferation of cancer cells. We found that Cho level was reduced in pancreatic cancer after radiotherapy, suggesting that proliferation of cancer cells was inhibited in response to radiotherapy. However, PC and GPC levels showed no significant change in tumor tissue after radiotherapy. This

Table 2 Relative integrals and their ratios from some selected metabolites contributing to the evaluation of pancreatic tumor tissues response to radiotherapy

		Untreated	10 Gy	20 Gy	30 Gy	*P*-value
Metabolites	Choline	3.99 ± 0.35	3.97 ± 0.43	3.77 ± 0.36	3.44 ± 0.36	0.0075[1]
						0.3740[2]
						0.9012[3]
	Taurine	13.63 ± 2.92	13.43 ± 3.25	11.45 ± 2.20	12.41 ± 3.03	0.4262[1]
						0.1141[2]
						0.9005[3]
	Betaine	1.58 ± 0.47	1.69 ± 0.38	1.23 ± 0.45	0.79 ± 0.30	0.0013[1]
						0.1466[2]
						0.6275[3]
	Glutamic acid	0.46 ± 0.13	0.38 ± 0.07	0.43 ± 0.10	0.48 ± 0.17	0.8408[1]
						0.6480[2]
						0.1366[3]
	Alanine	1.93 ± 0.16	2.10 ± 0.40	2.01 ± 0.27	1.96 ± 0.42	0.8818[1]
						0.4821[2]
						0.2890[3]
	Lactate	8.30 ± 1.02	7.79 ± 1.43	7.51 ± 1.33	7.55 ± 0.85	0.1316[1]
						0.2031[2]
						0.4259[3]
	Acetic acid	0.06 ± 0.03	0.15 ± 0.06	0.25 ± 0.07	0.27 ± 0.13	0.0025[1]
						0.0001[2]
						0.0013[3]
	Glycerophosphocholine + phosphocholine	16.61 ± 1.31	19.95 ± 5.87	20.59 ± 5.79	20.80 ± 5.44	0.0522[1]
						0.0783[2]
						0.1383[3]
Metabolites ratio	Glycerophosphocholine/Creatine	2.35 ± 0.58	2.19 ± 0.15	2.49 ± 0.83	2.11 ± 0.36	0.3312[1]
						0.7087[2]
						0.4487[3]
	Phosphocholine/Creatine	6.22 ± 1.52	5.92 ± 0.44	5.87 ± 1.09	6.51 ± 1.28	0.6805[1]
						0.6065[2]
						0.6096[3]

[1]Untreated *vs* 30 Gy; [2]Untreatment *vs* 20 Gy; [3]Untreatment *vs* 10 Gy.

might be explained by a blockage of Cho-kinase and PC transferase, or by the consumption of PC through the CDP-Cho pathway[30,31]. Thus, we may deduce that increasing Cho and unchanged PC and GPC could be used as a unique profile of pancreatic cancer response to radiotherapy. Bet donates methyl groups for the remethylation of homocysteine to methionine and dimethylglycine, which supports proper liver and pancreatic function, cellular replication, and detoxification reactions. Because Cho is the precursor of Bet, the decrease of both Bet and Cho levels in pancreatic cancer after radiation treatment must be interrelated. Interestingly, the Ace level showed no significant difference between the normal pancreas and pancreatic cancer. However, Ace level dramatically increased with the radiation dose. The underlying significance of this needs to be further investigated.

In summary, although the number of samples in our study was limited, the potential of HRMAS NMR for the *in vitro* investigation of pancreatic disease response to radiotherapy should not be ignored. The above results clearly demonstrate that the metabolic profile changes of pancreatic cancer after radiotherapy were closely correlated with therapeutic effect through HRMAS ^{1}H NMR and the PCA combined method. Because metabolite changes observed by HRMAS NMR always occur before morphological changes investigated by MRIS, HRMAS NMR will certainly be beneficial to pathological research, early diagnosis, and therapy evaluation of pancreatic diseases.

COMMENTS

Background

Therapeutic evaluations of radiotherapy are mainly: remission from the symptoms of pain and jaundice, solid tumor size and its survival time, and the lack of a specific targeted method. During the last three decades, there has been ongoing magnetic resonance spectroscopy research in malignant diseases. These studies provided valuable data on the biochemistry and metabolism of tumors, along with the effects on nutrients, hormones, and growth factors.

Research frontiers

High-resolution magic angle spinning proton magnetic resonance spectroscopy (HRMAS ^{1}H NMR) is a well-recognized technique in metabonomics studies *in vitro*, by which biopsy or postmortem samples of intact tissues are spun at the magic angle, resulting in a significant improvement in the resolution of the spectrum obtained for some of line-broadening factors such as dipole–dipole interactions and chemical shift anisotropy. Magnetic field inhomogeneities are also averaged out. This approach requires minimal sample preparation and, unlike convenient ^{1}H NMR spectroscopy of tissue extracts, both aqueous and lipid-soluble metabolites can be observed simultaneously *in situ*.

Innovations and breakthroughs

Although HRMAS ^{1}H NMR combined with principal components analysis (PCA) has demonstrated to be an efficient method for studying a wide variety of animal and human cancers, this combined method has not been reported to analyze the metabolic features of cancer response to therapy. Here, HRMAS ^{1}H NMR and PCA were combined to highlight metabolite profiles of pancreatic cancer after radiotherapy, and by which the correlation between radiotherapy effect and metabolic change was analyzed, and the therapeutic scheme optimized.

Applications

The study has important implication for a reference guide on therapeutic evaluation by nuclear magnetic resonance spectroscopy on pancreatic cancer *in vivo*.

Peer review

The authors investigated whether metabolic profile changes of pancreatic cancer after radiotherapy were closely correlated with therapeutic effect through the HRMAS ^{1}H NMR and PCA combined method. The outcome of the study is interesting and beneficial to pathological research, early diagnosis, and therapeutic evaluation of pancreatic diseases.

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BRIEF ARTICLE

Laparoendoscopic single-site cholecystectomy *vs* three-port laparoscopic cholecystectomy: A large-scale retrospective study

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Abstract

AIM: To perform a large-scale retrospective comparison of laparoendoscopic single-site cholecystectomy (LESSC) and three-port laparoscopic cholecystectomy (TPLC) in a single institution.

METHODS: Data were collected from 366 patients undergoing LESSC between January 2005 and July 2008 and were compared with the data from 355 patients undergoing TPLC between August 2008 and November 2011 in our department. Patients with body mass index greater than 35 kg/m^2, a history of major upper abdominal surgery, signs of acute cholecystitis, such as fever, right upper quadrant tenderness with or without Murphy's sign, elevated white blood cell count, imaging findings suggestive of pericholecystic fluid, gallbladder wall thickening > 4 mm, and gallstones > 3 cm, were excluded to avoid bias.

RESULTS: Altogether, 298 LESSC and 315 TPLC patients met the inclusion criteria. The groups were well matched with regard to demographic data. There were no significant differences in terms of postoperative complications (contusion: 19 *vs* 25 and hematoma at incision: 11 *vs* 19), hospital stay (mean ± SD, 1.4 ± 0.2 d *vs* 1.4 ± 0.7 d) and visual analogue pain score (mean ± SD, 8 h after surgery: 2.3 ± 1.4 *vs* 2.3 ± 1.3 and at day 1: 1.2 ± 0.4 *vs* 1.3 ± 1.2) between the LESSC and TPLC patients. Four patients required the addition of extra ports and 2 patients were converted to open surgery in the LESSC group, which was not significantly different when compared with TPLC patients converted to laparotomy (2 *vs* 2). LESSC resulted in a longer operating time (mean ± SD, 54.8 ± 11.0 min *vs* 33.5 ± 9.0 min), a higher incidence of intraoperative gallbladder perforation (56 *vs* 6) and higher operating cost (mean ± SD, 1933.7 ± 64.4 USD *vs* 1874.7 ± 46.2 USD) than TPLC. No significant differences in operating time (mean ± SD, 34.3 ± 6.0 min *vs* 32.7 ± 8.7 min) and total cost (mean ± SD, 1881.3 ± 32.8 USD *vs* 1876.2 ± 33.4 USD) were found when the last 100 cases in the two groups were compared. A correlation was observed between reduced operating time of LESSC and increased experience (Spearman rank correlation coefficient, -0.28). More patients in the LESSC group expressed satisfaction with the cosmetic result (98% *vs* 85%).

CONCLUSION: LESSC is a safe and feasible procedure in selected patients with benign gallbladder diseases, with the significant advantage of cosmesis.

Key words: Cholecystectomy; Laparoscopy; Single site;

Retrospective studies

Core tip: This is a large-scale retrospective randomized study aimed to explore the safety and feasibility of laparoendoscopic single-site cholecystectomy (LESSC) for the treatment of benign gallbladder diseases, compared with three-port laparoscopic cholecystectomy in clinical outcomes. It was found that LESSC is a safe and feasible procedure in selected patients, with the significant advantage of cosmesis.

Cheng Y, Jiang ZS, Xu XP, Zhang Z, Xu TC, Zhou CJ, Qin JS, He GL, Gao Y, Pan MX. Laparoendoscopic single-site cholecystectomy *vs* three-port laparoscopic cholecystectomy: A large-scale retrospective study. *World J Gastroenterol* 2013; 19(26): 4209-4213 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4209.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4209

INTRODUCTION

Laparoendoscopic single-site cholecystectomy (LESSC) has increased in popularity due to its potential cosmetic benefits and faster recovery. It is predicted that this technique may become a standard approach to cholecystectomy[1-3]. The aim of this study was to report our experience in the LESSC with the aid of suture suspension by performing a retrospective comparison with conventional three-port laparoscopic cholecystectomy (TPLC).

MATERIALS AND METHODS

Between January 2005 and November 2011, 366 patients underwent LESSC and 355 underwent TPLC in the Department of Hepatobiliary Surgery, Zhujiang Hospital, Guangzhou, China. Retrospective data were collected from both case notes and the operating theater database.

As the LESSC procedure is a new technique and we have performed TPLC for nearly 15 years at this hospital, to avoid bias, the exclusion criteria for both the LESSC and TPLC groups included patients with a body mass index greater than 35 kg/m^2, history of major upper abdominal surgery, signs of acute cholecystitis, such as fever, right upper quadrant tenderness with or without Murphy's sign, elevated white blood cell count, imaging findings suggestive of pericholecystic fluid, gallbladder wall thickening > 4 mm, and gallstones > 3 cm. This study protocol was approved by the Institutional Review Board of the Second Affiliated Hospital of Southern Medical University, Guangzhou, China in November 2009 (No. ZJYY-2012-GDEK-001). Written informed consent for the procedure was obtained from all patients.

Eligible patients were assigned to the LESSC group (n = 298) and the TPLC group (n = 315). Collected data included patient demographics, intra-operative data about estimated blood loss, intra-operative complications, conversion to multi-port laparoscopic cholecystectomy (LC) or open surgery, and operating time (in all patients and in the last 100 patients in both groups), and postoperative data about length of hospital stay, visual analogue pain score, post-operative complications (contusion: an injury around the port site and bruised skin; hematoma: a localized collection of blood in the port site), total cost (for all patients and for the last 100 patients in both groups) and cosmetic results. The total costs for all procedures in the study were calculated using hospital financial records, which consisted of the cost of operating room usage and hospital ward stay during the perioperative period. Postoperative pain was assessed using a standard visual analogue scale [range, 0 (no pain) to 10 (maximum pain)] at 8 h after surgery and on postoperative day 1. The cosmetic effect was evaluated at the 2-wk follow-up visit, where patients were asked to assess the cosmetic results (satisfied or not very satisfied) by identifying the number and site of scars[4]. All operations were performed by two experienced surgeons who had performed more than 200 LC procedures before this study.

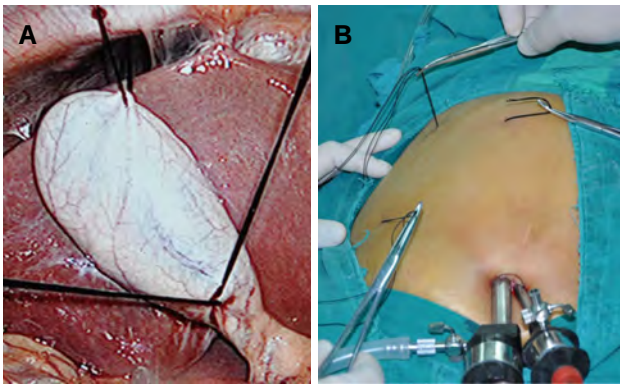

Figure 1 Suture suspension. A: The fundus and Hartmann's pouch were punctured and retracted by two sutures to expose Calot's triangle; B: Puncture spot at the superior chest wall along the costal margin in order to draw the liver up a bit more.

Surgical procedure

LESSC was performed with the help of 2 slings of sutures, and included the following steps.

Under general anesthesia, a single curved intra-umbilical 20-mm incision was made. One 10-mm trocar (Tonglu Kanger Medical Instrument Co., Ltd., Hangzhou, China) was placed to allow the insertion of a 30-degree laparoscope (Olympus, Tokyo, Japan) through the abdomen at the left side of the incision and a 5-mm trocar (Tonglu Kanger Medical Instrument Co., Ltd., Hangzhou, China) was inserted at the right side for the harmonic scalpel (Ethicon Endosurgery, 5 mm, Cincinnati, OH, United States). Tissues between the trocars were preserved to prevent air leakage. The first suture using a straight needle was inserted through the right 7th inter-costal space in the anterior axillary line, and the seromuscular layer of the gallbladder fundus was punctured and retracted toward the anterior abdominal wall. Hartmann's pouch was punctured and retracted using the second suture to expose Calot's triangle (Figure 1). A harmonic scalpel was used to dissect Calot's triangle. Once the cystic artery and duct were exposed, the cystic artery was cut using the

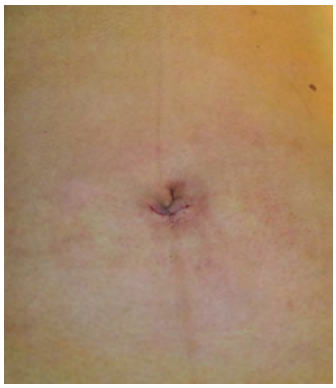

Figure 2 Umbilical incision was closed.

harmonic scalpel, and the cystic duct was ligated by three 5-mm titanium clips (Tonglu Kanger Medical Instrument Co., Ltd., Hangzhou, China) and divided. The harmonic scalpel was used to dissect the gallbladder from the gallbladder fossa. The specimen was placed into a specimen bag (TK Medical, Guangzhou, China), and removed through the umbilical incision. The umbilical incision was closed without a drainage tube in place (Figure 2)

In the TPLC procedure, the same instruments were used as in the LESSC procedure. A sub-umbilical incision, ultimisternal incision and right sub-costal incision were made. A 10-mm trocar was inserted into the sub-umbilical incision to allow introduction of the laparoscope, and another two trocars, a 10-mm and a 5-mm, respectively, were inserted for the grasp and harmonic scalpel. The operation was performed following the routine three-port cholecystectomy procedure[5], however, the cystic artery was divided and cut using the harmonic scalpel instead of being clipped and divided.

Statistical analysis

Statistical analysis was accomplished using the SPSS program for Windows 12.0 (SPSS, Chicago, IL, United States). The χ^2 test or t test was used as indicated. The Spearman rank correlation was used to investigate the relationship between operating time and experience. All data were presented as mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

There were no significant differences regarding demographic variables between the two groups (Table 1). In the LESSC group, four patients required additional ports (one or two) to adequately expose Calot's triangle. There were two conversions to open surgery in each group due to abnormal anatomy. There were no major intra- or post-operative complications such as bleeding, infection and bile leakage, however, LESSC resulted in a higher incidence of intraoperative gallbladder perforation than TPLC (56 cases *vs* 6 cases, $P < 0.001$). Overall, there were no significant differences in terms of surgical complications such as contusion (19 cases *vs* 25 cases, $P = 0.4540$) and hematoma at incision (11 cases *vs* 19 cases, $P = 0.1790$), hospital stay (1.4 ± 0.2 d *vs* 1.4 ± 0.7 d, $P = 1.0000$), and visual analogue pain score (8 h after surgery: 2.3 ± 1.4 *vs* 2.3 ± 1.3, $P = 1.0000$ and at day 1: 1.2 ± 0.4 *vs* 1.3 ± 1.2, $P = 0.2042$) between the LESSC and TPLC groups. LESSC resulted in a longer operating time (54.8 ± 11.0 min *vs* 33.5 ± 9.0 min, $P < 0.0010$). However, the operating time in the last 100 cases in the two groups was the same (34.3 ± 6.0 min *vs* 32.7 ± 8.7 min, $P = 0.1589$). A correlation was observed between reduced operating time and increased experience, with a Spearman rank correlation coefficient of -0.28.

Table 1 Demographic data

	LESSC (n = 298)	TPLC (n = 315)	P value
Age (yr)	41.5 ± 14.0	42.3 ± 11.0	0.3997
Female/male	170/128	191/124	0.3670
BMI (kg/m^2)	23.1 ± 4.0	23.5 ± 3.0	0.1279
ASA	1.4 ± 0.1	1.4 ± 0.2	1.0000
Clinical diagnosis			0.4530
Cholecystolithiasis	192	212	
Cystic polyps	106	103	

ASA: American Society of Anesthesiology; LESSC: Laparoendoscopic single site cholecystectomy; TPLC: Three-port laparoscopic cholecystectomy; BMI: Body mass index.

The total cost for LESSC per patient was 1933.7 USD compared with 1874.1 USD for the TPLC procedure (1933.7 ± 64.4 USD *vs* 1874.7 ± 46.2 USD, $P < 0.001$), and the overall cost of LESSC was approximately 57.8 USD more than the TPLC technique. However, no significant difference was found when the last 100 cases in the two groups were compared (1881.3 ± 32.8 USD *vs* 1876.2 ± 33.4 USD, $P = 0.0571$), suggesting that the cost difference was mainly due to the increased operating time.

Most patients were surprised by the reduced number of sites, and more patients who underwent LESSC satisfied with the cosmetic result than those who underwent TPLC (98% *vs* 85%, $P = 0.0010$) (Table 2).

DISCUSSION

Laparoendoscopic single-site surgery has attracted wide attention due to the decreased number of incisions needed and potentially good cosmetic results[6-13]. Recently, more studies have focused on comparing LESSC with multi-port LC and have reached an agreement that LESSC may become the gold standard treatment[14,15]. However, there is still a long way to go before this approach becomes the gold standard treatment as the standardization, safety, and other outcomes of LESSC require further validation[16-19].

Standardization is a prerequisite for clinical popularization of a surgical approach. Approaches to LESSC are technically immature. For example, to expose Calot' s triangle, trials on the use of sutures, Kirschner wires and loop retractors have been reported. The devices used in surgery vary from one surgeon to another: some use common trocars[20,21], some tend to use LESSC multi-ports[22] and others favor self-designed devices such as sterile gloves[23], in addition, there are differences in ma-

Table 2 Patient outcomes

	LESSC (n = 298)	TPLC (n = 315)	P value
Conversions to open surgery	2	2	1.0000
EBL (mL)	14 ± 6.0	15 ± 4.0	0.2643
Gallbladder perforation during surgery	56	6	< 0.001
Operating time (min)	54.8 ± 11.0	33.5 ± 9.0	< 0.001
Operating time of the last 100 cases (min)	34.3 ± 6.0	32.7 ± 8.7	0.1589
VAS (1–10)			
8 h after surgery	2.3 ± 1.4	2.3 ± 1.3	1.0000
Day 1	1.2 ± 0.4	1.3 ± 1.2	0.2042
Complications			
Contusion at incision	19	25	0.4540
Hematoma at incision	11	19	0.1790
Hospital stay (d)	1.4 ± 0.2	1.4 ± 0.7	1.0000
Cosmetic result	98%	85%	0.0010
Total cost (USD)	1933.7 ± 64.4	1874.7 ± 46.2	< 0.0010
Total cost of the last 100 cases (USD)	1881.3 ± 32.8	1876.2 ± 33.4	0.0571

EBL: Estimated blood loss; LESSC: Laparoendoscopic single site cholecystectomy; TPLC: Three-port laparoscopic cholecystectomy; VAS: Visual analogue score.

nipulative instruments such as routine instruments and reticulating instruments[22]. For example, to prevent air leakage, we have tried tri-ports and gel-ports at our center, but discontinued these due to high cost and longer trans-umbilical incision. We have used routine trocars because they are effective in preventing air leakage and are more cost-effective. With regard to surgical instruments, we have tried flexible forceps and laparoscopes, but have finally resorted to suture suspension assisted technology in LESSC, for which only one 30-degree laparoscope and one manipulative instrument are needed, eliminating the clashing of more instruments intra-operatively.

An appropriate method to place the sutures is essential for the operation. To achieve an ideal exposure of surgical site, we choose a puncture site at the superior chest wall along the costal margin so that the suture can draw the liver up a bit more, which is different from view of Piskun *et al*[23] that the puncture spot should be at the inferior costal margin. In addition, the use of harmonic scalpel is effective in occluding 3-mm blood vessels and dissecting tissues[24]. At our center, the cystic arteries were all cut using the harmonic scalpel, indicating the safety of this scalpel.

In this study, the groups were not randomized or operated on at the same time periods, thus inevitably increasing the risk of bias[25]. For example, TPLC was performed earlier than LESSC at our institution, suggesting a difference in operating experience between LESSC and TPLC. Many patients with signs of acute cholecystitis and other complications successfully underwent TPLC in our institution, but few patients with these complications successfully underwent LESSC during the study period. Therefore, exclusion criteria were applied, where patients with a history of major upper abdominal surgery, signs of acute cholecystitis, and gallstones > 3 cm, were excluded to minimize bias. However, despite the use of selection criteria, this study remains retrospective and was affected by the well-known bias due to this design.

Our results showed that the LESSC technique was more expensive and time-consuming than the TPLC technique. However, the comparisons in the last 100 patients between the two groups demonstrated that these differences were minimized through improvement of surgical skills. Analyses of operating time and total cost demonstrated a relationship between reduced operating time and increased experience, and a relationship between reduced total cost and increased experience. It is concluded that LESSC with the aid of suture suspension will not add a financial burden to the patient if the operator is skilled in this technique.

In conclusion, this large-scale retrospective trial demonstrated that LESSC with the aid of suture suspension is a safe and feasible procedure in selected patients. However, the limitations of the retrospective nature in this study preclude us from drawing a firm conclusion that LESSC is as safe as TPLC in terms of major complications, such as the bile duct injury, and from demonstrating its potential advantages, such as improved result, reduced postoperative pain and patient satisfaction. Therefore, more large-scale and multi-center randomized studies comparing LESSC with multi-port LC are needed to investigate the safety, potential benefits and clinical application of LESSC.

COMMENTS

Background

Recently, surgeons have begun performing laparoscopic cholecystectomy through a single umbilical incision, which is known as laparoendoscopic single-site cholecystectomy (LESSC). The potential benefits of this approach include reduced postoperative pain, improved cosmetic result and earlier return to normal life. Some investigators have predicted that LESSC may become an alternative standard approach for benign gallbladder diseases. However, there are still controversies with regard to its safety and efficiency, although increasing literatures demonstrate that single-incision laparoscopic surgery is a feasible and safe approach. This retrospective study explored the safety and efficiency of LESSC for the treatment of benign gallbladder diseases in selected patients compared with the three-port laparoscopic cholecystectomy (TPLC) in clinical outcomes.

Research frontiers

LESSC has attracted wide attention because of its potential advantages in cosmetic result and faster rehabilitation. However, whether LESSC could be an alternative to multi-port laparoscopic cholecystectomy remains unknown, and therefore it is necessary to compare the clinical outcome of LESSC and multiple-port laparoscopic cholecystectomy in a large cohort.

Innovations and breakthroughs

This is a large-scale retrospective study to explore the safety and efficiency of LESSC for the management of benign gallbladder diseases compared with the TPLC in selected patients.

Applications

LESSC is a safe and effective approach in selected patients with benign gallbladder diseases. LESSC has a better cosmetic benefit than TPLC.

Terminology

LESSC is a complementary approach to laparoscopic cholecystectomy, in which all operating procedures are completed through a single 15-25 mm incision around the navel. However, unlike the traditional multi-port laparoscopic approach, LESSC leaves only a single small scar.

Peer review

The authors have presented for an interesting manuscript in which they retrospectively compare a single incision laparoscopic cholecystectomy *vs* conventional 3 port cholecystectomy. The main strength of this study is the large simple size of considered groups of patients. The authors have compared the outcomes

of interest in a total of 613 eligible patients, 298 in the single incision group (LESSC) *vs* 315 in the three port group (TPLC). The procedures have been performed by two high experienced surgeons on laparoscopic cholecystectomy who have performed more than 200 laparoscopic cholecystectomy before this study. The authors have evaluated all necessary outcomes and they have accurately described the details of the performed surgical procedures. The study has concluded that LESSC is more expensive than TPLC, it requires longer operating time and it is a safe and feasible procedure in selected patients and in expert hands. Overall, the manuscript is well structured, clear and concise.

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BRIEF ARTICLE

Effect of amitriptyline on gastrointestinal function and brain-gut peptides: A double-blind trial

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Abstract

AIM: To study the effects of low-dose amitriptyline (AMT) on gastrointestinal function and brain-gut peptides in healthy Chinese volunteers.

METHODS: This was a double-blind, randomised, placebo-controlled, two-period cross-over trial. Twenty-eight healthy volunteers were randomised and administered 1-wk treatments of AMT (12.5 mg *tid*) or placebo. Before and during the final two days of treatment, gastric emptying, proximal gastric accommodation and visceral sensitivity were measured by drinking-ultrasonography test; the orocecal transit time (OCTT) was measured by lactulose hydrogen breath test, and fasting blood was collected. Plasma levels of ghrelin, motilin and neuropeptide Y (NPY) were measured by enzyme-linked immunosorbent assay kits.

RESULTS: AMT slowed the OCTT (109.2 ± 29.68 min *vs* 96.61 ± 23.9 min, $P = 0.004$) but did not affect liquid gastric emptying and had no effect on proximal gastric accommodation. AMT resulted in decreases in the visual analogue scale (VAS) for difficulty in drinking 600 and 800 mL of water (3.57 ± 0.94 *vs* 2.98 ± 0.85, 5.57 ± 0.82 *vs* 4.57 ± 0.98, $P < 0.01$ for both), although it had no significant effect on the VAS for difficulty in drinking 200 mL and 400 mL of water. AMT significantly increased the plasma ghrelin level (442.87 ± 176.79 pg/mL *vs* 526.87 ± 158.44 pg/mL, $P = 0.04$) and the neuropeptide-Y level (890.15 ± 131.46 pg/mL *vs* 965.64 ± 165.63 pg/mL, $P = 0.03$), whereas it had no effect on the MTL level.

CONCLUSION: Low-dose AMT could slow OCTT, make the stomach less sensitive and increase the plasma levels of ghrelin and NPY. Thus, we recommend the use of low-dose AMT for functional gastrointestinal disorders.

Key words: Amitriptyline; Orocecal transit time; Visceral hypersensitivity; Gastric emptying; Brain-gut peptides

Core tip: Low-dose amitriptyline has been used to treat functional gastrointestinal disorders for many years, but the precise mechanism is still not clear. Brain-gut peptides, such as motilin, ghrelin and neuropeptide Y, may regulate gastrointestinal functions. However, evidence indicating the possible effects of amitriptyline on the levels of brain-gut peptides in healthy Chinese volunteers is limited. In this study, we conclude that low-dose amitriptyline can slow orocecal transit time, make the stomach less sensitive and increase the plasma levels of ghrelin and neuropeptide Y. Thus, we recommend the use of low-dose amitriptyline for functional gastrointestinal disorders.

Huang W, Jiang SM, Jia L, You LQ, Huang YX, Gong YM,

Wang GQ. Effect of amitriptyline on gastrointestinal function and brain-gut peptides: A double-blind trial. *World J Gastroenterol* 2013; 19(26): 4214-4220 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4214.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4214

INTRODUCTION

Functional dyspepsia (FD) and irritable bowel syndrome (IBS) are the most common functional gastrointestinal disorders (FGIDs). The aetiology of FGIDs is unclear, and treatment options are limited[1,2]. Low-dose amitriptyline (AMT) is a tricyclic antidepressant that has been used to treat FGIDs for many years[3]; however, the exact mechanism of action is not clear.

Brain-gut peptides, including motilin (MTL), ghrelin, neuropeptide Y (NPY) and so on, also known as peptide hormones, can be found in the cerebral nervous system, enteric nervous system and endocrine cells in the gastrointestinal tract. Brain-gut peptides, can be neuropeptides and neuroendocrine and paracrine substances, regulate the secretory and motor functions of the gastrointestinal tract. MTL can reportedly accelerate gastric emptying and reduce the proximal gastric volume in patients with FD[4,5]. Ghrelin, the closest family member of MTL, was reported to be abnormal in FD[6]. NPY is a 36 amino-acid peptide in the central and peripheral nervous systems that can inhibit gastric emptying and stimulate colonic transit[7]. However, as far as we know, evidence indicating the possible effects of AMT on the levels of brain-gut peptides in healthy Chinese volunteers is limited.

We hypothesised that low-dose AMT is beneficial for FGIDs because of the changes in the gastrointestinal sensor, motor function and plasma levels of brain-gut peptides. Therefore, we aimed to explore the effects of low-dose AMT on liquid gastric emptying, proximal gastric accommodation, proximal gastric sensitivity, orocecal transit time (OCTT) and the plasma levels of MTL, ghrelin and NPY in healthy Chinese volunteers.

MATERIALS AND METHODS

Methods and drugs

This study was a randomised, double-blind, placebo-controlled, two-period cross-over trial in healthy Chinese volunteers (Clinical trial number: ChiCTR-TTRCC-12001967), which was approved by the ethics committee of the hospital. Written informed consent was obtained from healthy volunteers, which conformed to the Declaration of Helsinki.

Twenty-eight healthy volunteers were randomised to the two therapies: group A was treated for 1 wk with 12.5 mg AMT *tid* and then with placebo, while group B was treated with the opposite sequence. There was a 2-wk washout phase, followed by a crossover to the alternate treatment (Figure 1). AMT hydrochloride tablets were purchased from HuNan DongTing Pharmaceutical Co. Ltd. of China (batch number: B110824). The placebo

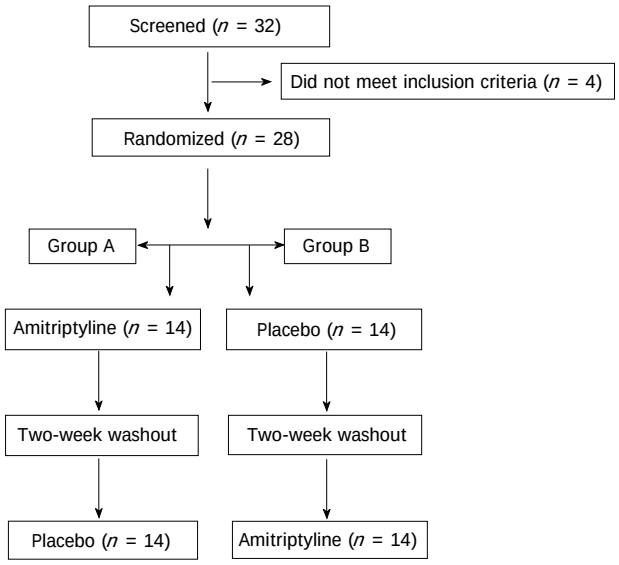

Figure 1 Consort diagram.

was supplied by ShenZhen WanHe Pharmaceutical Co. Ltd. of China. AMT and placebo tablets were similar, and the strength of each tablet was 25 mg. The investigators and patients were blinded to the treatment. The results were analysed by the investigators, and the original randomisation scheme was released after all of the analyses were performed.

Healthy volunteers

The exclusion criteria of healthy volunteers included: (1) history of FGIDs (in line with the definition of the Rome Ⅲ criteria) that may affect gastrointestinal motility; hypersensitivity or allergy to any tricyclic drug; (2) history of gastrointestinal surgery and psychiatric illness; (3) pregnancy or breast feeding; (4) use of medications that may affect gastrointestinal motor function (*e.g.*, prokinetics and anti-spasmotic agents) or the effect of AMT; (5) concomitant therapy with a monoamine oxidase inhibitor, history of urinary retention, known glaucoma, history of seizures and thyroid or liver dysfunction; and (6) participation in another clinical trial during the last two weeks.

Endpoints of the study

All healthy volunteers completed the Hamilton Anxiety and Depression Rating Scale before the treatment; a score less than 7 was defined as no anxiety or depression[8,9]. During the two days before therapy and the final two days of treatment, we assessed the following endpoints: (1) liquid gastric emptying, proximal gastric relaxation and visceral hypersensitivity by drinking- ultrasonography test; (2) OCTT by lactulose hydrogen breath test; and (3) plasma MTL, ghrelin and NPY levels by ELISA.

Drinking-ultrasonography test

The drinking-ultrasonography test was performed according to the method of Kato *et al*[10]. After an overnight

Table 1 Demographic and baseline characteristics of study in healthy volunteers

Variable	Group A (n = 14)	Group B (n = 14)	P value
Age (yr)	27.71 ± 8.56	32.5 ± 13.36	0.26
Sex (male)	7	7	1
BMI (kg/m^2)	20.09 ± 1.41	20.21 ± 1.42	0.82
HAMD	2.36 ± 1.28	1.71 ± 1.20	0.29
HAMA	2.71 ± 1.73	3.36 ± 1.45	0.18
Cross-sectional area of the proximal stomach (cm^2)			
200 mL	17.13 ± 4.53	18.75 ± 2.30	0.08
400 mL	29.19 ± 6.24	30.76 ± 6.59	0.95
600 mL	40.92 ± 11.5	40.66 ± 7.28	0.35
800 mL	46.21 ± 12.16	46.46 ± 6.81	0.24
Difficulty in drinking water VAS			
200 mL	0.79 ± 0.58	1.07 ± 0.62	0.23
400 mL	1.79 ± 0.58	1.86 ± 0.54	0.71
600 mL	3.21 ± 0.80	3.50 ± 0.65	0.31
800 mL	5.50 ± 0.76	5.43 ± 0.76	0.82
Gastric emptying			
5 min	77.65% ± 6.5%	81.46% ± 5.81%	0.67
10 min	62.61% ± 9.85%	65.18% ± 6.77%	0.55
OCTT (min)	88.93% ± 19.03%	81.43% ± 20.14%	0.46
Plasma levels (pg/mL)			
MTL	502.66 ± 127.52	440.85 ± 123.25	0.20
Ghrelin	460.06 ± 146.25	444.94 ± 202.43	0.82
NPY	888.88 ± 154.52	913.46 ± 139.32	0.66

Values are represented as mean ± SD. Symptom scores on 10-cm visual analogue scale. HAMD: Hamilton depression rating scale; HAMA: Hamilton anxiety rating scale; BMI: Body mass index; OCTT: Orocecal transit time; NPY: Neuropeptide Y; MTL: Motilin; VAS: Visual analogue scale.

fast, healthy volunteers ingested 200 mL of water (approximately 28 ℃) in 2 min for a total of four times with 2-min intervals. The subjects were in the supine position and ingested water through a straw. The emptying periods were calculated to be 5 and 10 min (by measuring the time) after drinking the total 800 mL of water. All of these examinations were performed by a single ultrasonography technician using a Philips IU22 ultrasound scanner (Philips Medical Systems, Bothell, Washington) and a Convex-type 5-20 MHz probe. The spleen served as an echo window, and the cross-section of the proximal stomach was measured *via* the 10th inter-costal space. The mucosal surface of the gastric lumen was traced from images acquired before the test at each 2 min interval after water consumption and 5 and 10 min after the end of the water consumption. The cross-sectional area was also calculated. Frozen images were saved on a hard disk. Before the test and every time after ingestion of water, abdominal symptoms were self-evaluated and recorded on a questionnaire using a visual analogue scale from 0 to 10 to investigate the difficulty (such as abdominal fullness) in drinking water.

Lactulose hydrogen breath test for OCTT

Subjects were placed on a low fibre diet 3 d before the test. After a 12-h overnight fast, two end-expiratory breath H_2 samples were collected as base values using a HHBT-01 breath hydrogen detector (Hydeway, China). After the subjects ingested 15 mL of lactulose syrup containing 10 g of lactulose, exhaled H_2 was recorded every 15 min for a total of three hours. OCTT was defined as the duration from the moment of lactulose administration to the moment when exhaled H_2 was increased over 12 ppm from the baseline[11].

Plasma ghrelin, MTL and NPY levels

After twelve-hours of fasting, the blood samples were collected and centrifuged at 3000 g for 10 min. Plasma samples were collected and stored at -70 ℃ until the procedure. We measured plasma levels of ghrelin, MTL and NPY using commercial ELISA kits (Shanghai Bluegene Biotech Co., Ltd., China).

Statistical analysis

Data analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago IL, United States), and the measurement data are reported as the mean ± SD; baseline parameters and differences between the two treatments were compared using Student's t test. Differences between the baseline and AMT or placebo treatment were compared by paired t test. $P < 0.05$ was considered statistically significant.

RESULTS

Study participants

Thirty-two healthy volunteers were selected initially by public advertisement. After a screening visit, four subjects were not appropriate for the study by the exclusion criteria (two subjects had a history of gastrointestinal surgery and two subjects experienced abdominal pain during the last three months). Twenty-eight subjects completed the study. There were no statistically significant differences in age, gender, body mass index, Hamilton depression scale, Hamilton anxiety scale scores, proximal gastric accommodation, liquid gastric emptying, proximal gastric sensitivity, OCTT or the levels of MTL, ghrelin and NPY between group A and group B ($P > 0.05$) (Table 1).

Proximal accommodation, visceral hypersensitivity and gastric emptying using the drinking-ultrasonography test

There was no statistically significant difference in the proximal gastric accommodation between the AMT and placebo groups after consumption of 200, 400, 600 or 800 mL water ($P > 0.05$). Similarly, no differences were found in the gastric emptying rate (%) at 5 and 10 min after the completion of the drinking test (all $P > 0.05$). Moreover, there were no statistically significant differences between the two groups for the VAS test for difficulty in drinking 200 or 400 mL water (all $P > 0.05$). However, there were significant differences in VAS results for difficulty in drinking 600 and 800 mL between the two groups (all $P = 0.001$) (Table 2).

There were no significant differences between the baseline and placebo treatment in proximal accommoda-

Table 2 Effects of amitriptyline on gastrointestinal function and brain-gut peptides

Variable	Amitriptyline (n = 28)	Placebo (n = 28)	P value
Cross-sectional area of the proximal stomach (cm^2)			
200 mL	16.51 ± 3.78	16.56 ± 3.98	0.97
400 mL	27.14 ± 5.71	27.84 ± 5.95	0.49
600 mL	34.11 ± 6.11	34.85 ± 6.61	0.39
800 mL	39.58 ± 7.35	40.86 ± 8.45	0.34
Difficulty in drinking water VAS			
200 mL	0.93 ± 0.65	0.96 ± 0.56	0.58
400 mL	1.93 ± 0.46	1.82 ± 0.54	0.29
600 mL	2.98 ± 0.85	3.57 ± 0.94	0.001
800 mL	4.57 ± 0.98	5.57 ± 0.82	0.001
Gastric emptying			
5 min	78.40 ± 11.71	78.84 ± 7.47	0.87
10 min	66.72 ± 11.63	64.54 ± 10.29	0.47
OCTT (min)	109.29 ± 29.68	96.61 ± 23.9	0.004
Plasma levels (pg/mL)			
MTL	461.88 ± 129.66	473.40 ± 122.75	0.61
Ghrelin	526.87 ± 158.44	442.87 ± 176.79	0.04
NPY	965.64 ± 165.63	890.15 ± 131.46	0.03

Values are represented as mean ± SD. Symptom scores on 10-cm visual analogue scale. OCTT: Orocecal transit time; MTL: Motilin; NPY: Neuropeptide Y; VAS: Visual analogue scale.

Table 3 Baseline and after treatment with placebo

Variable	Baseline (n = 28)	Placebo (n = 28)	P value
Cross-sectional area of the proximal stomach (cm^2)			
200 mL	17.95 ± 3.62	16.56 ± 3.98	0.19
400 mL	29.97 ± 6.35	27.84 ± 5.95	0.09
600 mL	40.78 ± 9.54	34.85 ± 6.61	0.06
800 mL	46.34 ± 9.67	40.86 ± 8.45	0.06
Difficulty in drinking water VAS			
200 mL	0.93 ± 0.59	0.96 ± 0.56	0.70
400 mL	1.82 ± 0.54	1.82 ± 0.54	1.00
600 mL	3.36 ± 0.72	3.57 ± 0.94	0.34
800 mL	5.46 ± 0.73	5.57 ± 0.82	0.56
Gastric emptying			
5 min	79.55 ± 6.35	78.84 ± 7.47	0.47
10 min	63.89 ± 8.39	64.54 ± 10.29	0.79
OCTT (min)	85.18 ± 19.60	96.61 ± 23.90	0.07
Plasma levels (pg/mL)			
MTL	471.75 ± 127.02	473.40 ± 122.75	0.75
Ghrelin	452.50 ± 173.46	442.87 ± 176.79	0.35
NPY	901.17 ± 144.91	890.15 ± 131.46	0.12

Values are represented as mean ± SD. Symptom scores on 10-cm visual analogue scale. OCTT: Orocecal transit time; MTL: Motilin; NPY: Neuropeptide Y; VAS: Visual analogue scale.

tion, gastric emptying and VAS results for difficulty in drinking 200, 400, 600 or 800 mL of water ($P > 0.05$) (Table 3).

There were no significant differences between the baseline and AMT treatment in the cross-sectional area of the proximal stomach (cm^2) after drinking 200, 400, 600 or 800 mL of water ($P > 0.05$) (Table 4). Similarly, no significant differences were found between the baseline and AMT treatment in the VAS after drinking 200 or 400 mL of water ($P > 0.05$). However, the VAS results significantly dropped from baseline in response to AMT treatment after drinking 600 and 800 mL water ($P = 0.001$) (Table 4). No differences in gastric emptying were observed between the baseline and AMT treatment ($P > 0.05$; Table 4).

OCTT with lactulose hydrogen breath test

AMT slowed the OCTT, and there was a significant difference between the AMT and placebo groups ($P = 0.004$; Table 2). OCTT was not different between the baseline and placebo treatment (Table 3), although there was a significant difference between the baseline and treatment with AMT ($P = 0.001$; Table 4).

Plasma levels of MTL, ghrelin and NPY using ELISA

The fasting plasma concentration of MTL was similar in the AMT and placebo groups ($P = 0.61$; Table 2). There were no significant differences in the MTL levels between the baseline and treatment with placebo ($P = 0.75$; Table 3) or between the baseline and treatment with AMT ($P = 0.11$; Table 4). However, in the AMT group, the fasting plasma ghrelin concentration was significantly greater than the placebo group ($P = 0.04$; Table 2). There was no difference in the ghrelin level between the baseline and treatment with placebo ($P = 0.35$; Table 3), but the ghrelin concentration was elevated following AMT treatment compared to the baseline values ($P = 0.001$; Table 4). Compared with the placebo group, the AMT group had higher fasting plasma NPY levels ($P = 0.03$; Table 2). There was no significant difference between the baseline and treatment with placebo ($P = 0.12$; Table 3), but the NPY level was significantly elevated after AMT treatment ($P = 0.001$; Table 4).

Adverse effects and safety

Table 5 shows the adverse effects that occurred during the treatment. There were no adverse effects which required emergency evaluation or hospitalisation. No subjects dropped out of the study.

DISCUSSION

In previous studies, low-dose AMT was useful for FD and IBS, especially for the improvement of abdominal pain[12-15], possibly because AMT reduces the visceral sensitivity and increases the pain threshold in FGID patients, although this is still controversial. Mertz *et al*[12] suggested that after AMT (50 mg/d) treatment for 4 wk in FD, the perception of gastric distension using the barostat test was not different from the placebo treatment. Conversely, Thoua *et al*[13] demonstrated that after 3 mo of treatment with AMT (25-50 mg/d) in IBS, the rectal hypersensitivity to electrical current stress was decreased, however the study was uncontrolled. Obviously, it is not due to the antidepressant effect of AMT as the doses were below the effective doses of the antidepressant; the benefits are in patients who are not depressive, with responses occurring before the antidepressant effect[16].

Here, we measured visceral sensitivity using the non-invasive drinking-ultrasonography test in healthy volun-

Table 4 Baseline and after treatment with amitriptyline

Variable	Baseline (n = 28)	Amitriptyline (n = 28)	P value
Cross-sectional area of the proximal stomach (cm^2)			
200 mL	17.95 ± 3.62	16.51 ± 3.78	0.22
400 mL	29.97 ± 6.35	27.14 ± 5.71	0.09
600 mL	40.78 ± 9.54	34.11 ± 6.11	0.06
800 mL	46.34 ± 9.67	39.58 ± 7.35	0.06
Difficulty in drinking water VAS			
200 mL	0.93 ± 0.59	0.93 ± 0.65	0.99
400 mL	1.82 ± 0.54	1.93 ± 0.46	0.36
600 mL	3.36 ± 0.72	2.98 ± 0.85	0.01
800 mL	5.46 ± 0.73	4.57 ± 0.98	0.001
Gastric emptying			
5 min	79.55 ± 6.35	78.40 ± 11.71	0.96
10 min	63.89 ± 8.39	66.72 ± 11.63	0.17
OCTT (min)	85.18 ± 19.60	109.29 ± 29.68	0.001
Plasma levels (pg/mL)			
MTL	471.75 ± 127.02	461.88 ± 129.66	0.11
Ghrelin	452.50 ± 173.46	526.87 ± 158.44	0.001
NPY	901.17 ± 144.91	965.64 ± 165.63	0.001

Values are represented as mean ± SD. Symptom scores on 10-cm visual analogue scale. OCTT: Orocecal transit time; MTL: Motilin; NPY: Neuropeptide Y; VAS: Visual analogue scale.

teers, which is different from previous studies. We found that low-dose AMT reduced gastric sensitivity immediately after the volunteers ingested 600 and 800 mL water, which was consistent with the result of Thoua *et al*[13]. This might contribute to the potential centrally mediated visceral analgesic properties of AMT. As Morgan *et al*[16] suggested, low-dose AMT has a central effect on pain-related cerebral activation in the anterior cingulate cortex and left posterior parietal complex in IBS patients during mental stress.

A variety of gastrointestinal motility disturbances have been implicated in FGIDs[17]. Bouras *et al*[18] demonstrated that low-dose AMT could slow solid gastric emptying in healthy individuals. Vahedi *et al*[14] observed that low-dose AMT was effective for the treatment of diarrhoea-predominant IBS; the reason might be the anti-cholinergic effect of the drug. In our research, AMT did not affect liquid gastric emptying but did significantly prolong the OCTT (which is also reflects the small bowel transit time[19]). This is consistent with previous investigations in which imipramine delayed OCTT in controls and IBS patients[20]. The reason for the differences in the current results compared to previous studies might be that liquid emptying is related to the proximal portion or fundus relaxation, but solid emptying is associated with the distal stomach[21]. In the current study, there were no effects on proximal gastric accommodation with low-dose AMT. Similar conclusions have been previously reported and showed that AMT had no effect on drinking capacity in healthy volunteers[18]. The gold standard for the measurement of proximal gastric accommodation is gastric barostat[22], although this procedure is invasive. In this study, we used the new drinking-ultrasonography test, which is non-invasiveness, safe, reproducible, better accepted by volunteers and relatively simple to administer.

Table 5 Adverse effects of amitriptyline and placebo

Adverse effect	Amitriptyline (n = 28)	Placebo (n = 28)
Sleepiness	10	2
Bitter taste	7	2
Dry mouth	6	3
Tired in early morning	2	1
Dizziness	2	0
Constipation	1	1

MTL levels were not significantly different between the placebo and AMT groups. However MTL levels have been reported to be significantly elevated in patients with constipation who are receiving tricyclic antidepressant drugs[23]. It is possible that we evaluated healthy volunteers rather than patients in this study. In healthy individuals, AMT might not have any effect on the normal levels of MTL because of intact reflex mechanisms. Ghrelin plays a role in regulating appetite[24]. Lee *et al*[6] reported that unusually low preprandial ghrelin levels occur in FD patients due to dysmotility. It is possible that FD patients with dysmotility may respond to AMT effectively. Caproni *et al*[25] found that the plasma levels of NPY were markedly increased in migraine patients receiving AMT treatment (25 mg/d) for 3 mo. The present study extends the previous finding by showing that the plasma level of NPY was significantly increased with low-dose AMT treatment. A previous study showed that NPY may help patients with stress on the gut-brain axis[26], so the increase in NPY levels might be a reason for the treatment of FD and IBS patients who are often hypersensitive to stress[27].

This study included a small sample size of healthy volunteers. Further studies consisting of larger sample sizes that are powered to find smaller differences may be required. As the duration of AMT administration in the clinic is typically 4-12 wk[12-15], it is possible that the course of medication in our study was too short. A longer trial might have different effects on gastrointestinal function and brain-gut peptides.

In summary, low-dose AMT slows OCTT, decreases gastric sensitivity and increases the plasma levels of ghrelin and neuropeptide Y in healthy Chinese individuals, which may be the cause of the beneficial effects of low-dose AMT in FGID patients.

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COMMENTS

Background

Low-dose amitriptyline (AMT) has been used to study functional gastrointestinal disorders for many years, although the precise mechanism of the action is still not clear. Evidence indicating the possible effects of AMT on gastrointestinal

function and brain-gut peptides in healthy Chinese volunteers is limited.

Research frontiers

Therapeutic options for functional gastrointestinal disorders are limited. Antidepressant agents such as low dose AMT are effective in functional gastrointestinal disorders.

Innovations and breakthroughs

Based on previous data, this study first explored the possible effects of low dose AMT on gastrointestinal function and brain-gut peptides in healthy Chinese volunteers. The results of the present study revealed that low-dose AMT could slow orocecal transit time (OCTT), decrease gastric sensitivity and increase the plasma levels of ghrelin and neuropeptide Y in healthy Chinese individuals, which may be the cause of the beneficial effects of low-dose AMT in functional gastrointestinal disorders (FGID) patients.

Applications

Low dose AMT plays a role in regulating gastrointestinal function, supporting its clinical applicability for gastrointestinal disorders in China.

Terminology

The drinking-ultrasonography test is a novel method to measure proximal accommodation, visceral hypersensitivity and gastric emptying. The test is non-invasive, safe, better accepted by volunteers, reproducible and relatively simple to administer.

Peer review

This manuscript has originality so far. Low-dose AMT slows OCTT, decreases gastric sensitivity and increases the plasma levels of ghrelin and neuropeptide Y in healthy Chinese individuals, which may be the cause of the beneficial effects of low-dose AMT in FGID patients.

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BRIEF ARTICLE

Magnified and enhanced computed virtual chromoendoscopy in gastric neoplasia: A feasibility study

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Abstract

AIM: To evaluate the feasibility of a new computed virtual chromoendoscopy (CVC) device (M i-scan) in the diagnosis of gastric neoplasia.

METHODS: Patients with superficial lesions no larger than 1.0 cm found during high definition endoscopy were included. Those with advanced or obviously protruded or depressed lesions, lesions larger than 1.0 cm and/or lesions which were not amenable to observation by zoom function were excluded. The endoscopist was required to give the real-time descriptions of surface pit patterns of the lesions, based on surface pattern classification of enhanced magnification endoscopy. According to previous reports, types Ⅰ-Ⅲ represent non-neoplastic lesions, and types Ⅳ-Ⅴ represent neoplastic lesions. Diagnosis with M i-scan and biopsy was performed before histopathological diagnosis. Magnified images of gastric lesions with and without enhancement were collected for further analysis. The diagnostic yield of real-time M i-scan and effects on magnification image quality by tone enhancement (TE), surface enhancement (SE) and color enhancement (CE) were calculated. The selected images were sent to another endoscopist. The endoscopist rated the image quality of each lesion at 3 levels. Ratings of image quality were based on visualization of pit pattern, vessel and demarcation line.

RESULTS: One hundred and eighty-three patients were recruited. Five patients were excluded for advanced gastric lesions, 1 patient was excluded for poor preparation and 2 patients were excluded for superficial lesions larger than 1.0 cm; 132 patients were excluded for no lesions found by high definition endoscopy. In the end, 43 patients with 43 lesions were included. Histopathology revealed 10 inflammation, 14 atrophy, 10 metaplasia, 1 low grade dysplasia (LGD), 5 high grade dysplasia (HGD) and 3 cancers. For 7 lesions classified into type Ⅰ, histopathology revealed 6 atrophy and 1 metaplasia; for 10 lesions classified into type Ⅱ, histopathology revealed 2 inflammation, 7 atrophy and 1 metaplasia; for 10 lesions classified into type Ⅲ, histopathology revealed 1 inflammation, 8 metaplasia and 1 LGD; for 9 lesions classified into type Ⅳ, histopathology revealed 4 inflammation, 1 atrophy and 4 HGD; for 7 lesions classified into type Ⅴ, histopathology revealed 3 inflammation, 1 HGD and 3 cancers. A total of 172 still images, including 43 images by white light (MWL) and 129 images by M i-scan (43 with TE, 43 with SE and 43 with CE), were selected and sent to the endoscopist who did the analysis. General image quality of M i-scan with TE and SE was significantly better than that

of MWL (TE, 4.55 ± 1.07; SE, 4.30 ± 1.02; MWL, 3.25 ± 0.99; $P < 0.001$). Visualization of pit pattern was significantly improved by M i-scan with SE (1.93 ± 0.25 *vs* 1.50 ± 0.50, $P < 0.001$). Microvessel visualization was significantly improved by M i-scan with TE (1.23 ± 0.78 *vs* 0.76 ± 0.73, $P < 0.001$). Demarcation line visualization was improved by M i-scan with both TE and SE (TE, 1.75 ± 0.52; SE, 1.56 ± 0.59; MWL, 0.98 ± 0.44; $P < 0.001$). M i-scan with CE did not show any significant improvements of image quality in general or in the 3 key parameters. Although M i-scan with TE and SE slightly increased the diagnostic yield of MWL, there was no significant difference ($P > 0.1$).

CONCLUSION: Although digital enhancement improves the image quality of magnification endoscopy, its value in improving the diagnostic yield seems to be limited.

Key words: Computed virtual chromoendoscopy; i-scan; Gastric neoplasia; Diagnosis

Core tip: In this study, the authors applied a new endoscopic device combining magnification endoscopy and virtual chromoendoscopy, equipped with surface enhancement, tone enhancement and color enhancement (M i-scan), in the diagnosis of 43 patients with small superficial gastric lesions. The results showed that real-time diagnosis of the gastric cancerous lesions by using M i-scan corresponded well with their histopathology. In comparisons between different enhancement capabilities using offline images, images with surface enhancement and tone enhancement were found to be slightly superior to those with color enhancement.

Li CQ, Li Y, Zuo XL, Ji R, Li Z, Gu XM, Yu T, Qi QQ, Zhou CJ, Li YQ. Magnified and enhanced computed virtual chromoendoscopy in gastric neoplasia: A feasibility study. *World J Gastroenterol* 2013; 19(26): 4221-4227 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4221.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4221

INTRODUCTION

Since conventional endoscopy has limited definition and magnification, detection and characterization of early gastric cancer are still challenging in daily practice. Recently, advanced endoscopy techniques have been introduced to improve the diagnosis of early gastric cancer, such as chromoendoscopy with dyes[1], acetic acid-enhanced endoscopy[2,3], magnification endoscopy[4] and dyeless virtual chromoendoscopy[5]. Incorporation of magnification endoscopy and chromoendoscopy[6] or enhanced endoscopy[7] into one instrument is perfect, because chromoendoscopy and enhanced endoscopy serve as the red flag in detection, while magnification endoscopy serves in characterization. Magnified virtual chromoendoscopy is more preferable than dye spraying magnification chromoendoscopy for efficiency and safety[5]. One example is magnified narrow band imaging (M-NBI)[8-16]. Clinical trials suggest that M-NBI is helpful in the diagnosis of Barrett's esophagus[17-21], small colorectal lesions[22] and early gastric cancer[8,23]. Along with NBI, multi-band imaging virtual chromoendoscopy, such as Fuji Intelligence Chromoendoscopy (FICE)[24-30] and Pentax i-scan, are also available in clinical practice.

Unlike NBI, FICE and i-scan use reflection band filtering to achieve color enhancement of the mucosa. The instrument in this study not only incorporates color enhancement but also surface enhancement and magnification (M i-scan). The principle of surface enhancement is to adjust the dark-to-light contrast of the nearby pixels in order to show sharper surface details.

The aim of this study is to assess the accuracy of a real-time M i-scan in the diagnosis of gastric neoplasia (primary outcome). A comparison between magnified virtual chromoscopy and non-magnified virtual chromoscopy was made by using post-endoscopy still images (secondary outcome).

MATERIALS AND METHODS

Patients

From January 1st to March 31st 2012, consecutive patients who underwent high definition gastroscopy in Shandong University Qilu Hospital were recruited into this study. Patients aged 18-80 years, having superficial lesions with diameter less than 1 cm were included. Those with advanced or obviously protruded or depressed lesions, lesions larger than 1.0 cm and/or lesions which were not amenable to observation by zoom function (poor preparation, difficult positions, and non-cooperation of patients) were excluded. This study was approved by the local ethics committee (Ethics Committee of Shandong University Qilu Hospital) and adhered to the Declaration of Helsinki for Medical Research involving Human Subjects-Ethical Principles for Medical Research Involving Human Subjects. All the patients who participated in this study have provided their written informed consents.

Endoscopic procedure

The instruments applied in this study were an EG-2990Zi endoscope (Pentax, Tokyo, Japan) and an EPK-i endoscopic system (Pentax, Tokyo, Japan). This high definition endoscope incorporated surface enhancement (at +2, +4 and +6 levels), color enhancement (+4, +5 and +6 levels) and tone enhancement functions. It is also equipped with an adjustable image magnification in a continuous range up to 100-fold. The diameter and the length of the insertion tube of this instrument are the same as those of a standard upper endoscope. To achieve the maximum magnification, a transparent hood was attached to the distal tip of the endoscope to fix the distance between endoscope and gastric mucosa at 2 mm.

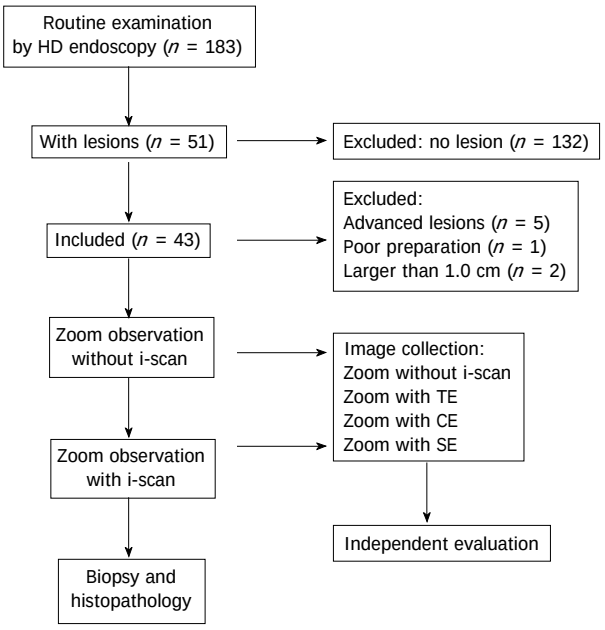

Figure 1 Study flow diagram. TE: Tone enhancement; SE: Surface enhancement; CE: Color enhancement.

All the patients underwent routine preparation before the procedure. The detected lesions were observed with magnification endoscopy in white light (MWL) mode and in enhancement (M i-scan) mode consecutively. The endoscopic procedures were performed by an experienced endoscopist who was familiar with magnification endoscopy diagnosis of early gastric cancer. The endoscopist was required to give the real-time descriptions of surface pit patterns of the lesions, based on surface pattern classification of enhanced magnification endoscopy. The surface pattern classification includes 5 types: type Ⅰ, small round pits of uniform size and shape; type Ⅱ, slit-like pits; type Ⅲ, gyrus and villous patterns; type Ⅳ, irregular arrangement and size; and type Ⅴ, destructive pattern. According to previous reports, types Ⅰ-Ⅲ represent non-neoplastic lesions, and types Ⅳ-Ⅴ represent neoplastic lesions[7]. Real-time diagnoses to determine neoplasia or non-neoplasia were not required from the endoscopist. Instead, the diagnoses were made by another investigator according to the diagnostic strategy and real-time description above. Images of MWL [without tone enhancement (TE), surface enhancement (SE) and color enhancement (CE)] and i-scan (with "g" TE, +2 SE or +4 CE) were collected and stored on USB devices during the procedures. Four best quality images per lesion were selected and sorted randomly by the investigator.

Post-endoscopy still image analysis

The selected images were sent to another endoscopist who did not participate in any of the endoscopic procedures. The endoscopist was kept blind to the clinical and endoscopic information of the patients. The endoscopist rated the image quality of each lesion at 3 levels. Ratings of image quality were based on visualization of pit pattern, vessel, and demarcation line[22], which are key parameters to detect and characterize the gastric neoplasia. Rating scales of image quality were: pit pattern, 0 for unassessable, 1 for fine, 2 for excellent; vessel, 0 for invisible, 1 for visible, 2 for clearly visible; demarcation line, 0 for unassessable, 1 for fine, 2 for clear. The endoscopist then recorded the descriptions of the still images according to the same standards as applied in the real time observation[7].

Biopsy and histopathology

The lesions were routinely biopsied, and the specimens were placed in 10% formalin solution and processed in the routine manner. The slices were examined by an experienced pathologist who had specific training in gastrointestinal pathology. The pathologist was kept blind to the clinical and endoscopic information of the patients. The histology report was based on the WHO (World Health Organization) classification of gastrointestinal tumors. The study flow diagram is illustrated in Figure 1.

Statistical analysis

Diagnostic accuracy of gastric neoplasia by using real-time M i-scan was presented with sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and likelihood ratio (LR). The agreement between real time M i-scan and histopathology was presented with kappa values (0.1-0.2 were considered slight agreement, 0.21-0.4 fair agreement, 0.41-0.6 moderate agreement, 0.61-0.8 substantial agreement and 0.81-0.99 almost perfect agreement). Parameters of still image quality were presented as mean ± SD, and differences of magnification image quality between MWL and i-scan were determined by one-way ANOVA test. A P value < 0.05 is considered to be significant. All data were analyzed by SPSS 13.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Patients

One hundred and eighty-three patients were recruited. Five patients were excluded for advanced gastric lesions, 1 patient was excluded for poor preparation and 2 patients were excluded for superficial lesions larger than 1.0 cm; 132 patients were excluded for no lesions found by high definition endoscopy. In the end, 43 patients with 43 lesions were included. The average age of the patients was 47.5 (18-74) years, of which 32 were males. Locations of the lesions were: 5 in cardia and fundus, 2 in body, 4 on angle and 32 in antrum. All the lesions could be easily identified and zoomed. Histopathology revealed 10 inflammation, 14 atrophy, 10 metaplasia, 1 low grade dysplasia (LGD), 5 high grade dysplasia (HGD) and 3 cancers.

Real-time diagnosis by M i-scan

For 7 lesions classified into type Ⅰ, histopathology revealed 6 atrophy and 1 metaplasia; for 10 lesions classified into type Ⅱ, histopathology revealed 2 inflammation, 7 atrophy and 1 metaplasia; for 10 lesions classified into type Ⅲ, histopathology revealed 1 inflammation, 8 meta-

Table 1 Histopathology and pit patterns of the lesions classified by M i-scan

Pit	Histology						Total
	Inflammation	Atrophy	Metaplasia	LGD	HGD	Cancer	
Type Ⅰ	0	6	1	0	0	0	7
Type Ⅱ	2	7	1	0	0	0	10
Type Ⅲ	1	0	8	1	0	0	10
Type Ⅳ	4	1	0	0	4	0	9
Type Ⅴ	3	0	0	0	1	3	7
Total	10	14	10	1	5	3	43

HGD: High grade dysplasia; LGD: Low grade dysplasia.

plasia and 1 LGD; for 9 lesions classified into type Ⅳ, histopathology revealed 4 inflammation, 1 atrophy and 4 HGD; for 7 lesions classified into type Ⅴ, histopathology revealed 3 inflammation, 1 HGD and 3 cancers. The real-time descriptions of pit patterns and the corresponding histopathology are shown in Table 1. Typical images representing pit patterns of types Ⅰ-Ⅴ are illustrated in Figure 2.

When the histopathology was re-classified into 2 categories (as non-cancerous lesions including inflammation, atrophy, metaplasia and LGD, or cancerous lesions including HGD and cancer) and the pit patterns re-classified into 2 categories as described above, sensitivity, specificity, PPV, NPV and likelihood ratio of M i-scan regarding gastric neoplasia were 100%, 77.1%, 50%, 100% and 4.37% respectively. Kappa value calculated from agreement between M i-scan and histopathology was 0.557 (moderate agreement). The diagnostic yield after re-classification is shown in Table 2.

Post-endoscopy still image analysis

A total of 172 still images, including 43 images by MWL and 129 images by M i-scan (43 with TE, 43 with SE and 43 with CE), were selected and sent to the endoscopist who did the analysis. General image quality of M i-scan with TE and SE was significantly better than that of MWL (TE, 4.55 ± 1.07; SE, 4.30 ± 1.02; MWL, 3.25 ± 0.99; $P < 0.001$). Regarding the 3 key parameters, visualization of pit pattern was significantly improved by M i-scan with SE (1.93 ± 0.25 *vs* 1.50 ± 0.50, $P < 0.001$). Microvessel visualization was significantly improved by M i-scan with TE (1.23 ± 0.78 *vs* 0.76 ± 0.73, $P < 0.001$). Demarcation line visualization was improved by both M i-scan with TE and SE (TE, 1.75 ± 0.52; SE, 1.56 ± 0.59; MWL, 0.98 ± 0.44; $P < 0.001$). M i-scan with CE did not show any significant improvements of image quality in general or in the 3 key parameters.

Descriptions of the still images based on lesions demonstrated that diagnosis by MWL revealed a sensitivity, specificity, PPV, NPV and LR of 87.5%, 71.4%, 41.2%, 96.2% and 3.06%, respectively. Although M i-scan with TE and SE slightly increased the diagnostic yield, there was no significant difference ($P > 0.1$). M i-scan with CE did not change the diagnostic yield by MWL. M i-scan with SE perfectly matched the results of real-time

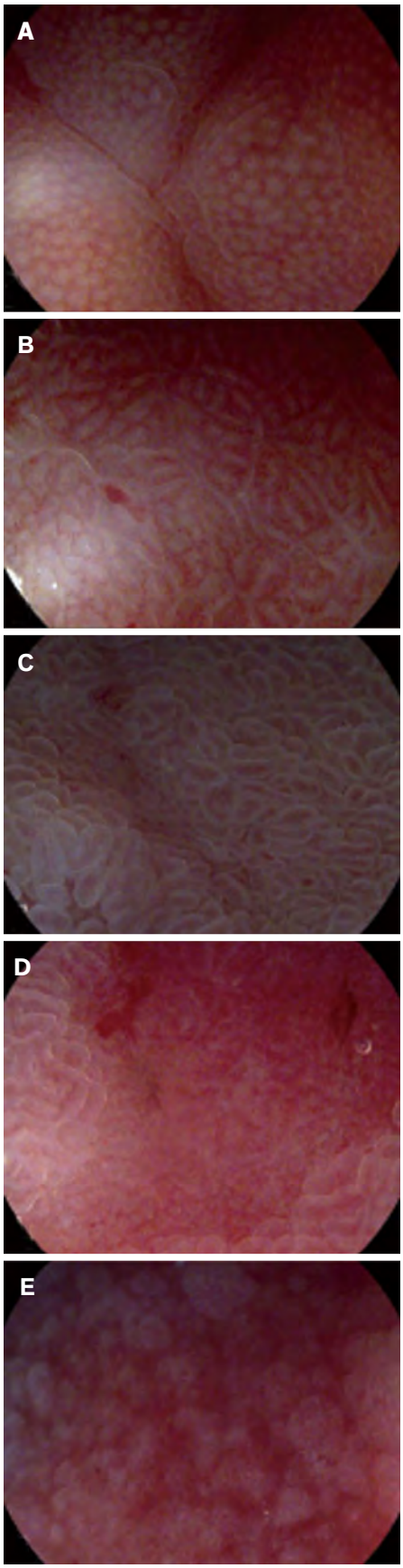

Figure 2 Images representing typical pit pattern classification by M i-scan. A: Type Ⅰ, small round pits of uniform size and shape; B: Type Ⅱ, slit-like pits; C: Type Ⅲ, gyrus and villous patterns; D: Type Ⅳ, irregular arrangement and size; E: Type Ⅴ, destructive pattern.

Table 2 Diagnostic yield of gastric neoplasia by real time M i-scan

M i-scan	Histopathology		Total
	Cancerous	Non-cancerous	
Neoplasia	8	8	
Non-neoplasia	0	27	
Total	8	35	43

Sensitivity: 100%; Specificity: 77.1%; Positive predictive value: 50%; Negative predictive value: 100%; Likelihood ratio: 4.37.

descriptions. Representative images showing image quality differences among different modes are illustrated in Figure 3.

DISCUSSION

Detection and characterization of early gastric cancer by dyeless virtual chromoendoscopy, such as NBI and computed virtual chromoendoscopy (CVC), are preferable for the endoscopist, because of time, labor and potential risks reduction[31,32]. Virtual chromoendoscopy with magnification is thought to be the dream team, since the former provides the detection "red flag" followed by real-time characterization by the latter. It was reported that real-time characterization of Barrett's esophagus[33,34], gastric cancer[35,36] and colorectal adenoma[37,38] can be improved by dyeless virtual chromoendoscopy, such as NBI or FICE[25-29,39]. In this pilot feasibility study, we aimed to evaluate application of M i-scan in the diagnosis of small superficial gastric lesions, both in real-time investigation and post-endoscopy still image analysis. The preliminary results showed that M i-scan is helpful for the *in vivo* prediction of small gastric superficial lesions with excellent sensitivity and NPV, acceptable specificity and LR, and poor PPV. The post-endoscopy still image analysis showed that M i-scan with TE and SE can slightly increase the image quality.

One feature of M i-scan is to mimic the surface enhancement of EME by acetic acid spraying. In this study, the still image analysis showed that SE significantly improves visualization of surface pit pattern and demarcation line compared to MWL. Although there were excellent sensitivity and NPV, and acceptable specificity results, the PPV was poor, just as the results of enhanced magnification endoscopy[7]. This is partly due to the low percentage of neoplastic lesions in the sample (18.6%, 8/43). On the other hand, erosion is sometimes difficult to be differentiated from neoplasia by surface pit pattern evaluation, as in both lesions surface pits could be lost. In these cases, evaluation of microvessel pattern in addition to surface pit pattern may be helpful. However, observation of microvessels is not satisfactory by M i-scan. Although still image analysis shows that TE significantly improves the visualization of microvessels, which only happens in cases with visible microvessels (visible to clearly visible), visualization of those cases with invisible microvessels (41.2%) remains unchanged.

This study has several limitations. Firstly, this is a feasibility study with small sample size and no sample size calculation. Secondly, the detection rate of small superficial gastric lesions was not evaluated. There has not been any report on the detection rate of small gastric lesions by CVC yet. In our own practice, CVC is not suitable for screen gastroscopy with insufficient luminous intensity. Thirdly, only one endoscopist performed the real-time and still image analysis, so there was no interobserver agreement analysis. However, the perfect match between surface classification of real-time and still image with

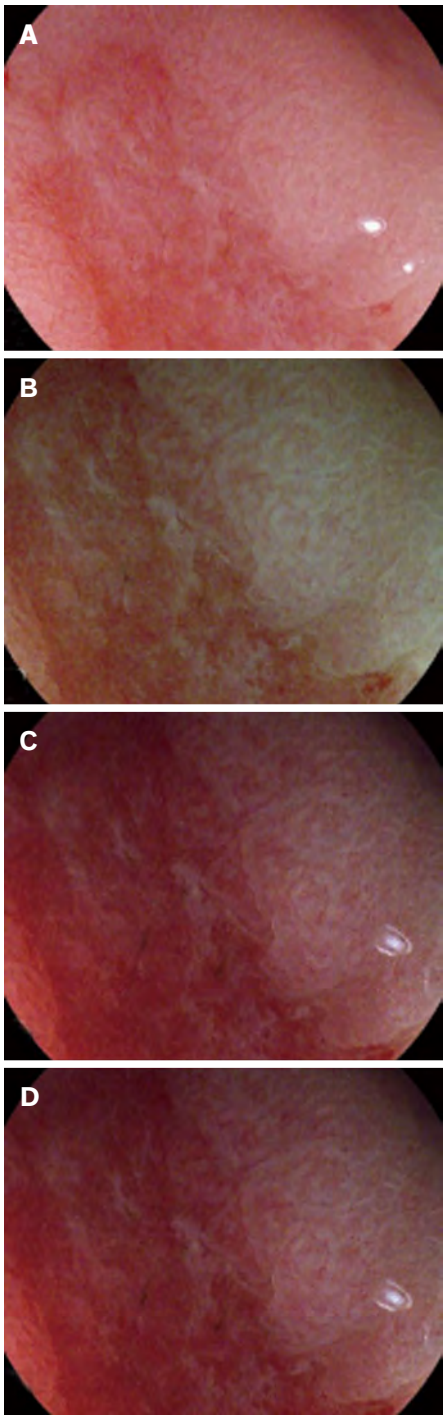

Figure 3 Representative images showing white light (A), M i-scan with tone enhancement (B), surface enhancement (C) and color enhancement (D), respectively.

SE suggests an excellent consistency, which should be validated in future studies. Fourthly, there was no comparison between M i-scan and magnification chromoendoscopy with indigo carmine or other contrast agents. And finally (the last may not be the least), gold standard histopathology was only performed by biopsy. Although we only included lesions smaller than 1.0 cm to minimize the heterogeneity, a discrepancy between biopsy and autopsy still remains.

In conclusion, real-time prediction of the histopathology of small superficial gastric lesions by M i-scan is feasible. Although digital enhancement increases image quality, its value in the diagnosis of gastric neoplasia seems to be limited.

COMMENTS

Background

Magnified chromoendoscopy is a promising tool in the surveillance and diagnosis of gastric neoplasia. Enhanced magnification endoscopy is superior to conventional endoscopy with detailed surface characterization.

Research frontiers

Dyeless virtual chromoendoscopy with magnification might be preferable for reduction of labor and health risks. The endoscope used in this study is a magnification endoscope with both color and surface enhancement.

Innovations and breakthroughs

To date, this is the first endoscopic device with surface enhancement mimicking acetic acid spraying enhanced magnification endoscopy. With the surface enhancement, the gastric pit patterns can be classified into 5 categories according to the classification from enhanced magnification endoscopy, which enables the detailed characterization of the gastric mucosa. With classification of gastric pits, different common gastric pathologies such as atrophy, intestinal metaplasia and neoplasia can be identified in real-time procedures or by still image analysis. The margin of gastric lesions can be more easily identified although the differences were not significant.

Terminology

Although digital enhancement improves the image quality of magnification endoscopy, its value in improving the diagnostic yield seems to be limited.

Peer review

This is a quite interesting study on virtual chromoscopy on gastric neoplasia. However, data are limited.

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BRIEF ARTICLE

Effects of propranolol or propranolol plus isosorbide-5-mononitrate on variceal pressure in schistosomiasis

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Abstract

AIM: To compare the effects of propranolol (PR) to that of PR plus isosorbide-5-mononitrate (ISMN) on variceal pressure in patients with schistosomiasis.

METHODS: Forty-eight patients with schistosomiasis who had no previous variceal bleeding were treated with PR alone or PR plus ISMN. Seven patients refused variceal pressure manometry (3 receiving PR and 4 receiving PR plus ISMN). One patient withdrew from the trial due to headache after taking ISMN. At the time of termination, twenty patients were randomly assigned to treatment with PR plus ISMN or PR alone. The dose of PR was adjusted until the resting heart rate had been reduced by 25% or was less than 55 bpm. In the PR plus ISMN group, after PR was titrated to the same target, the dose of ISMN was increased up to 20 mg orally twice a day. Variceal pressure was measured using a noninvasive endoscopic balloon technique at the end of the 6-mo treatment period.

RESULTS: In 40 patients (20 in the PR group and 20 in the PR plus ISMN group), variceal pressure was measured before treatment and at the end of the 6-mo treatment period. PR or PR plus ISMN treatment caused a significant reduction in variceal pressure (PR group: from 24.15 ± 6.05 mmHg to 22.68 ± 5.70 mmHg, P = 0.001; PR plus ISMN group: from 25.69 ± 5.26 mmHg to 20.48 ± 5.43 mmHg; P < 0.001). The percentage decrease in variceal pressure was significant after PR plus ISMN compared with that after PR alone (15.93% ± 8.37% *vs* 6.05% ± 3.67%, P = 0.01). One patient in the PR plus ISMN group and two patients in the PR group had variceal bleeding during follow-up. There were no significant differences between the two groups regarding the incidence of variceal bleeding. In the PR plus ISMN group, three patients had headache and hypotension. The headache was mild and transient and promptly disappeared after continuation of the relevant drug in two patients. Only one patient withdrew from the trial due to severe and lasting headache after taking ISMN. No side effects occurred in the PR group.

CONCLUSION: PR plus ISMN therapy may be an alternative treatment for patients with schistosomiasis who have a high risk of bleeding.

Key words: Esophageal varices; Schistosomiasis; Portal hypertension; Bleeding; Propranolol; Variceal pressure; Isosorbide-5-mononitrate

Core tip: The results of the present study suggested that the combination of propranolol and isosorbide-5-mononitrate was more effective than propranolol alone in decreasing variceal pressure. This drug combination will reduce the rate of bleeding in patients with schistosomiasis, high-risk esophageal varices and no previous history of variceal bleeding.

Kong DR, Ma C, Wang M, Wang JG, Chen C, Zhang L, Hao JH, Li P, Xu JM. Effects of propranolol or propranolol plus isosorbide-5-mononitrate on variceal pressure in schistosomiasis. *World J Gastroenterol* 2013; 19(26): 4228-4233 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4228.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4228

INTRODUCTION

Variceal bleeding is the most frequent and severe complication of portal hypertension in patients with cirrhosis. Identification of those who have a high risk of variceal hemorrhage is effective for preventive therapy in patients with a high disease predisposition[1]. Variceal size and the red color sign are considered to be the most important endoscopic parameters in predicting variceal bleeding[2]. However, endoscopic findings alone can not be used to reliably predict the risk of variceal bleeding. The formation of esophageal varices depends on an elevation in portal pressure; a hepatic venous pressure gradient (HVPG) greater than 10 mmHg is necessary for the development of and bleeding from esophageal varices[3-6]. On the other hand, a more rational approach would be to guide pharmacologic therapy based on hemodynamic response, defined as a decrease in HVPG to < 12 mmHg or a decrease of > 20% from baseline levels[7]. However, limitations to the generalized use of HVPG measurement are the lack of local expertise and poor adherence to guidelines that will ensure reliable and reproducible measurements, and its invasive nature[5]. In the majority of published studies, the dose of nonselective β-blockers was titrated to decrease the heart rate by 25% from baseline or maximal tolerated doses[5,7].

Propranolol (PR) or isosorbide-5-mononitrate (ISMN) is effective in preventing the first variceal bleeding in patients with cirrhosis[1,5]. ISMN enhances the reductive effect of PR on variceal pressure in cirrhotic patients[1,5]. In contrast to liver cirrhosis, published data regarding the effect of PR on schistosomiasis-related portal hypertension are scarce and contradictory, and the effect of ISMN plus PR treatment is unknown in these patients[8,9]. A short-term study in patients with schistosomiasis and previous variceal bleeding after PR treatment found that the portal pressure was not decreased[8]. Moreover, the required mean dose to achieve a 20%-25% reduction in heart rate from baseline was up to 400 mg/d[8]. Cohort studies indicated that PR treatment achieved a reduction in rebleeding rates and increased the survival of patients with no serious side effects[9]. Recently, a study from Brazil found that PR significantly reduced variceal pressure in schistosomiasis patients who had never bled[10]. However, it is not clear whether ISMN plus PR is better than PR alone in the treatment of schistosomiasis patients who had never bled. In this study, we will ascertain whether the combination of PR and ISMN is more effective than PR alone in decreasing variceal pressure.

MATERIALS AND METHODS

Selection of patients

From September 2007 to October 2010, patients admitted to our hospital due to schistosomiasis-related portal hypertension were assessed for inclusion in the trial. The diagnosis of schistosomiasis was established in accordance with the World Health Organization criteria[11]. The eligibility criteria were age between 18 and 65, schistosome eggs in stool specimens, the characteristic ultrasound criteria, and endoscopic evidence of esophageal varices. The exclusion criteria were previous treatment for portal hypertension (*e.g.*, beta-blockers, sclerotherapy, or endoscopic band ligation), severe hepatic disease (*e.g.*, Child-Pugh score higher than 12 points or hepatorenal syndrome), previous variceal bleeding, presence of any neoplastic disease, portal vein thrombosis, inability to attend follow-up, contraindications to beta-blockers (severe chronic pulmonary obstructive disease, asthma, severe insulin-dependent diabetes mellitus, heart failure, grade II atrioventricular block, sinus bradycardia < 50 bpm, aortic stenosis, peripheral arterial disease, arterial hypotension with systolic pressure < 85 mmHg), or long-acting nitrates (glaucoma). The study was approved by the Ethics Committee of Anhui Medical University, and all patients gave written informed consent to participate in the study. Patients were assigned to one of two treatment groups according to the sequential method of randomization.

Treatment

Patients who fulfilled the inclusion and exclusion criteria were immediately randomized into the two treatment groups using consecutively numbered envelopes that contained the treatment assignments, which were generated by a system using computer-allocated random digit numbers. PR was given orally at an initial dose of 20 mg 3 times daily. The dose was subsequently adjusted over a period of 5 d until the resting heart rate had been reduced by 25% or was less than 55 bpm. In the PR plus ISMN group, after PR was titrated to the same target in resting heart rate, the dose of ISMN was increased up to an oral dose of 20 mg twice a day.

Methods

Measurement of variceal pressure was performed after

Table 1 Demographic profile of the study population

	PR group (n = 20)	PR + ISMN group (n = 20)	P value
Sex			0.619
Male	12	11	
Female	8	9	
Age (yr)	47.87 ± 15.16	44.14 ± 9.51	0.585
Child-Pugh grade			1.000
A	9	8	
B	11	12	
Child-Pugh score	8.87 ± 1.88	8.00 ± 1.63	0.358
Albumin (g/L)	30.63 ± 3.82	33.34 ± 5.30	0.271
Total bilirubin (μmol/L)	29.45 ± 17.02	25.11 ± 11.26	0.577
Prothrombin time (s)	16.80 ± 1.82	16.65 ± 1.59	0.875
VP (mmHg)	24.15 ± 6.05	25.69 ± 5.26	0.248
Varix grade			0.608
F2	10	9	
F3	10	11	
Red color signs	12	14	1.000

VP: Variceal pressure; PR: Propranolol; ISMN: Isosorbide-5-mononitrate.

an overnight fast during upper gastrointestinal endoscopy. Variceal pressure was assessed with a previously described noninvasive technique using an esophageal variceal manometer (EVM; Esophageal Varix Manometer; Treier Endoscopie AG, Beromünster, Switzerland) and recorded by the workstation which was developed by our group[12,13]. To minimize esophageal tonus and peristalsis, all patients received premedication with 5 mg diazepam and 20 mg *n*-butylscopolamine intravenously. The reliability of the endoscopic measurement of variceal pressure was determined in a previous study which found a good correlation with needle puncture measurement[13-15]. In the current study, endoscopic measurement of variceal pressure was used because of the unique hemodynamic pattern of pre-sinusoidal portal hypertension. The largest varix situated above the cardia was chosen for measurement of variceal pressure. The pressure in each patient was measured five times. Variceal pressure was calculated as the mean of five satisfactory measurement periods recorded.

After variceal pressure measurement, the size of the varix was estimated in the absence of peristaltic waves, by comparing the varix with the scales in the balloon variceal markers (5-mm intervals). The maximal size of the varices and the red color signs were recorded as proposed by the Japanese Research Society for portal hypertension[16].

Follow-up and endpoints

All patients were followed in the outpatient clinics at 3-month intervals and assessed for adverse events, compliance (direct questioning, prescription renewal, and reinforcement), variceal bleeding, and progression of liver disease. Variceal pressures in all patients were measured before and after 6 mo of continuous PR or PR plus ISMN therapy. The primary end point was variceal bleeding and secondary end points were treatment-related complications and mortality. Variceal bleeding was defined as hemetemesis or melena, with an associated drop in hematocrit by 10%, in the absence of any other source of gastrointestinal bleeding on endoscopy. In the case of variceal bleeding, physicians were free to choose endoscopic treatment to prevent rebleeding.

Statistical analysis

Statistical analyses were performed with SPSS (version 10; SPSS, Inc., Chicago, IL, United States). All quantitative data were tested for normal distribution. Quantitative data were expressed as mean ± SD if the data were normally distributed. Each continuous parameter was analyzed with the independent-samples t-test. The paired-samples t-test was used to examine change from baseline to follow-up. Categorical data were examined using Fisher's exact test. P-values < 0.05 were considered statistically significant.

RESULTS

Baseline data

Twenty-five patients received PR plus ISMN and 23 patients received PR alone (dosage of PR: 60 to 160 mg/d, median: 80 mg; dosage of ISMN: 20 mg/d). Seven patients refused to variceal pressure manometry (3 receiving PR and 4 receiving PR plus ISMN). One patient withdrew from the trial due to headache after taking ISMN. Therefore, there were 20 patients in each treatment group. Clinical and endoscopic data of the patients in the subsets are shown in Table 1. There were no significant differences between the two groups at baseline with regard to clinical and demographic characteristics or baseline variceal pressure (Table 1, PR group = 24.15 ± 6.05 mmHg; PR plus ISMN = 25.69 ± 5.26 mmHg).

Changes in variceal pressure

In 40 patients (20 in the PR group and 20 in the PR plus ISMN group), variceal pressure was measured again the end of a 6-mo continuous treatment period. PR or PR plus ISMN caused a significant reduction in variceal pressure (PR group: from 24.15 ± 6.05 mmHg to 22.68 ± 5.70 mmHg, P = 0.001; PR plus ISMN group: from 25.69 ± 5.26 mmHg to 20.48 ± 5.43 mmHg; P < 0.001). The percentage decrease in variceal pressure after PR plus ISMN was more significant than that after PR alone (Table 2, 15.93% ± 8.37% *vs* 6.05% ± 3.67%, P = 0.01).

Bleeding

One patient in the PR plus ISMN group and two patients in the PR alone group had variceal bleeding during the 6-mo follow-up period. There were no significant differences between the two groups regarding the incidence of variceal bleeding.

Adverse effects

In the PR plus ISMN group, three patients had headache and hypotension. The headache was mild and transient and promptly disappeared after continuation of the rel-

Table 2 Effects of propranolol and propranolol plus isosorbide-5-mononitrate on variceal pressure, liver function and systemic hemodynamics in patients with 6 mo of follow-up

	PR		PR + ISMN	
	Baseline	6 mo	Baseline	6 mo
(ΔVP)%	0	15.93 ± 8.37	0	6.05 ± 3.67[a]
ALB (g/L)	30.63 ± 3.82	31.14 ± 3.08	33.34 ± 5.30	34.30 ± 5.09
TB (umol/L)	29.45 ± 17.02	27.26 ± 12.27	25.11 ± 11.26	26.74 ± 12.96
SBP (mmHg)	132 ± 20	124 ± 21[d]	130 ± 19	125 ± 19[d]
DBP (mmHg)	77 ± 10	72 ± 11[d]	74 ± 10	70 ± 13[d]

[a]$P < 0.05$ *vs* PR group, [d]$P < 0.01$ *vs* baseline. (ΔVP)%: Percentage difference in variceal pressure from baseline; PR: Propranolol; ISMN: Isosorbide-5-mononitrate; ALB: Albumin; TB: Total bilirubin; SBP: Systolic blood pressure; DBP: Diastolic blood pressure.

evant drug in two patients. One patient withdrew from the trial due to severe and lasting headache after taking ISMN. No side effects occurred in the PR group. There was no worsening of liver function or impairment of renal function in the 2 groups within the 6-mo treatment period (Table 2).

DISCUSSION

Nonselective β-blockers are the most commonly used drugs to prevent variceal bleeding in patients with cirrhosis and esophageal varices[6,14]. Although many trials have shown that variceal hemorrhage risk was reduced with β-blockers, these drugs do not protect all treated patients, probably due to an inadequate decrease in the HVPG[5,17]. Most published studies have shown that PR and ISMN have a synergistic effect on reducing portal pressure and a combination of the two could be more effective than PR alone[17,18]. Recently, PR was found to significantly reduce variceal pressure and wall tension in patients with schistosomiasis[10]. However, it is uncertain whether the combination of PR and ISMN is more effective than PR alone in decreasing variceal pressure in schistosomiasis patients who have never bled.

This study investigated the efficacy of PR compared with PR plus ISMN in schistosomiasis patients that had never bled. Our approach was to assess variceal pressure in patients with high-risk varices, using the same methodology reported for cirrhotic patients[12]. Variceal bleeding is believed to occur when the tension exerted over the thin wall of the varices increases beyond a critical value determined by the elastic limit of the vessel[5]. Variceal pressure and size are key factors determining variceal wall tension. Not only is variceal pressure the best parameter for predicting rupture of varices and consequent complications, but it is also a useful guide for studying the effect of the pharmacotherapy of portal hypertension and a measure of the effects of transjugular intrahepatic portosystemic shunting[6,19-21]. As confirmed by one study, the measurement of variceal pressure can efficiently monitor the direct effect of the prophylaxis of variceal bleeding compared with the rate of bleeding in cirrhotic patients[17].

In the present study, we observed that PR and PR plus ISMN administration caused a significant reduction in variceal pressure in patients with schistosomiasis. After a 6-mo continuous treatment period, the percentage decrease in variceal pressure was more obvious in patients receiving PR plus ISMN than PR alone (15.93% ± 8.37% *vs* 6.05% ± 3.67%, $P = 0.01$). Thus, the results of our study suggest that PR plus ISMN is superior to PR alone in reducing variceal pressure in patients with schistosomiasis. These results are consistent with the data from different randomized clinical trials which show that the effect in patients treated with combined pharmacological therapy was greater than that obtained with PR alone[17,18]. Therefore, the pharmacological therapy of choice in the prevention of variceal bleeding is probably the combination of PR and ISMN.

The mean dosage of PR used in our study was lower than that in other studies for cirrhotic patients and schistosomiasis patients[1,5]. However, the low dosage of PR in the current study was expected because it is well know that the metabolism of this drug is different between Asian and European patients[22]. In a previous study, Lay *et al*[23] found that the mean daily dosage of PR was 68.2 ± 32.8 mg, which was sufficient to reduce the heart rate by 25%. Therefore, it is possible that a lower dosage of PR to reach a target heart rate reduction of 25% would have enough power to result in a lower bleeding rate in a Chinese population.

Three patients treated with PR plus ISMN experienced side effects. Most reported side effects caused by β-blockers (hypotension, tiredness, breathlessness, poor memory, insomnia) can be easily managed by adjusting the dose of the medication, which does not affect the treatment effect. ISMN may increase vasodilatation leading to more side effects such as headache and hypotension[1,5]. In a trial performed in patients with cirrhosis and ascites which compared β-blockers with ISMN, the latter medication was associated with more side effects[24]. Furthermore, other studies also found a trend toward more side effects requiring withdrawal of the combination therapy compared with PR alone[17,18,25]. In our study, two patients experienced mild and transient headache on the first administration of ISMN, which disappeared after continuation of the relevant medication. One patient withdrew from the trial due to severe and lasting headache after taking PR plus ISMN. When the resting heart rate was reduced by 25% or was less than 55 bpm, most patients showed a significant reduction in variceal pressure (15.93% ± 8.37%) after receiving PR plus ISMN.

We are aware of the limitations of the current study. First, we found that the measurement of variceal pressure is technically difficult and time consuming in patients with small varices, which may reduce the applicability of measurements in clinical practice. However, because very large varices and red color signs indicate imminent bleeding, these patients are at high risk of bleeding and require prophylactic measures even though their variceal pressure

is not high[26,27]. On the other hand, the measurement of variceal pressure is probably not very important in patients with very small varices due to rare bleeding[28-31]. Second, patients not suitable for PR plus ISMN therapy need to be investigated in future studies. Third, future randomized controlled studies with a larger number of patients are warranted to confirm these findings and to demonstrate the long-term decrease in the frequency of bleeding episodes and mortality.

In conclusion, we found that combination treatment with PR plus ISMN compared with PR alone, more effectively decreased variceal pressure in schistosomiasis patients. Future randomized controlled studies with a larger number of patients are warranted to demonstrate the long-term decrease in variceal pressure, to determine when side effects will outweigh the benefits, and monitor the frequency of bleeding episodes and mortality.

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COMMENTS

Background

Non-cirrhotic portal hypertension and gastrointestinal bleeding are complications of the infection caused by the intravascular parasitic trematode *Schistosoma mansoni*. The prophylactic treatment of variceal bleeding is therefore crucial in the management of these patients.

Research frontiers

Treatment with propranolol plus isosorbide-5-mononitrate resulted in a synergistic decrease in variceal pressure compared with propranolol alone in cirrhotic patients. However, this has not been demonstrated in non-cirrhotic portal hypertension caused by *Schistosoma mansoni* infection.

Innovations and breakthroughs

In this study, the authors found that the combination of propranolol and isosorbide-5-mononitrate was more effective than propranolol alone in decreasing variceal pressure, which is important in reducing the rate of bleeding in patients with schistosomiasis, high-risk esophageal varices and no previous history of variceal bleeding.

Applications

The results suggest that the combination of propranolol plus isosorbide-5-mononitrate should be recommended as the first prophylaxis of variceal bleeding in non-cirrhotic portal hypertension caused by *Schistosoma mansoni* infection. Additional studies with long-term follow-up are needed to confirm the results concerning mortality.

Peer review

The authors have compared the effect of propranolol alone with the combination of propranolol and isosorbide-5-mononitrate on variceal pressure in patients with portal hypertension due to schistosomiasis. The results suggested that the combination led to a more pronounced decrease of variceal pressure than propranolol did.

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META-ANALYSIS

Hepatitis B or C viral infection and risk of pancreatic cancer: A meta-analysis of observational studies

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Abstract

AIM: To investigate if there is an association between hepatitis B virus (HBV) or hepatitis C virus (HCV) infection and the risk of pancreatic cancer.

METHODS: All relevant studies published before 11 October, 2012 were identified by a systematic search of MEDLINE, EMBASE, BIOSIS Previews and the Cochrane Library databases and with cross-referencing. The observational studies that reported RR or OR estimates with 95%CIs for the association between HBV or HCV and pancreatic cancer were included. A random-effects model was used to summarize meta-analytic estimates. The Newcastle-Ottawa quality assessment scale was applied to assess the quality of the methodology in the included studies.

RESULTS: A total of 8 eligible studies were selected for meta-analysis. Overall, chronic hepatitis B and inactive hepatitis B surface antigen (HBsAg) carrier state (HBsAg positive) had a significantly increased risk of pancreatic cancer with OR of 1.20 (95%CI: 1.01-1.39), especially in the Chinese population (OR = 1.30, 95%CI: 1.05-1.56). Past exposure to HBV (possible occult HBV infection) had an increased OR of pancreatic cancer risk (OR = 1.24, 95%CI: 1.05-1.42), especially among those patients without natural immunity [anti hepatitis B core (HBc) positive/hepatitis B surface antibody (anti HBs) negative], with OR of 1.67 (95%CI: 1.13-2.22). However, past exposure to HBV with natural immunity (anti-HBc positive/anti-HBs positive) had no association with pancreatic cancer development, with OR 0.98 (95%CI: 0.80-1.16), nor did the HBV active replication (hepatitis B e antigen positive status), with OR 0.98 (95%CI: 0.27-1.68). The risk of pancreatic cancer among anti-HBs positive patients was significantly lower than among anti-HBs negative patients (OR = 0.54, 95%CI: 0.46-0.62). Past exposure to HCV also resulted in an increased risk of pancreatic cancer (OR = 1.26, 95%CI: 1.03-1.50). Significant between-study heterogeneity was observed. Evidence of publication bias for HBV/HCV infection-pancreatic cancer association was not found.

CONCLUSION: Chronic HBV and HCV infection increases pancreatic cancer risk. Our findings underscore the need for more studies to confirm this potential relationship.

Key words: Hepatitis B; Hepatitis C; Pancreatic cancer; Observational studies; Meta-analysis

Core tip: Based on the meta-analysis, we identified that chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection is associated with pancreatic cancer, especially among Chinese population. Patients with past exposure to HBV/HCV should be screened for hepatocellular carcinoma and other malignancies, especially pancreatic cancer.

Xu JH, Fu JJ, Wang XL, Zhu JY, Ye XH, Chen SD. Hepatitis B or C viral infection and risk of pancreatic cancer: A meta-analysis of observational studies. *World J Gastroenterol* 2013; 19(26): 4234-4241 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4234.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4234

INTRODUCTION

Pancreatic cancer is one of the most lethal and devastating human malignancies and the fourth leading cause of cancer-related fatality worldwide. Because of absence of early symptoms, lack of sensitive and specific tests to screen the cancer in the initial phases, limited therapeutic options and rapid progression, nearly all patients die of the disease within one year of diagnosis with the overall 5-year survival rate being less than 5%[1]. Therefore, it is crucial to identify the intrinsic genes as well as other risk factors, that may influence the progression of the cancer, and to develop more accurate screening programs for early monitoring and intervention strategies. Although several risk factors associated with pancreatic cancer have been explored, the causative factors for pancreatic cancer are far from being understood, one of which is chronic hepatitis infection[2,3].

The prevalence of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection varies worldwide, ranging from less than 0.5% in Western countries to 7% and 25% in East Asian and African countries[4,5]. Infection with HBV is a huge global public health concern, especially in China. According to the World Health Organization, there are 2 billion people infected with HBV globally, with China accounting for 65% of the HBV infective public health burden of the world[6]. HBV/HCV have been detected not only in hepatotropic tissue but also in extrahepatic sites such as the pancreas[7,8]. Several studies reported conflicting results regarding the association between HBV infection and the risk of subsequent pancreatic cancer. Studies by Berrington de Gonzalez *et al*[9] and Hong *et al*[10] found no relationship between chronic infection with HBV and the development of pancreatic cancer, while several published studies found an association between presence of HBV infection and incidence of pancreatic cancer, not only in countries with a lower prevalence of HBV infection such as the United States but also in countries with a higher number of HBV infections such as China[11,12]. Meanwhile, little is known about the association between the presence of HCV infection and the risk of pancreatic cancer[3].

The purpose of the present study is to summarize all available evidence of observational studies in order to better define the impact of HBV and HCV infection on the risk of pancreatic cancer in patients following the meta-analysis of observational studies in epidemiological guidelines.

MATERIALS AND METHODS

Data sources and searches

A comprehensive literature search was carried out on observational studies and trials, and no language or time restriction was applied. All literature from January 1, 1980 to October 18, 2012 was searched using the following databases: Pubmed, ISI web of Science, Embase and Cochrane library. The following main keywords or corresponding MeSH terms were used: hepatitis, virus, viral, pancreas, cancer OR adenocarcinoma OR neoplasm OR tumor. A manual search was also performed for references cited in the selected articles.

Study selection

Studies were included in the meta-analysis if (1) they were the principal published reports of original data from case-control or cohort studies; (2) they were independent from other studies to avoid giving double weight to estimate the same study; (3) the exposure of interest was a history of HBV/HCV infection; (4) the outcome of interest was pancreatic cancer incidence or mortality; and (5) they had sufficient information to allow adequate estimation of OR or RR and 95%CI to estimate cancer risk under HBV/HCV exposure. Two authors (Fu JJ and Xu JH) independently evaluated all of the studies retrieved from database, then compared their results. Any disagreements were resolved by consensus.

Data extraction

The following data were extracted from each study: authors, publication year, study design, country of origin, sample size, measure of outcome, duration of follow-up, marker of hepatitis serostatus, covariates adjusted for in the analysis, and the effect estimates with corresponding 95%CIs.

Quality evaluation

The Newcastle-Ottawa quality assessment scale (NOS)[13] was applied to assess the quality of the methodology in the included studies. A star system was used to judge the data according to the selection populations, comparability of groups and exposure/outcome of interest. The NOS scale consists of 8 questions with 9 possible points. The assessment score ranged from 0 to 9. Studies with a total score of 6 or lower indicated low quality while study

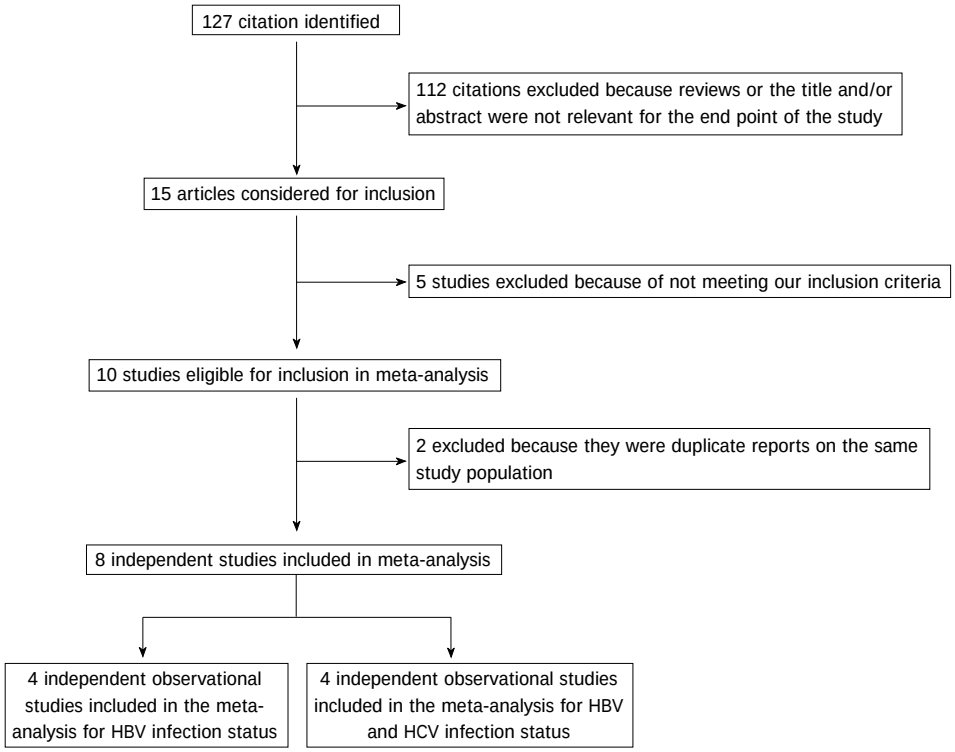

Figure 1 Flowchart of selection of studies for inclusion in meta-analysis. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Table 1 Assessment of study quality

Ref.	Quality indicators from NOS									Score
	Selection				Comparability		Exposure/outcome			
	1	2	3	4	5	6	7	8	9	
Hong *et al*[10]	Yes	Yes	No	Yes	Yes	No	Yes	Yes	No	6
Hassan *et al*[11]	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	7
Wang *et al*[18]	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	7
Zhu *et al*[17]	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	7
Ben *et al*[12]	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	7
Berrington de Gonalez *et al*[9]	Yes	Yes	Yes	Yes	Yes	No	Yes	No	No	6
El-Serag *et al*[3]	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	8
Iloeje *et al*[2]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	9

NOS: Newcastle-Ottawa quality assessment Scale. For case-control studies: (1) represents cases with independent validation; (2) cases are consecutive or representative; (3) controls are community; (4) controls have no history of pancreatic cancer; (5) study controls are comparable for age and sex; (6) study controls for any additional factor(s); (7) cases and controls have the same method of ascertainment; (8) was follow-up long enough for outcomes to occur; and (9) cases and controls have complete follow-up. For cohort studies: (1) indicates the exposed cohort study representative of the population; (2) the non exposed cohort drawn from the same population; (3) the exposure ascertainment are from secure record or structured interview; (4) the pancreatic cancer was not present at start of study; (5) cohorts are comparable for age and sex; (6) cohorts are comparable for any additional factor(s); (7) assessment of pancreatic cancer is from secure record; (8) follow-up long enough for pancreatic cancer to occur; and (9) complete follow-up.

scores of 7 or higher were considered to be of high quality. Two reviewers (Xu JH and Fu JJ) independently evaluated and cross-checked the qualities of the included studies (Table 1).

Statistical analysis

Statistical analyses were completed with STATA version 10.0 (STATA, College Station, TX, United States). Summary odd ratio estimates with the corresponding 95%CIs were combined and weighted to produce pooled ORs using a random-effects model, which considers both within- and between-study variations[14]. Q and I^2 statistics were both examined to investigate the source of heterogeneity across studies. I^2 values of 25, 50 and 75% were assigned to low, moderate, and high heterogeneities, respectively[15]. The Begg's adjusted rank correlation test and the Egger's regression test (significant at $P < 0.1$) were performed to test for evidence of publication bias[16].

RESULTS

Description of the studies

The participant flow diagram for the study inclusion in the meta-analysis is shown in Figure 1. A total of 8 articles were retrieved and checked for relevance in terms of infectious status, population studied, and reporting of pancreatic cancer risk data[2,3,9-12,17,18]. Seven of other articles were not included in the meta-analysis for the following reasons: (1) two referred to the same cohort[19,20]; (2) two were editorials responding to originated studies[21,22]; (3) one reported HCV infection and pancreatic cancer incidence in the abstract but no OR (95%CI) information was found[23]; and (4) two studies were manual search cited in the selected articles, but did not meet our inclusion criteria after reading the text[24,25].

The main characteristics of the 8 studies pooled in

Table 2 Characteristics of studies included in the meta-analysis

Ref.	Population	Study design	Country	Ethnicity	Case (*n*)	No. of control	Confirmation of HBV/HCV	Confirmation of PC	Matching criteria
Berrington de Gonalez *et al*[9]	Population-based	Cohort	South Korea	Asian	2194	628978	HBsAg	clinic diagnosed	Age, sex
Hong *et al*[10]	Hospital-based	CC	South Korea	Asian	506	1008	Anti-HCV, Anti-HBs, HBsAg	histologically confirmed	Age, sex
Hassan *et al*[11]	Hospital-based	CC	United States	White	476	879	Anti-HCV,Anti-HBs, Anti-HBc	pathologically confirmed	Age, sex, race
El-Serag *et al*[3]	Population-based	Cohort	United States	Asian	140	477	Anti-HCV, HBV	ICD-9	Age, sex
Iloeje *et al*[2]	Population-based	Cohort	Taiwan	Asian	48	22471	HBsAg, HBV DNA	pathologically confirmed	Age, sex, smoking, alcohol
Wang *et al*[18]	Hospital-based	CC	China	Asian	645	711	HBsAg, Anti-HBs, Anti-HBc	pathologically confirmed	Age, sex
Zhu *et al*[17]	Hospital-based	CC	China	Asian	80	77	HBsAg, Anti-HCV, Anti-HBc	pathologically confirmed	Age, sex
Ben *et al*[12]	Hospital-based	CC	China	Asian	943	1128	HBsAg, Anti-HBs, Anti-HBc	pathologically confirmed	Age, sex, smoking, DM, BMI

CC: Case-control study; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HBsAg: Hepatitis B surface antigen; Anti-HBs: Anti-hepatitis B surface antigen; Anti-HBc: Anti-hepatitis B core antigen; Anti-HCV: Anti-hepatitis C virus; ICD-9: International Classification of Diseases, Ninth Revision; DM: Diabetes mellitus; BMI: Body mass index.

the present analysis are reported in Table 2. All studies except one prospective study were retrospective. Five studies were case-control, and three were cohort studies conducted between 1988 and 2010 and published between 2008 and 2012. These studies included a total of 744 120 investigated patients and 3758 cases of pancreatic cancer events. Four studies were conducted in China, two in South Korea and two in the United States.

Quantitative data synthesis

Hepatitis B surface antigen (HBsAg) was seropositive in 4492 patients across all of the groups. Meta-analysis of 6 studies in a random-effects model found that compared to individuals without a history of chronic hepatitis B, those with chronic hepatitis B and in inactive HBsAg carrier state (HBsAg positive) had a 20% greater risk of pancreatic cancer (OR = 1.20, 95%CI: 1.01-1.39), with moderate heterogeneity among studies (test for heterogeneity I^2 = 29.6%, P = 0.213). To further evaluate the HBsAg carrier state associated with pancreatic cancer in the Chinese population, a subgroup type was used to analyze the data. As shown in Figure 2, 4 studies conducted in the Chinese population revealed that the odds ratio of pancreatic cancer for HBsAg positivity was 1.30 (95%CI: 1.05-1.56). Moderate heterogeneity (I^2 = 40.2%, P = 0.171) was seen across studies.

Prior infection with hepatitis B, as determined by the presence of anti- hepatitis B core (HBc), resulted in a significantly increased risk of pancreatic cancer showing OR of 1.24 (95%CI: 1.05-1.42) (I^2 = 71.4%, $P_{heterogeneity}$ = 0.015) which is summarized in Figure 2. Furthermore, an increased risk of pancreatic cancer was observed for hepatitis B surface antibody (anti-HBs)-seronegative/anti-HBc-seropositive carriers who were previously exposed to HBV without natural immunity, with OR of 1.67 (95%CI: 1.13-2.22) (I^2 = 0.0%, $P_{heterogeneity}$ = 0.329), but not for past exposure to HBV carriers with natural immunity (anti-HBs-seropositive/anti-HBc-seropositive), with OR of 0.98 (95%CI: 0.80-1.16) (I^2 = 82.3%, $P_{heterogeneity}$ = 0.003).

We observed non-significant positive associations between markers of active viral replication and pancreatic cancer risk, as illustrated in Figure 2. The risk of developing pancreatic cancer was 0.98 (95%CI: 0.27-1.68) (I^2 = 0.0%, $P_{heterogeneity}$ = 0.399) for HBsAg-seropositive/hepatitis B e antigen (HBeAg)- seropositive subjects compared with that of HBsAg-seronegative subjects while a significant positive association between the protective markers of HBV, anti-HBs and pancreatic cancer risk was found for studies conducted in the pooled analysis with OR of 0.54 (95%CI: 0.46-0.62) (I^2 = 92.7%, $P_{heterogeneity}$ = 0.000).

As summarized in Figure 2, the incidence of pancreatic cancer risks were also significantly increased in previously HCV infected population, with OR of 1.26 (95%CI: 1.03-1.50) (I^2= 0.0%, $P_{heterogeneity}$ = 0.949).

We also carried out stratified analyses to assess the impact of confounding factors of the RRs on the chronic carriers of HBV subgroup. As shown in Table 3, when we restricted the meta-analysis to those studies adjusted for smoking, the association between chronic HBV infection and pancreatic cancer risk was positive, the pooled OR was 1.32 (95%CI: 1.08-1.56). No positive association between chronic HBV infection and pancreatic cancer risk was found in the studies that were not adjusted for smoking, the pooled OR was 0.99 (95%CI: 0.68-1.30). In the stratified analysis, the association between chronic HBV infection and pancreatic cancer risk was also similar between the studies that were adjusted for alcohol drinking (the pooled OR = 1.51, 95%CI: 1.17-1.85) and those that were not adjusted for alcohol drinking (the pooled OR = 1.05, 95%CI: 0.83-1.28). Meanwhile, there was no positive association between chronic HBV infection and pancreatic cancer risk in the studies adjusted for diabetes (the pooled OR = 1.25, 95%CI: 0.95-1.55), neither was

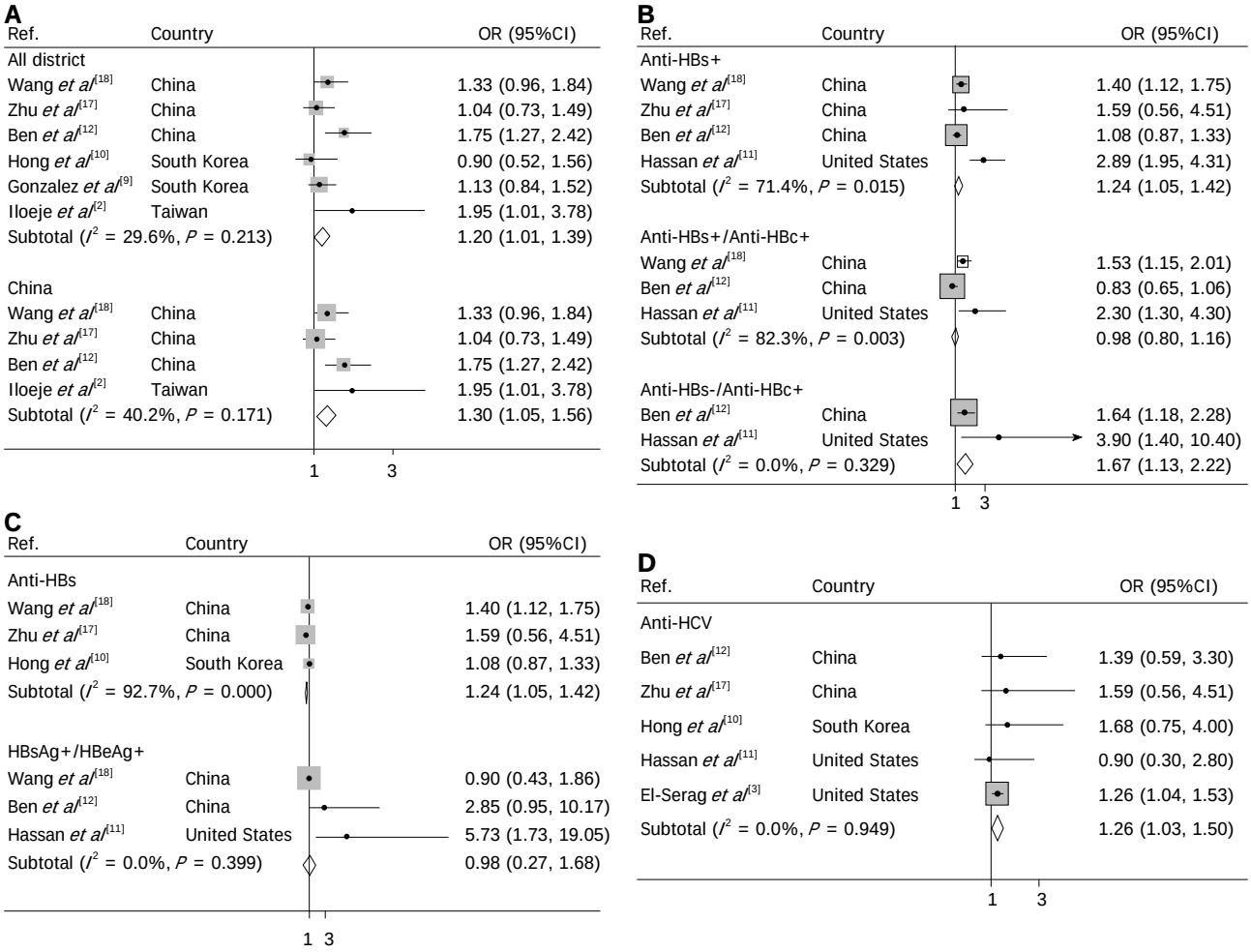

Figure 2 Forest plots of risk of pancreatic cancer. A: Associated with chronic hepatitis B (HBsAg carrier state) around the world and Chinese population; B: Associated with anti-HBc status; C: Associated with active hepatitis B virus viral replication and anti-HBs status; D: Associated with hepatitis C virus infection.

Table 3 Stratified analysis of pancreatic cancer risk by adjusted covariates

Stratifying variables	Studies (*n*)	OR (95%CI)	Tests for heterogeneity		
			χ^2	*P* value	I^2
Adjusted for smoking					
Yes	4	1.32 (1.08-1.56)	4.14	0.246	27.60%
No	2	0.99 (0.68-1.30)	0.18	0.670	0.00%
Adjusted for drinking					
Yes	3	1.51 (1.17-1.85)	1.70	0.428	0.00%
No	3	1.05 (0.83-1.28)	0.53	0.766	0.00%
Adjusted for diabetes					
Yes	4	1.25 (0.95-1.55)	5.22	0.156	42.50%
No	2	1.24 (0.50-1.98)	1.54	0.214	35.20%

in the studies that were adjusted for diabetes (the pooled OR, 1.24, 95%CI: 0.50-1.98).

Publication bias

No publication bias was apparent following an assessment by funnel plot (Figure 3, Begg's test $P = 0.711$, Egger's test $P = 0.868$).

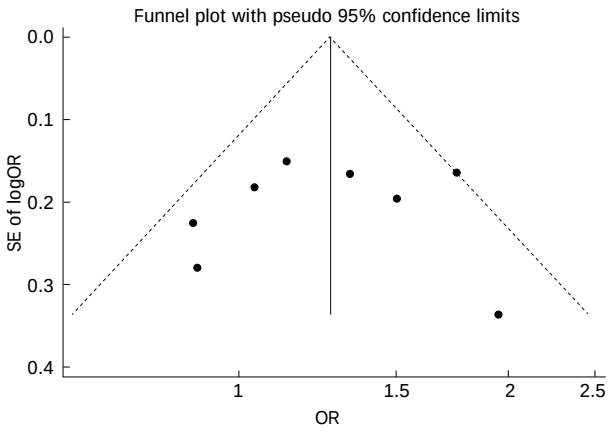

Figure 3 Begg's funnel plot with 95% confidence limits to detect publication bias. Each point represents a separate study for the indicated association.

DISCUSSION

This is the first comprehensive meta-analysis of observational studies on the association between chronic hepa-

titis viral infection and pancreatic cancer risk. We found that HBV and HCV infection is associated with 20% and 23% higher risk of pancreatic cancer, respectively. Our results reveal that prior infection with hepatitis B, especially in those without natural immunity would significantly increase the risk of pancreatic cancer. However, active hepatitis B viral replication does not increase the pancreatic cancer incidence. These observations provide evidence supporting the importance role of chronic HBV and HCV infection in the development of pancreatic cancer. In light of the fact that pancreatic cancer is a highly fatal tumor with a 5-year survival rate of less than 5% and that the number of people with HBV is 2 billion[6,26], our findings have substantial clinical and public significance on a global scale. It points to the need for further investigation on the etiological causes involved in human pancreatic carcinogenesis, the recognition of pancreatic damage mechanisms caused by chronic hepatic viral infection, and for long-term, large scale clinical studies to confirm this clinical association.

If the positive association between the chronic or inactive HBV or HCV carriers and the development of pancreatic cancer is a true, what mechanism could explain such a link? From the anatomical point of view, the proximity of the liver to the pancreas, as well as the sharing of the two organs blood vessels and ducts may make the pancreas a potential reservoir of hepatitis viruses. HBV or HCV may travel through the blood stream and be deposited in non-liver tissue[27,28]. In fact, by means of *situ* hybridization and immunohistochemical techniques, the serological markers of present or past HBV infection, HBsAg was detected in chronic inflammatory pancreatic acinar cells and in the pancreatic duct epithelia with pancreatic adenocarcinoma[29]. The same was true with HCV antigen, which was also found in pancreatic acinar cells[30]. These findings demonstrated the possibility of HBV infection and evidence of a chronic inflammatory reaction in non-hepatic tissues.

HBV and HCV replication intermediates in pancreatic cells support the assumption that the permissiveness of these extrahepatic cells for viral replication might also induce the chronic inflammatory response, thus eventually promoting tumor development. Anti-HBc-positive status, the sero biological marker of past exposure to HBV had an increased risk of developing pancreatic cancer. The observation provides some biological plausibility to the idea that long-lasting persistence of viral infection could indeed replicate in the pancreas. In fact, HBV and HCV are oncogenic viruses, and both are able to integrate the viral RNA or DNA into the genome of the infected cells[31,32]. DNA integration may play a key role in the regulation of the cell cycle, inducing carcinogenesis associated with HBV infection[33].

The third reason why our finding of a relationship between HBV infection and pancreatic cancer incidence may not be surprising is that the presence of HBV infection protection marker, seroconversion from HBsAg to anti-HBs, which is considered a sign of disease protection, leads to a significantly decreased risk of pancreatic cancer showing an OR of 0.54 in the pooled studies.

As with all meta-analyses of observational studies, our findings might have some limitations. First, because five of eight studies used a case-control design[10-12,17,18], the findings provided by this meta-analysis should be viewed with caution since more recall and selection bias might be seen in case-control studies. In addition, all the case-control studies were hospital-based and therefore may not fully represent the general population of pancreatic cancer patients, thereby introducing potential for selection bias into our meta-analysis.

Second, when investigating the association between hepatic virus infection and the risk of pancreatic cancer, the potential residual confounding and the allocation bias, with hepatic virus infection being at different stage and baseline risk of pancreatic cancer would affect the results. For example, it is difficult to understand the biological explanation for finding the cancer risk in subjects with chronic infection and not in those with current and active replication of the virus as shown by a positive HBeAg. The progression from active hepatitis virus infection to chronic inflammatory response targeted to pancreatic carcinogenesis is still unknown, for there is a lack of such data[34]. This incomplete information on HBV/HCV in the pathogenesis of progressive stages limits our knowledge on the true relationship of these oncogenic viruses with pancreatic cancer development. Although an increased risk of pancreatic cancer was observed for anti-HBs-seronegative/anti-HBc-seropositive carriers who were previously exposed to HBV without natural immunity, it is very difficult to interpret a pooled analysis of only 2 studies. Another issue is that none of the studies directly tested for the presence of markers of hepatitis virus infection in the pancreatic tissue. Therefore, a correlation between the level of the markers of HBV/HCV infection in peripheral blood and that in pancreatic tissue could not be established, which would throw some doubt into the reliability of the summary of RRs. More research is necessary to assess a dose-response association to examine the influence of viral load on the progression of pancreatic cancer to support biological plausibility.

Third, possible confounding factors and biases that may not have been fully adjusted for in this study exist. In fact, risk factors such as cigarette smoking, alcohol intake and diabetes, all of which could increase risk of pancreatic cancer associated with a history of chronic hepatitis virus infection. Only 4[2,10-12], 3[2,11], and 4[10-12,17] studies provided risk estimated adjusting for smoking, alcohol intake, and diabetes, respectively. The positive association between chronic HBV infection and pancreatic cancer risk was found after adjustment by smoking and alcohol drinking, but no positive correlation was maintained after adjustment by diabetes, suggesting that residual confounding by diabetes modified the association between chronic HBV infection and pancreatic cancer risk.

Finally, it is also important to realize that there is still a significant heterogeneity observed across studies, mostly

due to the diversity of the study designs and the varying incidence of pancreatic cancer and HBV/HCV infection rates may vary from continent to continent.

Even with these limitations, our meta-analysis supports the hypothesis that chronic HBV and HCV infection may significantly increase pancreatic cancer risk. The findings of this study raise the question of whether the early detection and provision of aggressive antiviral treatment for chronic hepatitis virus infection could prevent the development of pancreatic cancer, and whether patients with past exposure to HBV/HCV should be screened for malignancies other than HCC particularly in patients at high risk of HBV/HCV infection.

In conclusion, our meta-analysis favors the association between HBV/HCV infection and pancreatic cancer risk. However, observational studies were moderately heterogeneous and biased. Additional long-term prospective evidence for HBV/HCV infection among higher risk of pancreatic disease patients should be monitored and new evaluations on the effects of early intervention including HBV/HCV treatment, especially in occult HBV infection (anti-HBc-seropositive status), on the molecular carcinogenesis of pancreatic cancer are warranted.

COMMENTS

Background

Data from epidemiological studies related to the association of hepatitis B virus (HBV) and hepatitis C virus (HCV) and pancreatic cancer risk are inconsistent, with some studies supporting the excess pancreatic cancer with HBV/HCV infection compared to non-infected controls, and some studies showing differently. The aim of this meta-analysis was to clarify the association of chronic hepatitis viruses with the risk of pancreatic cancer.

Research frontiers

To date, several studies have assessed the association between the chronic HBV/HCV and pancreatic cancer risk in different ethics; however, the results are inconsistent and inconclusive. No quantitative summary of the evidence has ever been performed.

Innovations and breakthroughs

Based on the meta-analysis, the authors identified that chronic HBV and HCV infection is associated with pancreatic cancer, especially among Chinese population. Early intervention of HBV and HCV infection might decrease pancreatic cancer incidence.

Applications

The results support the hypothesis that chronic HBV/HCV infection significantly increases pancreatic cancer risk. Findings of this analysis are comparable with previous studies, and long-term prospective studies. Patients with past exposure to HBV/HCV should be screened for hepatocellular carcinoma and other malignancies, especially pancreatic cancer.

Terminology

Anti-hepatitis B core antigen (HBc)+/anti-hepatitis B surface antigen (HBs)± are serum biomarkers of possible occult HBV infection. Anti-HBc+/anti-HBs+ is the status of past exposure to HBV with evidence of HBV immunity or recovery, but possibly harboring persistent HBV infection. Anti-HBc+/ anti-HBs- is the status of past exposure to HBV without natural immunity.

Peer review

The meta-analysis was aimed at assessing the association between HBV/HCV chronic infection and risk of pancreatic cancer. This is an appealing issue, leading to interesting results.

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META-ANALYSIS

Association of *Helicobacter pylori babA2* with peptic ulcer disease and gastric cancer

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Abstract

AIM: To investigate the association between *babA2* gene and peptic ulcer disease (PUD) and gastric cancer (GC) in *Helicobacter pylori*-infected populations.

METHODS: We evaluated the relationship between *babA2* and clinical outcomes (PUD and GC) using a meta-analysis. A literature search was performed using the PubMed and Web of Science databases for relevant case-control studies that met the defined inclusion criteria. The ORs and 95%CIs were calculated to estimate the association between *babA2* genotype and clinical outcomes. A fixed-effect or random-effect model was performed depending on the absence or presence of significant heterogeneity.

RESULTS: A total of 25 articles with 38 studies met the inclusion criteria and were finally included in this meta-analysis. The results showed that the *babA2* genotype was significantly associated with an increased risk of PUD (OR = 2.069, 95%CI: 1.530-2.794, $P < 0.001$) and especially in the subgroup of duodenal ulcer (OR = 1.588, 95%CI: 1.141-2.209, $P = 0.006$). Moreover, a significant association between *babA2* gene and PUD and duodenal ulcer (OR = 2.739, 95%CI: 1.860-4.032, $P < 0.001$; OR = 2.239, 95%CI: 1.468-3.415, $P < 0.001$, respectively) was observed in western countries but not in Asian countries.

CONCLUSION: We demonstrated that the presence of *babA2* may be associated with increased risks for PUD, especially duodenal ulcer, in western countries.

Key words: *Helicobacter pylori*; *babA2*; Peptic ulcer; Gastric cancer; Risk

Core tip: BabA encoded by *babA2* gene is an outer member protein of *Helicobacter pylori* (*H. pylori*), which plays a key role in facilitating bacterial colonization in the stomach. The association between *babA2* and *H. pylori*-related gastroduodenal diseases is still controversial. We summarized a total of 25 case-control articles with 38 studies in this meta-analysis and evaluated the relationship between *babA2* and clinical outcomes. The presence of *babA2* may contribute to increased risk of peptic ulcer disease (PUD), especially duodenal ulcer, in western countries. In Asians, *babA2* genotype only showed a marginal association with PUD risk, which requires further investigation.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative spiral bacterium that may colonize the human gastric mucosa and establish a life-long infection[1]. Although *H. pylori* infects approximately half of the population worldwide, especially in developing countries, the majority of infected people remain asymptomatic. Only 15%-20% of those infected develop severe gastroduodenal diseases, such as peptic ulcer disease (PUD), gastric cancer (GC), and mucosa-associated lymphoid tissue lymphoma[2,3]. In addition to the host and environmental factors, another important reason for the diverse clinical outcomes is the differences in virulence factors among *H. pylori* strains[3]. For example, *H. pylori* strains harboring the vacuolating toxin A (*vacA*) and the cytotoxin-associated antigen (*cagA*) have been proposed as possible risk factors for PUD and GC[4].

Successful colonization in the stomach is the most important step for the pathogenicity of *H. pylori* infection. It is generally accepted that bacterial attachment to the gastric epithelium is the first critical stage of colonization by *H. pylori*[5]. The blood group antigen binding adhesin (BabA) is a well-described outer member protein of *H. pylori* that targets fucosylated Lewis^b blood group antigens presented on gastric epithelium[6,7]. Three *bab* allelic types have been identified, including *babA1*, *babA2* and *babB*; however, only the product of the *babA2* gene is necessary for endowing the bacteria with Lewis^b binding activity[6]. In 1999, Gerhard *et al*[8] first reported a positive association between a *babA2*-gene-positive strain and duodenal ulcer (DU) and GC. Subsequently, a series of studies of the association between *babA2* gene and PUD and GC have been done, but with inconsistent or conflicting conclusions[9-11].

We proposed a hypothesis that bacterial adherence factor BabA mediating close attachment to the epithelium may contribute to pathogenesis of PUD and/or GC. So far, it has not been possible to draw any causal conclusion about the relationship between the *babA2* gene and specific diseases, partly because of the small size of individual studies. Therefore, in the present study, we conducted a meta-analysis, combining available data from published case-control studies, to obtain a more precise estimate of the association between *babA2* gene and PUD and GC in *H. pylori*-infected populations.

MATERIALS AND METHODS

Literature search strategy

A literature search was performed using the PubMed and Web of Science databases for articles estimating the association between *babA2* gene and clinical outcomes in *H. pylori*-infected populations. All enrolled studies were published from January 1997 to October 2012 and retrieved using one of the keywords "*babA*" or "*babA2*" in combination with "*Helicobacter pylori*". The search was performed without restriction on language.

Inclusion criteria

The criteria used to select studies for this meta-analysis were as follows: (1) fully published case-control studies [case group included DU, gastric ulcer (GU), PUD or GC, and the control group included gastritis or nonulcer disease (NUD)]; (2) studies described the relationship between *babA2* gene status and clinical outcomes; (3) the presence of *babA2* was examined by polymerase chain reaction (PCR); and (4) the papers were written in English.

Exclusion criteria

The exclusion criteria were as follows: (1) the results came from review articles; (2) there was no integrated raw data; (3) *in vitro* studies or animal experiments; (4) studies with abstract only; and (5) studies with children.

Data extraction

Evaluation of all potentially relevant articles and extraction of raw data were independently performed by two investigators (Chen MY and He CY). Disagreements were resolved through discussion. We collected information on the following items from each study: first author's name, year of publication, countries and areas of the study population, *babA2* status and clinical outcomes (DU, GU, PUD and GC), and the total number of cases and controls.

Statistical analysis

All statistical analyses were performed using STATA version 11.0 (College Station, TX, United States). Two-sided *P* values were evaluated in this meta-analysis and $P < 0.05$ was considered statistically significant. The strength of the association between the *babA2* gene and clinical outcomes was estimated by OR and corresponding 95%CIs. The statistical heterogeneity among the included studies was assessed by χ^2-based Q and I^2 statistics. If the heterogeneity was considered not significant (with $P > 0.1$ for Q test) among studies, a fixed-effects model based on the Mantel-Haenszel method[12] was used to calculate the pooled OR. On the contrary, a random-effects model based on the DerSimonian and Laird method[13] was used to assess the pooled OR when the P value of the Q test was < 0.1. In addition, a sensitivity analysis was performed to estimate the effects of each included study on the overall risk of clinical outcomes. ORs and 95%CIs were recalculated when any single study was excluded in turn. Begg's test[14] and Egger's test[15] were performed to estimate the publication bias.

RESULTS

Characteristics of selected studies

According to the literature search strategy, a total of 220

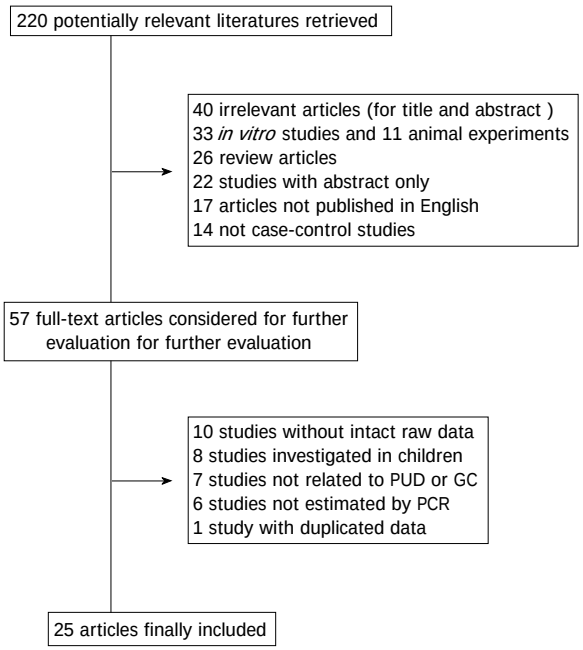

Figure 1 Flowchart of literature search and studies selection. PUD: Peptic ulcer disease; GC: Gastric cancer; PCR: Polymerase chain reaction.

possibly relevant studies were retrieved and 195 were excluded. The main reasons for exclusion were that the articles were reviews, *in vitro* studies, irrelevant to the theme of our research, or did not meet our inclusion criteria (Figure 1). Twenty-five case-control studies met the inclusion criteria[8-11,16-36]. Four of these studies[10,11,19,32] investigated the association between *babA2* gene and clinical outcomes in several different countries. Considering that these data partially evaluated the geographic variation of the influence of *babA2* gene status on the risk of *H. pylori*-related gastroduodenal diseases, data that came from different countries were treated as a separate study. Therefore, with respect to geographical location, 16 studies[11,17,19,22-26,29,33-36] were concerned with Asian populations, and 23[10,16,18,20,21,27,28,30-32] analyzed western populations. One of the latter group, which involved a study from Sweden[10], was excluded because of insufficient data. Finally, a total of 38 independent studies with 4556 patients were included in this meta-analysis (Table 1).

Association between babA2 gene and PUD

There were 36 studies[8-11,17-20,22-36] that investigated the distribution difference of *babA2* genotypes between patients with PUD and gastritis and/or NUD, which consisted of 1859 cases and 1909 controls. The overall prevalence of *babA2* gene was 73.96% (1375/1859) in PUD patients and 57.94% (1106/1909) in control subjects. Data from Oleastro *et al*[19] (Japan, South Korea, Brazil population), Sheu *et al*[26] and Lai *et al*[34] showed that the prevalence of *babA2* gene was 100% in both case and control groups, and the OR and standard error could not be estimated; thus, these studies were excluded. We found that the *babA2* gene significantly increased the risk of PUD in a random-effects model, with a pooled OR of 2.069 (95%CI: 1.532-2.794, $P < 0.001$), and moderate heterogeneity was observed ($I^2 = 62.8$, $P < 0.001$) (Figure 2).

To explore the source of heterogeneity, subgroup analysis was performed. PUD was classified into DU and GU. Among the total of 36 PUD-related studies, 19[8,9,11,17,22-24,26,27,29,31-35] could be used to evaluate risk for DU and eight[22,24,26,27,29,33-35] for GU. For DU analysis, the overall prevalence of *babA2* gene in DU and control subjects was 77.20% (813/1053) and 71.77% (811/1130), respectively. After removal of two studies with 100% prevalence of *babA2* genotype[26,27], the pooled OR based on the random-effects model was 1.588 (95%CI: 1.141-2.209, $P = 0.006$), and mild heterogeneity was observed ($I^2 = 45.8$, $P = 0.021$) (Figure 2). For GU analysis, the overall prevalence of *babA2* genotype seemed to be lower in GU (73.73%, 174/236) than in controls (80.89%, 402/497). Two studies with 100% prevalence of *babA2* genotype were also excluded because of statistical limitation[26,34]. No significant association was observed between *babA2* genotype and GU in a fixed-effects model (OR = 0.755, 95%CI: 0.496-1.150, $P = 0.191$), and there was no heterogeneity among the studies ($I^2 = 0.0\%$, $P = 0.845$) (Figure 2).

When geographical location was considered, data from different countries were subdivided into Asian and western groups. For PUD, the overall prevalence of *babA2* gene was 78.36% (822/1049) in Asian countries and 68.27% (553/810) in western countries. Furthermore, in western countries, the presence of *babA2* substantially increased PUD risk, with a pooled OR of 2.739 (95%CI: 1.860-4.032, $P < 0.001$), while in Asian countries, the *babA2* genotype was only borderline associated with PUD (OR = 1.370, 95%CI: 0.941-1.994, $P = 0.100$) (Figure 2). For DU, the *babA2* genotype significantly increased the risk of DU in western countries (OR = 2.239, 95%CI: 1.468-3.415, $P < 0.001$), but not in Asian countries (OR = 1.158, 95%CI: 0.802-1.672, $P = 0.433$) (Figure 2). The results suggested that differences in geographical distribution of *babA2* genotype may also confer heterogeneity to the studies. Only one study with a small sample size investigated the relationship of *babA2* gene and GU in a western country[27]; therefore, we did not perform subgroup analysis according to geographical area.

Sensitivity analysis was conducted to assess the influence of individual studies on the overall risk of PUD and DU by excluding any single study in turn and recalculating the pooled OR and 95%CI. A similar OR and 95%CI were generated, which indicated high stability of the results (Figure 3).

Association between babA2 and GC

A total of 16 studies[8,9,16,17,21-24,29,31-36] investigated the association between *babA2* gene and GC. The overall prevalence of *babA2* gene was 70.72% (384/534) in GC cases and 60.64% (607/1001) in gastritis or NUD controls. One study with both 100% prevalence of *babA2* in cases and controls was excluded from our meta-analysis[34]. In a random-effects model, the risk of GC increased 1.972-fold (95%CI: 1.103-3.525, $P = 0.022$) in the pres-

Table 1 Characteristics of studies included in the meta-analysis *n* (%)

Ref.	Population	Gastritis or NUD	PUD	GU	DU	GC
		babA2 +	*babA2* +	*babA2* +	*babA2* +	*babA2* +
Asian						
Saxena *et al*[36]	India	35 (26.32)	19 (52.78)			10 (28.57)
Talebi Bezmin Abadi *et al*[9]	Iran	17 (26.15)	10 (18.18)		10 (18.18)	38 (95.00)
Safaei *et al*[17]	Iran	30 (68.18)	20 (74.07)		20 (74.07)	8 (80.00)
Oleastro *et al*[19]	Japan	28 (100.00)	42 (100.00)			
Oleastro *et al*[19]	South Korea	37 (100.00)	28 (100.00)			
Chomvarin *et al*[24]	Thai	57 (91.94)	31 (91.18)	17 (85.00)	14 (100.00)	15 (93.75)
Erzin *et al*[23]	Turkey	7 (23.33)	14 (46.67)		14 (46.67)	29 (87.88)
Zhang *et al*[22]	China	89 (66.92)	89 (60.14)	28 (59.57)	61 (60.40)	54 (68.35)
Sheu *et al*[26]	Taiwan	85 (100.00)	60 (100.00)	30 (100.00)	30 (100.00)	
Zheng *et al*[25]	China	11 (37.93)	17 (39.53)			
Han *et al*[29]	China	28 (65.12)	50 (64.94)	15 (50.00)	35 (74.47)	12 (57.14)
Lai *et al*[34]	Taiwan	41 (100.00)	46 (100.00)	15 (100.00)	31 (100.00)	14 (100.00)
Maeda *et al*[33]	Japan	52 (96.30)	40 (95.24)	20 (100.00)	20 (90.91)	11 (100.00)
Yamaoka *et al*[32]	Korea	47 (88.68)	111 (96.52)		111 (96.52)	
Yamaoka *et al*[32]	Japan	112 (88.89)	172 (95.56)		112 (88.89)	
Mizushima *et al*[35]	Japan	34 (80.95)	73 (84.88)	38 (84.44)	35 (85.37)	36 (90.00)
Western						
Mattar *et al*[16]	Brazil	22 (64.71)				14 (41.18)
Oleastro *et al*[18]	Portugal	7 (11.67)	27 (47.37)			
Bartchewsky *et al*[21]	Brazil	102 (79.07)				40 (78.43)
Oleastro *et al*[19]	Portugal	16 (32.00)	25 (50.00)			
Oleastro *et al*[19]	France	3 (50.00)	22 (81.48)			
Oleastro *et al*[19]	Sweden	4 (40.00)	10 (83.33)			
Oleastro *et al*[19]	Germany	6 (60.00)	7 (77.78)			
Oleastro *et al*[19]	United States	12 (92.31)	10 (100.00)			
Oleastro *et al*[19]	Brazil	12 (100.00)	10 (100.00)			
Oleastro *et al*[20]	Portugal	18 (32.14)	25 (50.00)			
Gatti *et al*[27]	Brazil	16 (43.24)	20 (40.00)	11 (37.93)	9 (42.86)	
Gatti *et al*[28]	Brazil	37 (54.41)	3 (20.00)			
Olfat *et al*[10]	Finland	12 (46.15)	22 (70.97)			
Olfat *et al*[10]	Portugal	12 (19.67)	19 (63.33)			
Olfat *et al*[10]	Germany	19 (28.36)	22 (88.00)			
Oliveira *et al*[31]	Brazil	24 (31.58)	43 (53.75)		43 (53.75)	29 (55.77)
Zambon *et al*[30]	Italy	26 (27.96)	20 (48.78)			
Yamaoka *et al*[32]	Colombia	28 (70.00)	34 (85.00)		34 (85.00)	34 (82.93)
Yamaoka *et al*[32]	United States	28 (70.00)	35 (85.37)		35 (85.37)	19 (63.33)
Yamaoka *et al*[11]	United States	66 (71.74)	123 (84.83)		123 (84.83)	
Yamaoka *et al*[11]	Colombia	37 (71.15)	53 (82.81)		53 (82.81)	
Gerhard *et al*[8]	Munich	13 (37.14)	23 (100.00)		23 (100.00)	21 (77.78)

NUD: Nonulcer disease; PUD: Peptic ulcer disease; GU: Gastric ulcer; DU: Duodenal ulcer; GC: Gastric cancer.

ence of *babA2* compared with the controls; however, high heterogeneity among studies was observed (I^2 = 76.8%, $P < 0.001$). Meta-analyses were conducted repeatedly when each study was omitted. As showed in Figure 4, two studies[9,23] showed larger differences in the risk estimates compared with other studies in the sensitivity analysis. Sensitivity analysis excluding these studies generated an OR of 1.303 (95%CI: 0.881-1.927, $P = 0.185$) among homogeneous studies (I^2 = 45.0%, $P = 0.040$), which was different from the OR of 1.972 (95%CI: 1.103-3.525, $P = 0.022$) before the removal of those studies (Figure 4). In terms of geographical area, no statistically significant findings were found among the Asian or western subpopulations, with a pooled OR of 1.132 (95%CI: 0.763-1.680, $P = 0.539$) in the former and 1.303 (95%CI: 0.881-1.927, $P = 0.349$) in the latter (Figure 4).

Publication bias analysis

Publication bias was preliminarily estimated by Begg's and Egger's tests. No significant publication bias was observed in all the comparisons based on Begg's test (all $P > 0.1$), but P value was 0.08 in Egger's test, suggesting a slight publication bias.

DISCUSSION

The Gram-negative bacterium *H. pylori* is known to have a remarkably high level of genetic diversity, and is implicated in human diseases after decades of persistence in the stomach[37-39]. A crucial virulence factor BabA, encoded by the *babA2* gene, facilitates colonization by *H. pylori* in the stomach and may be involved in the pathogenesis of different *H. pylori*-related gastroduodenal diseases, such as PUD and gastric malignancy[8]. To date, there have been numerous relevant studies published but with divergent results on the relationship between the *babA2* gene and PUD and GC[9-11]; moreover, there is no comprehensive meta-analysis on the significance

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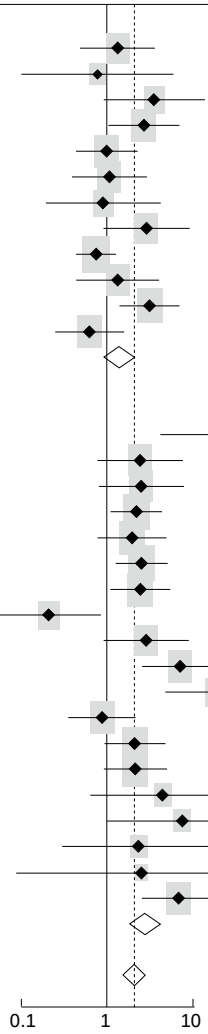
Ref.	Country	PUD *babA2*+/-	NUD/GS *babA2*+/-	OR (95%CI)	%Weight
Asian					
Mizushima *et al*[35]	Japan	73/13	34/8	1.32 (0.50, 3.49)	3.61
Maeda *et al*[33]	Japan	40/2	52/2	0.77 (0.10, 5.70)	1.62
Yamaoka *et al*[32]	South Korea	111/4	47/6	3.54 (0.96, 13.13)	2.75
Yamaoka *et al*[33]	Japan	172/8	112/4	2.69 (1.09, 6.61)	3.80
Han *et al*[29]	China	50/27	28/15	0.99 (0.45, 2.17)	4.15
Zheng *et al*[25]	China	17/26	11/18	1.07 (0.41, 2.82)	3.61
Chomvarin[24]	Thai	31/3	57/5	0.91 (0.20, 4.05)	2.37
Erzin *et al*[23]	Turkey	14/16	7/23	2.88 (0.95, 8.72)	3.23
Zhang *et al*[22]	China	89/59	89/44	0.75 (0.46, 1.22)	5.01
Safaei *et al*[17]	Iran	20/7	30/14	1.33 (0.46, 3.88)	3.34
Saxena *et al*[36]	India	19/17	35/98	3.13 (1.46, 6.69)	4.22
Talebi Bezmin Abadi *et al*[9]	Iran	10/45	17/48	0.63 (0.26, 1.51)	3.86
Subtotal (I^2 = 46.3%, P = 0.039)				1.37 (0.94, 1.99)	41.58
Western					
Gerhard *et al*[8]	Munich	23/0	13/22	78.33 (4.39, 1397.21)	0.92
Yamaoka *et al*[32]	Colombia	34/6	28/12	2.43 (0.81, 7.30)	3.26
Yamaoka *et al*[32]	United States	35/6	28/12	2.50 (0.83, 7.50)	3.26
Yamaoka *et al*[11]	United States	123/22	66/26	2.20 (1.16, 4.18)	4.57
Yamaoka *et al*[11]	Colombia	53/11	37/15	1.95 (0.81, 4.73)	3.85
Oliveira *et al*[31]	Brazil	43/37	24/52	2.52 (1.31, 4.84)	4.45
Zambon *et al*[30]	Italy	20/21	26/67	2.45 (1.15, 5.26)	4.21
Gatti *et al*[28]	Brazil	3/12	37/31	0.21 (0.05, 0.81)	2.66
Olfat *et al*[10]	Finland	22/9	12/14	2.85 (0.96, 8.51)	3.27
Olfat *et al*[10]	Portugal	19/11	12/49	7.05 (2.66, 18.69)	3.59
Olfat *et al*[10]	Germany	22/3	19/48	18.53 (4.96, 69.22)	2.74
Gatti *et al*[27]	Brazil	20/30	16/21	0.88 (0.37, 2.07)	3.92
Oleastro *et al*[20]	Portugal	25/25	18/38	2.11 (0.96, 4.64)	4.13
Oleastro *et al*[19]	Portugal	25/25	16/34	2.13 (0.94, 4.79)	4.06
Oleastro *et al*[19]	France	22/5	3/3	4.40 (0.68, 28.60)	1.78
Oleastro *et al*[19]	Sweden	10/2	4/6	7.50 (1.04, 54.12)	1.65
Oleastro *et al*[19]	Germany	7/2	6/4	2.33 (0.31, 17.54)	1.60
Oleastro *et al*[19]	United States	10/0	12/1	2.52 (0.09, 68.60)	0.72
Oleastro *et al*[18]	Portugal	27/30	7/53	6.81 (2.65, 17.52)	3.68
Subtotal (I^2 = 59.1%, P = 0.001)				2.74 (1.86, 4.03)	58.42
Overall (I^2 = 62.8%, P = 0.000)				2.07 (1.53, 2.79)	100.00
Note: Weights are from random effects analysis					



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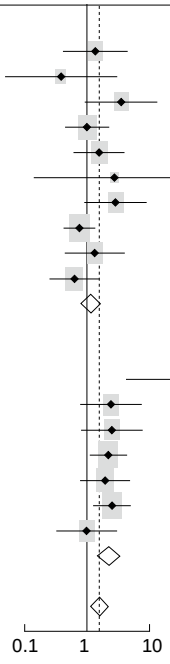
Ref.	Country	DU *babA2*+/-	NUD/GS *babA2*+/-	OR (95%CI)	%Weight
Asian					
Mizushima *et al*[35]	Japan	35/6	34/8	1.37 (0.43, 4.37)	5.18
Maeda *et al*[33]	Japan	20/2	52/2	0.38 (0.05, 2.92)	2.24
Yamaoka *et al*[32]	South Korea	111/4	47/6	3.54 (0.96, 13.13)	4.40
Yamaoka *et al*[32]	Japan	112/4	112/14	1.00 (0.46, 2.19)	7.92
Han *et al*[29]	China	35/12	28/15	1.56 (0.63, 3.87)	6.89
Chomvarin *et al*[24]	Thai	14/0	57/5	2.77 (0.14, 53.09)	1.15
Erzin *et al*[23]	Turkey	14/16	7/23	2.88 (0.95, 8.72)	5.48
Zhang *et al*[22]	China	61/40	89/44	0.75 (0.44, 1.29)	10.40
Safaei *et al*[17]	Iran	20/7	30/14	1.33 (0.46, 3.88)	5.73
Talebi Bezmin Abadi *et al*[9]	Iran	10/45	17/48	0.63 (0.26, 1.51)	7.10
Subtotal (I^2 = 23.1%, P = 0.231)				1.16 (0.80, 1.67)	56.49
Western					
Gerhard *et al*[8]	Munich	23/0	13/22	78.33 (4.39, 1397.21)	1.20
Yamaoka *et al*[32]	Colombia	34/6	28/12	2.43 (0.81, 7.30)	5.53
Yamaoka *et al*[32]	United States	35/6	28/12	2.50 (0.83, 7.50)	5.54
Yamaoka *et al*[11]	United States	123/22	66/26	2.20 (1.16, 4.18)	9.31
Yamaoka *et al*[11]	Colombia	53/11	37/15	1.95 (0.81, 4.73)	7.07
Oliveira *et al*[31]	Brazil	43/37	24/52	2.52 (1.31, 4.84)	9.19
Gatti *et al*[27]	Brazil	9/12	16/21	0.98 (0.33, 2.90)	5.65
Subtotal (I^2 = 28.1%, P = 0.214)				2.24 (1.47, 3.42)	43.51
Overall (I^2 = 45.8%, P = 0.021)				1.59 (1.14, 2.21)	100.00
Note: Weights are from random effects analysis					



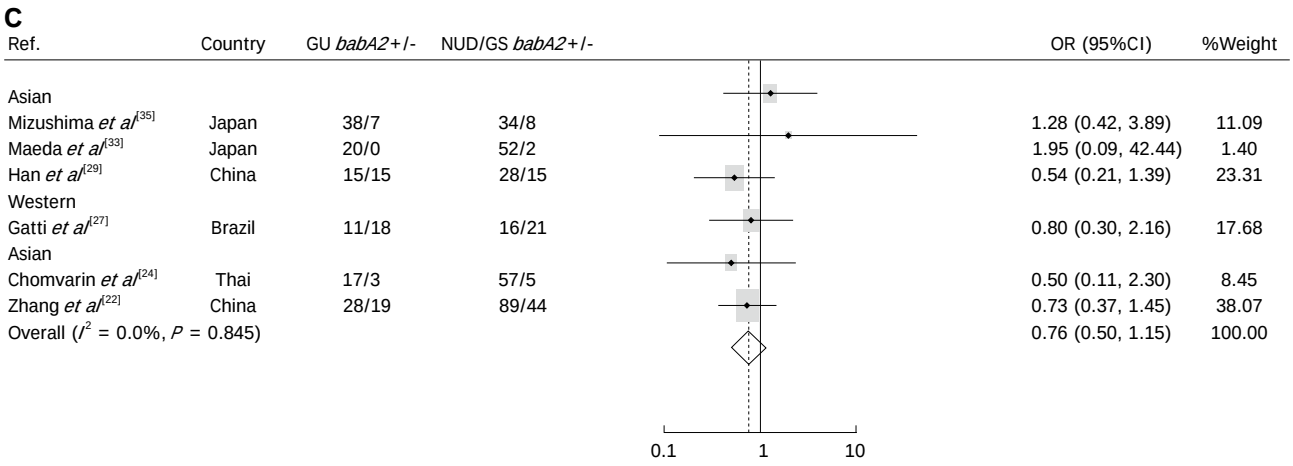


Figure 2 Results of the association between *babA2* gene and peptic ulcer disease, duodenal ulcer and gastric ulcer risk. A: Association between *babA2* and peptic ulcer disease (PUD); B: Association between *babA2* and duodenal ulcer (DU); C: Association between *babA2* and gastric ulcer (GU). ORs and 95%CIs were calculated by a random-effect (A, B) and fixed-effect (C) model. NUD: Nonulcer disease.

of *babA2*. Therefore, we performed the present meta-analysis of the available published literature to obtain a more precise conclusion. Our meta-analysis showed that *babA2* was significantly associated with increased risks of PUD, especially DU, with corresponding ORs of 2.069 and 1.588; moreover, statistically significant findings were more apparent in western populations with ORs of 2.739 for PUD and 2.239 for DU. The summary ORs for PUD and DU in Asians, however, were relatively small (1.370 and 1.158, respectively) and without statistical significance. No significant risk association was observed for GU and GC, but a decreased tendency was noted for GU with a pooled OR of 0.755.

Over the past 20 years, there has been marked progress in our understanding of the role of *H. pylori* infection in the etiology of gastroduodenal diseases. It is well known that *H. pylori* infection increases the risk of developing PUD, including both GU and DU subtypes[40]. Our meta-analysis confirmed a positive association of *H. pylori* with *babA2* genotype with PUD development. Among the major outer membrane proteins of *H. pylori*, BabA has significance not only in triggering bacterial colonization of the gastric epithelium, but also in regulating its functional interaction with host cells, which mainly acts through binding to Lewis^b and fucosylated ABO blood group antigens present in the stomach[41,42]. Gene inactivation experiments have demonstrated that only the product of *babA2* gene is essential for Lewis^b binding activity[7]. Rad *et al*[43] have reported a high density of *H. pylori* colonization in the stomach in the presence of *babA2* genotype, which increases interleukin-8 secretion and granulocytic infiltration, resulting in intense mucosal inflammation. In addition, Ishijima *et al*[41] have demonstrated that *babA2*-positive strains with Lewis^b binding activity are potentiators of the type IV secretion system (T4SS), implying a possible combined effect of *babA2* and other virulence factors related to T4SS. Although the detailed mechanism of the pathogenicity of *babA2* in PUD development has not been fully established, our meta-analysis suggests an important role of *babA2* genotype in distinguishing *H. pylori*-related PU and especially DU from NUD.

Intriguing findings in this study further suggested that individuals infected with *babA2*-positive pathogens have a unique pathogenicity in DU development; conversely, there was no significant association between *babA2* and GC. This difference may be partially due to the distinct etiologies of DU and GC development. Generally, *H. pylori*-related chronic severe gastritis could progress in two different directions[44]. One possibility is that *H. pylori*-related gastritis, predominating in the antrum as well as generating gastric acid, usually induces DU[45]. Patients with DU rarely develop atrophic gastritis of the corpus, and therefore GC risk may decrease in such cases[46]. Another possibility is that patients with extensive gastritis in the corpus and antrum, involving decreased acid output, tend to develop intestinal metaplasia, atrophic gastritis, and even GC[45]. It is speculated that *babA2* combined with other virulence factors may also lead to GC development. Studies conducted by Gerhard *et al*[8] and Erizin *et al*[47] have suggested that triple-positive *H. pylori* strains with *cagA, vacAs1* and *babA2* coexpression increase the risk of developing GC. Zambon *et al*[30] have also reported that infections with these triple-positive strains carry a higher risk of intestinal metaplasia, known as a gastric precancerous lesion. The different risk associations between GC and DU should be interpreted with caution, which should be further investigated in the future.

Our stratified analysis according to geographical areas demonstrated that *babA2* genotype is closely involved in the risk of PUD, especially DU in western populations, but not in Asian populations. This important information about geographical difference in the *babA2* gene suggests a potential biomarker distinguishing PUD, especially DU, from other NUDs in western populations, and reveals a phylogenetic difference between Asian and western *H. pylori* strains. Previous studies have also reported divergence in genes accounting for BabA and other virulence genes, such as *cagA* and *vacA*, between Asian and western strains[48-50]. The above-mentioned findings support the

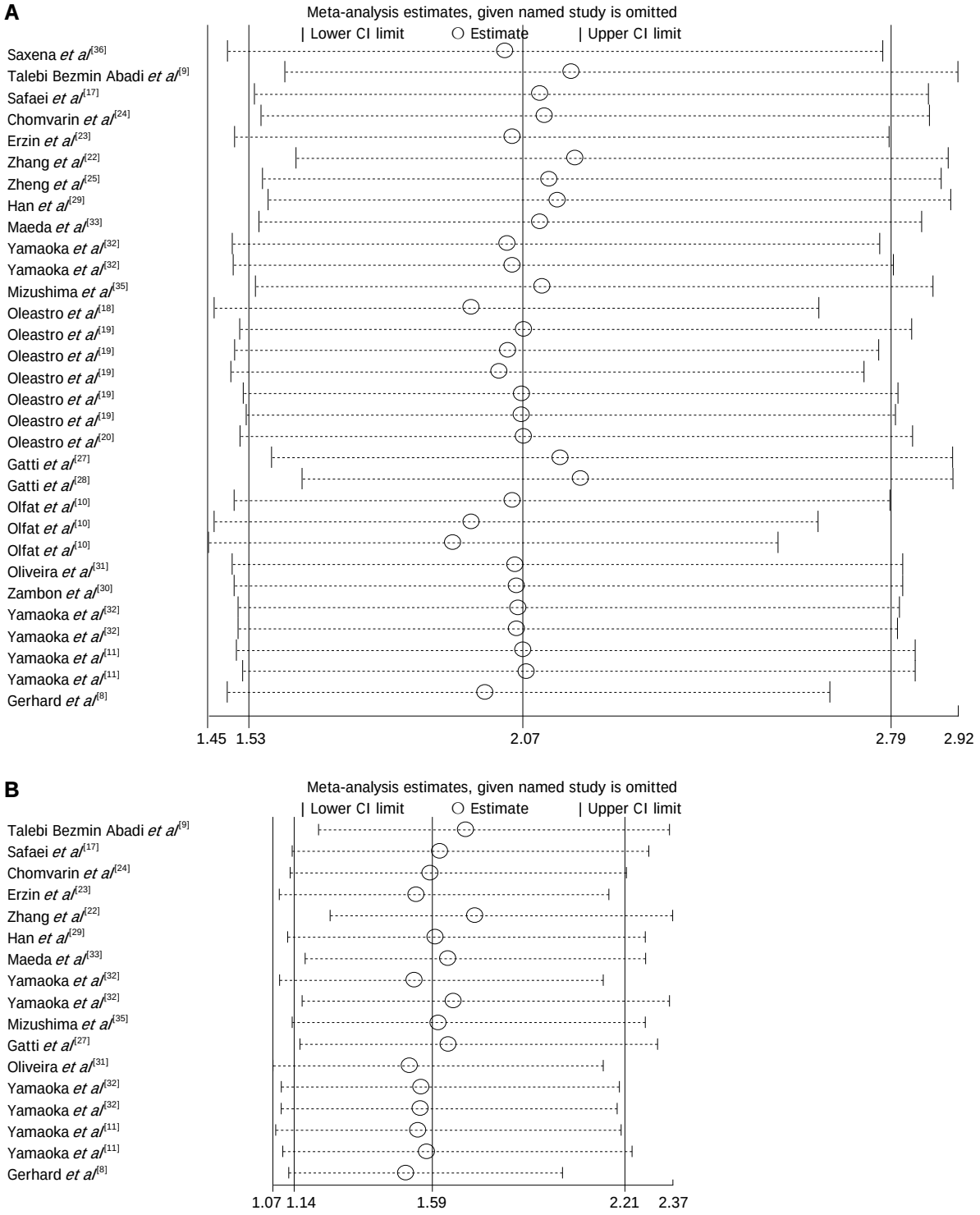
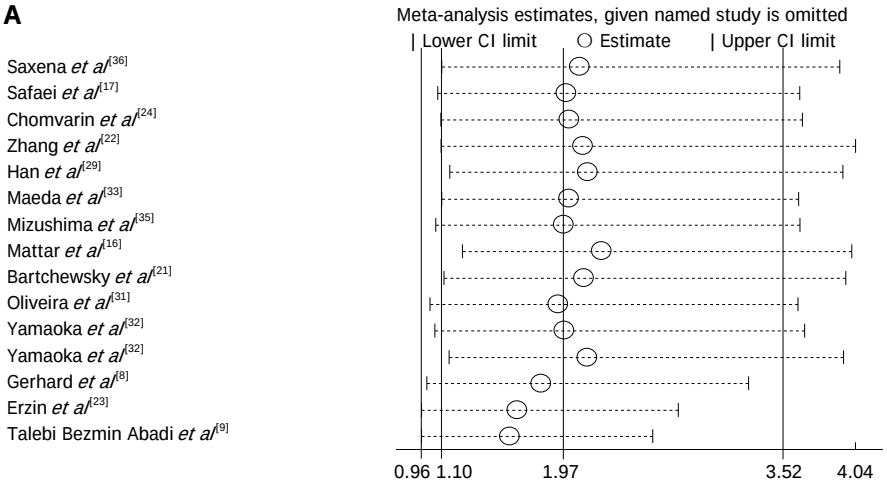


Figure 3 Influence of the summary OR coefficients on the association between *babA2* genotype and peptic ulcer disease and duodenal ulcer risk. A: *babA2* genotype and peptic ulcer disease (PUD) risk; B: *babA2* genotype and duodenal ulcer risk. Results were calculated by omitting each study (on the left) in turn. Bars, 95%CI. Meta-analysis random-effects estimates (exponential form) were used.

suggestion that genetic variability within the *H. pylori* genome, especially in probable host interaction genes, plays a critical role in its different adaptive ability and pathogenicity among different ethnicities[49].

There were several unavoidable limitations to our meta-analysis that should be considered. First, 17 studies[10,18-20,25,28,30,36] related to PUD lacked information about the distribution of DU and GU, which may have influenced the results of the stratified analysis. Second, a lack of original data on histopathological types of GC limited the subgroup analysis according to differences in these types. An unstable result was obtained according to the sensitivity analysis that assessed the relationship between *babA2* and GC, but there were insufficient data to explore

A



B

Ref.	Country	GC *babA2*+/-	NUD/GS *babA2*+/-	OR (95%CI)	%Weight
Asian					
Mizushima *et al*[35]	Japan	36/4	34/8	2.12 (0.58, 7.68)	6.19
Maeda *et al*[33]	Japan	11/0	52/5	1.10 (0.05, 24.38)	1.47
Han *et al*[29]	China	12/9	28/15	0.71 (0.25, 2.08)	7.84
Chomvarin *et al*[24]	Thai	15/1	57/5	1.32 (0.14, 12.13)	2.67
Zhang *et al*[22]	China	54/25	89/44	1.07 (0.59, 1.94)	13.11
Safaei *et al*[17]	Iran	8/2	30/14	1.87 (0.35, 9.96)	4.24
Saxena *et al*[36]	India	10/25	35/98	1.12 (0.49, 2.57)	10.21
Subtotal (I^2 = 0.0%, P = 0.918)				1.13 (0.76, 1.68)	45.73
Western					
Gerhard *et al*[8]	Munich	21/6	13/22	5.92 (1.90, 18.47)	7.27
Yamaoka *et al*[32]	Colombia	34/7	28/12	2.08 (0.72, 6.00)	7.93
Yamaoka *et al*[32]	United States	19/11	28/12	0.74 (0.27, 2.02)	8.41
Oliveira *et al*[31]	Brazil	29/23	24/52	2.73 (1.32, 5.67)	11.38
Bartchewsky *et al*[21]	Brazil	40/11	102/27	0.96 (0.44, 2.12)	10.65
Mattar *et al*[16]	Brazil	14/20	22/12	0.38 (0.14, 1.02)	8.63
Subtotal (I^2 =73.8%, P = 0.002)				1.43 (0.68, 3.00)	54.27
Overall (I^2 = 45.0%, P = 0.040)				1.30 (0.88, 1.93)	100.00

Note: Weights are from random effects analysis

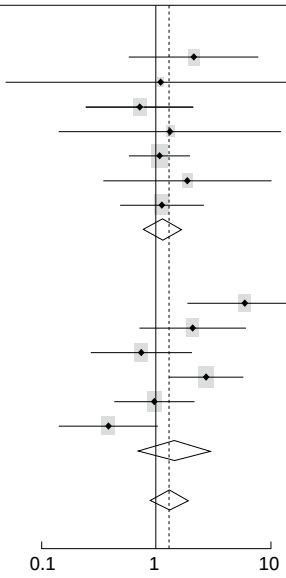


Figure 4 Influence of summary OR coefficients and results on the association between *babA2* genotype and gastric cancer risk. A: Influence analysis. Results were calculated by omitting each study (on the left) in turn. Bars, 95%CI. Meta-analysis random-effects estimates (exponential form) were used; B: Results. ORs and 95%CIs were calculated by a random-effect model.

the source of heterogeneity related to histopathological types. Third, most of the studies had a relatively small sample size.

In conclusion, our results suggest that the presence of *babA2* may contribute to increased risk of PUD, especially DU development, in western countries. In Asians, *babA2* genotype only showed a marginal association with PUD risk, which requires further investigation in the future.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) is a common bacterium with a high prevalence rate and severe pathogenicity, which has been identified as a major cause of severe gastroduodenal diseases, such as peptic ulcer disease (PUD) and gastric cancer (GC). The genome of various *H. pylori* strains demonstrates significant genetic diversity. Genetic variation in specific virulence genes of *H. pylori* may participate in the pathogenic process of *H. pylori* infection in the stomach, thereby contributing to the variable risk of diverse clinical outcomes.

Research frontiers

BabA encoded by the *babA2* gene is a crucial virulence factor of *H. pylori*, which may be involved in the pathogenesis of PUD and GC. Although a few studies have focused on the association between *babA2* gene and the risks of *H. pylori*-related gastroduodenal diseases, those studies showed discrepant results. Moreover, there is no comprehensive meta-analysis integrating the currently available data on the relationship between *babA2* gene and PUD and GC.

Innovations and breakthroughs

This meta-analysis investigated the association between *babA2* gene and PUD and GC. They observed that the presence of *babA2* may contribute to increased risk of PUD, especially duodenal ulcer (DU) development, in western countries. However, in Asians, the presence of *babA2* only showed a marginal association with PUD risk, which requires further investigation. This meta-analysis achieved a relatively comprehensive conclusion on the relationship between *babA2* and clinical outcomes.

Applications

The study suggested that individuals infected with *H. pylori* harboring *babA2* gene were associated with increased risk of PUD, especially DU, in western countries. Eradication of *H. pylori*, in particular *H. pylori* harbouring *babA2*, may contribute to a lower incidence of PUD.

Terminology

babA2: Three *bab* allelic types have been identified, including *babA1*, *babA2* and *babB*, and only the product of the *babA2* gene is necessary for endowing *H. pylori* with Lewis^b antigen binding activity. *babA2* encodes the blood group antigen binding adhesion that binds to fucosylated Lewis^b blood group antigens on gastric epithelial cells.

Peer review

This was a well-performed meta-analysis of currently available studies on the association between *babA2* gene and PUD and GC, and concluded that the presence of *babA2* may be associated with increased risk of PUD, with an emphasis on DU and in western countries. This study was well designed and performed, and the results are well discussed.

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CASE REPORT

Microscopic colitis: Is it a spectrum of inflammatory bowel disease?

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Abstract

Lymphocytic and collagenous colitis are forms of microscopic colitis which typically presents in elderly patients as chronic watery diarrhea. The association between microscopic colitis and inflammatory bowel disease is weak and unclear. Lymphocytic colitis progressing to ulcerative colitis has been previously reported; however there is limited data on ulcerative colitis evolving into microscopic (lymphocytic or collagenous) colitis. We report a series of six patients with documented ulcerative colitis who subsequently were diagnosed with collagenous colitis or lymphocytic colitis suggesting microscopic colitis could be a part of the spectrum of inflammatory bowel disease. The median duration of ulcerative colitis prior to being diagnosed with microscopic colitis was 15 years. We noted complete histological and/or symptomatic remission in three out of six cases while the other three patients reverted back into ulcerative colitis suggesting lymphocytic or collagenous colitis could present as a continuum of ulcerative colitis. The exact molecular mechanism of this histological transformation or the prognostic implications is still unclear. Till then it might be prudent to follow up these patients to assess for the relapse of inflammatory bowel disease as well as for dysplasia surveillance.

Key words: Ulcerative colitis; Lymphocytic colitis; Microscopic colitis; Collagenous colitis; Inflammatory bowel disease

Core tip: Lymphocytic colitis (LC), together with collagenous colitis (CC) is a part of the spectrum of "microscopic colitis" (MC) characterized by profuse non-bloody watery diarrhea, without endoscopic or radiological lesions, but with histological abnormalities. The association between LC and inflammatory bowel disease (IBD) is weak and unclear. The case reports of CC progressing to ulcerative colitis (UC) and vice versa has been previously reported but however to our knowledge we report the first case series of six patients with chronic UC subsequently developing into CC or LC suggesting MC could be a part of the spectrum of IBD.

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INTRODUCTION

Lymphocytic colitis (LC), together with collagenous

colitis (CC) is a part of the spectrum of "microscopic colitis" (MC) characterized by profuse non-bloody watery diarrhea, without endoscopic or radiological lesions, but with histological abnormalities. LC is characterized by increased lymphocytic infiltration of the colonic epithelium and lamina propria. CC in addition to the inflammatory infiltrate is characterized by a markedly thickened sub epithelial collagen band adjacent to the basal membrane. The association between LC and inflammatory bowel disease (IBD) is weak and unclear. The case reports of CC progressing to ulcerative colitis (UC) and vice versa has been previously reported but however to our knowledge we report the first case series of six patients with chronic UC subsequently developing into CC or LC suggesting MC could be a part of the spectrum of IBD[1-4].

We evaluated more than 1000 UC patients from a retrospectively collected UC colonoscopy database who had more than 3000 colonoscopies at our institution from 1998-2011. We identified a total of six patients with documented UC who subsequently were diagnosed with biopsy proven CC or LC from this database. All six patients were seen at our institution with the underlying UC for further management and the diagnosis of UC was reconfirmed by colonoscopic study in our institution. When these patients were followed up either for the change in symptoms or surveillance with the colonoscopic studies, colonic biopsies revealed CC or LC with no evidence of UC (Table 1).

Diagnostic criteria used by our pathologists to diagnose LC is increased intraepithelial lymphocytes (IELs > 20/100 colonic surface epithelial cells) in an architecturally normal colonic mucosa, accompanied by surface epithelial damage and a mixed mononuclear inflammatory infiltrate in the lamina propria[5]. These patients were treated for LC and on subsequent follow up three out of six patients reverted back to UC. All the slides were re-examined by a single pathologist and the diagnosis was confirmed (Liu X).

CASE REPORT

Patient 1

A 79-year-old female with a history of UC for 14 years presented with complaints of left lower quadrant abdominal pain for 1 month duration. She also had intermittent watery diarrhea and fecal urgency over the past 2 years. She denied bloody diarrhea or recent weight loss. She was on maintenance mesalamine, and UC was kept under complete remission. She was evaluated with colonoscopy for the present complaints which revealed very mild generalized redness throughout colon. Random colonic biopsies suggested marked surface epithelial lymphocytosis with collagen deposition in all areas of the colon consistent with the diagnosis of CC without any evidence of UC. She was treated with mesalamine 2400 mg/d and complete resolution of symptoms occurred within a month. She was asked to continue mesalamine and follow up if symptoms recur again. She has not had any recurrence of any symptoms.

Patient 2

A 75-year-old male with a history of UC for 25 years came for the surveillance colonoscopy to our institution. The surveillance colonoscopy with random colon biopsies revealed chronic quiescent UC involving the entire colon with no dysplasia. Subsequently, the patient developed intermittent watery diarrhea with fecal urgency. He denied nocturnal symptoms, bloody diarrhea and recent weight loss. He had a history of aspirin intake but there was no temporal relationship between aspirin intake and onset of diarrhea. Further work up with colonoscopy revealed mild erythema in left colon. The colon biopsies were consistent with LC throughout the colon with a significant increase in the number of intraepithelial lymphocytes without any evidence of UC. He was started on sulfasalazine and symptoms resolved within a month. Surveillance colonoscopy done 2 years later was macroscopically normal. Histopathological studies were negative both for UC and LC. He was asymptomatic to follow up till date.

Patient 3

A 61-year-old male with a long standing history of left-sided UC maintained on remission with sulfasalazine and 6-mercaptopurine had a surveillance colonoscopy performed for UC at our institution 20 years later and was macroscopically normal. Colonic biopsies revealed evidence of LC in the right colon. He was not treated for LC since he was asymptomatic on maintenance treatment. Four years later another surveillance colonoscopy revealed chronic quiescent UC. He was completely asymptomatic through these years.

Patient 4

A 59-year-old female was diagnosed with left-sided UC at the age of 46 and was maintained on mesalamine with complete symptomatic remission. She subsequently developed symptoms of intermittent watery diarrhea accompanied by abdominal cramps. She was treated with prednisone and mesalamine with no symptomatic improvement. She had a history of ibuprofen intake for back pain for a long time and there was no association between ibuprofen and the development of new symptoms. She was further evaluated with colonoscopy which revealed increased intraepithelial lymphocytes with collagen deposition throughout the colon consistent with CC. She was treated with bismuth and her symptoms resolved within 2 mo. The follow up colonoscopy a year later was negative for CC.

Patient 5

A 53-year-old female with a history of left-sided UC for 16 years had surveillance colonoscopy performed which revealed inactive UC in rectum and a tubular adenoma in the descending colon. She was referred to our institution for an opinion regarding UC and tubular adenoma. She

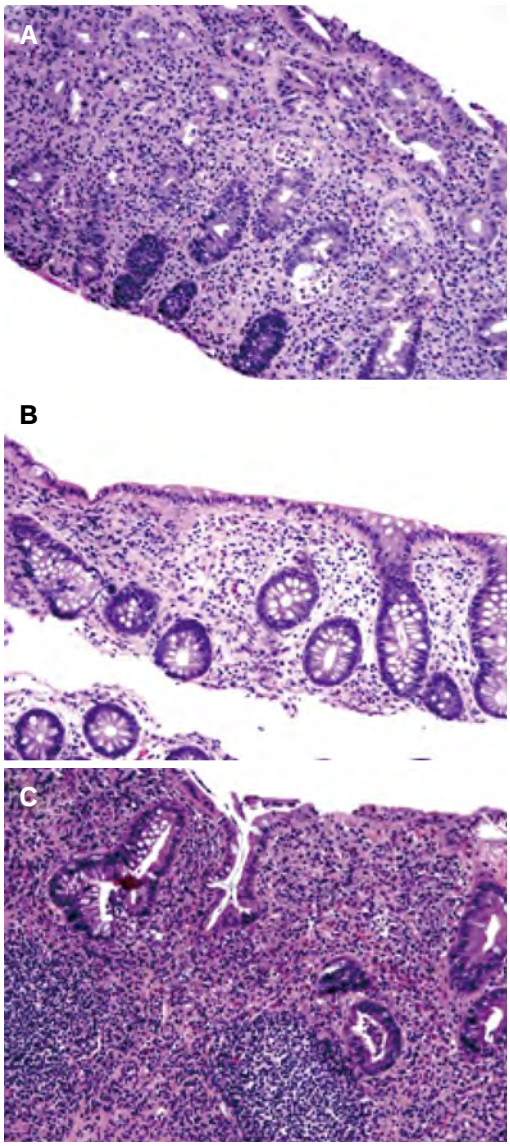

Figure 1 Histologic evolution of colitis (hematoxylin and eosin stain, × 200). A: Active colitis manifested by epithelial injury and cryptitis, in the context of clinical history of ulcerative colitis (UC) and lack of other etiologies for active colitis, this is consistent with early exacerbation of UC; B: Lymphocytic colitis (LC) (3 years after the exacerbation depicted in A). There is surface intraepithelial lymphocytosis and epithelial injury but without significant chronic information. The crypt architecture is normal; C: Reverting to active ulcerative colitis manifested by basal lympho plasmacytosis, architectural distortion, and cryptitis (3 years after an episode of LC-pattern of injury).

was treated with mesalamine for UC in the past and she was kept under complete remission. She was evaluated with a colonoscopy in our institution which suggested macroscopically normal colon with a histop anthology consistent with LC without any evidence of dysplasia. There was no change in treatment. Subsequently, surveillance colonoscopy done a year later revealed mild inactive UC in rectum and sigmoid colon without any evidence of LC and dysplasia. Two subsequent surveillance colonoscopies were positive for chronic UC in sigmoid colon and rectum without any dysplasia.

Patient 6

A 36-year-old female had a long standing history of extensive colitis for 18 years. She developed steroid dependent UC and required 6-mercaptopurine and mesalamine for symptom control (Figure 1A). She was maintained on complete remission on these medications. She had a surveillance colonoscopy performed which revealed mild erythema in distal rectum. The colonic biopsy studies were consistent with LC (Figure 1B) in all other areas of the colon. She had a follow up colonoscopy three years later which showed left-sided chronic active UC without any evidence of LC (Figure 1C). She has been asymptomatic at the time of last follow-up.

DISCUSSION

LC is characterized by chronic watery diarrhea and specific histopathological changes in a macroscopically normal colonic mucosa. The incidence has been reported to be 5.5 per 100000 and prevalence is 63.7 per 100000[6], but the incidence and prevalence appears to be increasing over time. The median age at diagnosis is 59 years with female: male ratio of 2.4:1. The most frequent symptoms at presentation are diarrhea, abdominal pain, weight loss and fecal urgency. Most patients are in remission with a limited disease duration of 6 mo; it can be chronic intermittent or chronic continuous in minority of patients. 7 However, relatively limited data has been published on the relationship between inflammatory bowel disease (IBD) and LC[7-11].

The etiology of LC is largely unknown and probably multifactorial. At present, it is thought to be caused by immunological reaction to different mucosal insults in predisposed individuals. The frequent association of LC with other autoimmune disorders (thyroid disease, diabetes mellitus, celiac disease, psoriasis, and rheumatoid arthritis), inflammation in the lamina propria with increased intraepithelial lymphocytes and the fair response to steroids support this theory. Infectious agents, drugs, or food antigen such as gluten may be precipitating factors. The importance of genetic factors in LC is still unclear but Olesen *et al*[7] suggested a family history of IBD in patients with LC. We describe six cases of UC that subsequently evolved into CC or LC. The median age at the time of UC diagnosis was 38 years in our series. The median duration of UC prior to being diagnosed with CC or LC was 15 years. The median age at the time of LC diagnosis was 51 years. We noted complete histological and/or symptomatic remission in three out of six cases while the other three patients reverted back into UC suggesting that LC could present as a continuum of UC. The triggering factor for this transformation is still unknown. The association between MC and IBD is found predominantly in patients with extensive colitis[12]. However, in our case series only 50% of patients had extensive colitis when LC or CC was diagnosed.

The relationship between LC and IBD is unclear. Sur-

Table 1 Clinical characteristic of patients

Variables	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Age at the time of last follow-up (yr)	81	75	62	60	52	36
Gender	Female	Male	Male	Female	Female	Female
Age at diagnosis of UC	65	42	28	46	34	18
Age at diagnosis of LC/CC	79	72	48	54	45	33
Duration of UC when LC/CC was diagnosed	14	30	20	8	11	15
Extent of UC when LC/CC was diagnosed	Extensive colitis	Extensive colitis	Left-sided	Left-sided	Left-sided	Extensive colitis
Extent of LC/CC at diagnosis	Extensive colitis	Extensive colitis	Right-sided	Extensive colitis	Extensive colitis	Extensive colitis
Endoscopic findings at the time of LC/CC diagnosis	Mild generalized redness	Mild erythema at left colon	Normal	Normal	Normal	Mild erythema at distal rectum
Medication at the time of LC/CC diagnosis	Mesalamine	None	Sulfasalazine, 6-mercaptopurine	Mesalamine	Mesalamine	Mesalamine, 6-mercaptopurine
Medications added when LC/CC was diagnosed	None	Sulfasalazine	None	Bismuth	None	None
Outcome on subsequent follow up	Asymptomatic	Asymptomatic	Ulcerative colitis	Asymptomatic	Ulcerative colitis	Ulcerative colitis
Family history of IBD/LC/CC	UC	None	None	None	None	UC
Smoking History	Yes	Ex-smoker	No	Yes	Yes	Ex-smoker
Alcohol use history	Yes alcoholic	Yes	No	No	Yes	No
History of aspirin/NSAIDS intake	No	Aspirin	No	NSAIDS	No	No
Period of follow up after LC/CC was diagnosed	3 mo	3 yr	10 yr	3 yr	8 yr	3 yr

NSAIDS: Non steroidal anti-inflammatory drugs; CC: Collagenous colitis; LC: Lymphocytic colitis; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

veillance colonoscopic biopsies from IBD patients with inactive disease may show a collagenous colitis pattern in UC and a focal LC-like pattern with Crohn's disease[1,12,13]. In our patients, the histopathological diagnosis of LC when UC was in complete remission further raises the question whether the observed LC pattern is an expression of healing and inactive UC disease in reality. However, the presence of symptoms in some patients and lack of IBD flare immediately prior to LC diagnosis makes this "healing" theory unlikely. A previous study had demonstrated that the transcriptional factor nuclear factor κB activation occurs in both UC and CC patients. However in CC patients, nuclear factor κB activation occurs only in epithelial cells whereas it occurs both in epithelial cells and lamina propria macrophages in UC patients[14]. Hence it is possible that the site of nuclear factor κB activation determines the pathological manifestation of the disease. Either UC or MC may precede the onset of the other. Based on these data, it seems reasonable to assume that UC and MC could represent both ends of the spectrum of the same disorder.

With the aggregate of cases we have reported along with other few case reports it seems highly reasonable to assume that LC or CC could be a part of the spectrum of IBD. Whether it is a random coincidence of MC and IBD in the same patient remains to be answered. Since there was no specific cause of LC in our patients such as an infection or drugs, along with the absence of autoimmune conditions usually associated with LC further supports our current view. The prognostic implication of this histological transformation to LC in inactive UC patients is still unknown and has to be further evaluated with prospective studies. Until then it might be prudent to consider MC as a part of the natural history of IBD, at least in some cases, and follow up these patients to assess for the relapse of IBD as well as for dysplasia surveillance.

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CASE REPORT

Alveolar echinococcosis-spreading disease challenging clinicians: A case report and literature review

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Abstract

Human alveolar echinococcosis (AE) is a potentially deadly disease; recent studies have shown that the endemic area of *Echinococcus multilocularis*, its causative agent, is larger than previously known. This disease has low prevalence and remains underreported in Europe. Emerging clinical data show that diagnostic difficulties are still common. We report on a 76-year old patient suffering from AE lesions restricted to the left lobe of the liver who underwent a curative extended left hemihepatectomy. Prior to the resection a liver biopsy under the suspicion of an atypical malignancy was performed. After the intervention he developed a pseudoaneurysm of the hepatic artery that was successfully coiled. Surprisingly, during surgery, the macroscopic appearance of the tumour revealed a growth pattern that was rather typical for cystic echinococcosis (CE), *i.e.*, a gross tumour composed of multiple large vesicles with several centimeters in diameter. In addition, there were neither extensive adhesions nor infiltrations of the neighboring pancreas and diaphragm as was expected from previous imaging results. The unexpected diagnosis of AE was confirmed by definite histopathology, specific polymerase chain reaction and serology results. This is a rare case of unusual macroscopic presentation of AE that posed immense diagnostic challenges and had an eventful course. To our knowledge this is the first case of an autochthonous infection in this particular geographic area of Germany, the federal state of Saxony. This report may provide new hints for an expanding area of risk for AE and emphasizes the risk of complications in the scope of diagnostic procedures and the limitations of modern radiological imaging.

Key words: Alveolar echinococcosis; *Echinococcus multilocularis*; Autochthonous infection; Liver resection; Hemihepatectomy

Core tip: We describe a rare case of uncommon macroscopic presentation of autochthonous infection with *Echinococcus multilocularis* that posed immense diagnostic challenges and had an eventful course. To our knowledge this is the first case of an autochthonous infection in this geographic area. This report may deliver new hints for an expanding area of risk for alveolar

echinococcosis and emphasizes the risk of complications in the scope of diagnostic procedures and the limitations of modern imaging techniques.

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INTRODUCTION

The fox-tapeworm *Echinococcus multilocularis* (*E. multilocularis*) is the causative agent of alveolar echinococcosis (AE), a potentially deadly parasitic disease. AE is prevalent in the northern hemisphere and central Europe is an endemic focus[1-3]. In the era prior to anthelmintic treatment the cumulative lethality for AE was about 90% ten years after a diagnosis has been established[4]. Imaging techniques of hepatic involvement by AE commonly reveal an ill-defined lesion of the liver parenchyma and contrast computer tomography (CT) and magnetic resonance imaging (MRI) are considered to clearly demonstrate infiltrative structure and extension of the parasitic tumour to adjacent structures[5-8]. In macroscopic sections of the human liver the larval parasite usually exhibits an alveolar (spongy) structure composed of numerous irregular vesicles with diameters between less than 1 and up to 20 mm[9]. Biologically, the lesions behave like a slow-growing liver cancer, without sharp boundaries between the parasitic tissue and the liver parenchyma.

Publications on surgical procedures and results are rare but essential, and prospective studies are not available because the incidence of the disease is low. According to current treatment guidelines, surgery should be the first choice if the parasitic mass is resectable *in toto*[10]. Complete resections of the parasitic lesion can cure the patient while available drugs are only parasitostatic[10-12].

Emerging clinical data indicate that the parasite's geographic range has widened in recent years[13,14]. Growing fox populations in Europe, especially in urban zones, have drawn attention to a potentially increased infection risk for humans with a phase lag of 10-20 years[15-17]. In addition, since AE is not adequately considered as a differential diagnosis, the disease remains thus underdiagnosed in Europe[18].

We report on a 76-year old patient with AE of the liver, who underwent a curative resection. Prior to resection the patient had an eventful course due to the development of a postinterventional pseudoaneurysm (aneurysm spurium) of the hepatic artery following a biopsy of the liver lesion. Interestingly, during surgery the parasitic mass appeared typical for cystic echinococcosis (CE) and revealed no infiltrations or extensive adhesions to adjacent structures as contrastingly expected from the preoperative imaging results of the abdomen. Nevertheless, the definite histology as well as referral evaluations [serology and polymerase chain reaction (PCR)] confirmed the diagnosis of AE. The patient has not been abroad for the last 20 years and at his farm he has been in constant contact to various animal species, including dogs and wild foxes. To our knowledge this is the first case of autochthonous AE infection in the federal state of Saxony, Germany.

CASE REPORT

In April 2010 a 76-year old farmer from Saxony was presented in the emergency department of a general district hospital with a severe abdominal pain under the suspicion of a biliary colic and pancreatitis, respectively. In the performed imaging of the abdomen a large cystic tumour in the left sided liver was detected. In the further course, suspecting a highly malignant atypical primary liver cancer, a biopsy for the presumed confirmation of the diagnosis was performed. After the intervention the patient was discharged in good clinical condition. Surprisingly, the histological findings from the tumour biopsy were consistent with the larval stage of *E. multilocularis*. Subsequent serological investigation by a referral laboratory for echinococcosis confirmed specific antibodies for AE in the patient's serum. Moreover, a pan-cestode 12S rRNA gene-PCR from the paraffin block was positive and sequencing of the amplicon revealed 100% identity with *E. multilocularis*. Therefore, an anti-infective drug treatment with albendazole was initiated.

Several days after liver biopsy the patient presented again with severe abdominal pain and jaundice. In the emergency CT scan of the abdomen the gross tumour revealed no progression. Though, a postjunctional pseudoaneurysm of a branch of the left hepatic artery in segment 4a in direct proximity to the tumour tissue was newly diagnosed. A subsequent coil-embolization of the aneurysm was successfully performed.

One month later, in May 2010, the patient was referred to our centre as a potential candidate for abdominal surgery. A thorough examination of patient data and history files revealed that the farmer has not been abroad in the last few decades. In his farm he has been in a constant contact and exposure to numerous domestic and wild animals, including dogs and foxes. The patient had neither B-symptoms nor further major ailments and was in good general condition. The blood tests revealed normal findings for alpha-1-fetoprotein, carbohydrate-antigen 19-9 and carcinoembryonic antigen. Preoperatively, due to pronounced cholestasis and hyperbilirubinaemia, an endoscopic retrograde cholangiography (ERC) with stenting of the main bile duct was performed. Clinical imaging prior to surgery revealed that the echinococcal lesion infiltrated liver segments 2 and 3 and showed a maximum diameter of 13.5 cm (Figure 1A and B). In addition, CT suggested the presence of adhesions in the

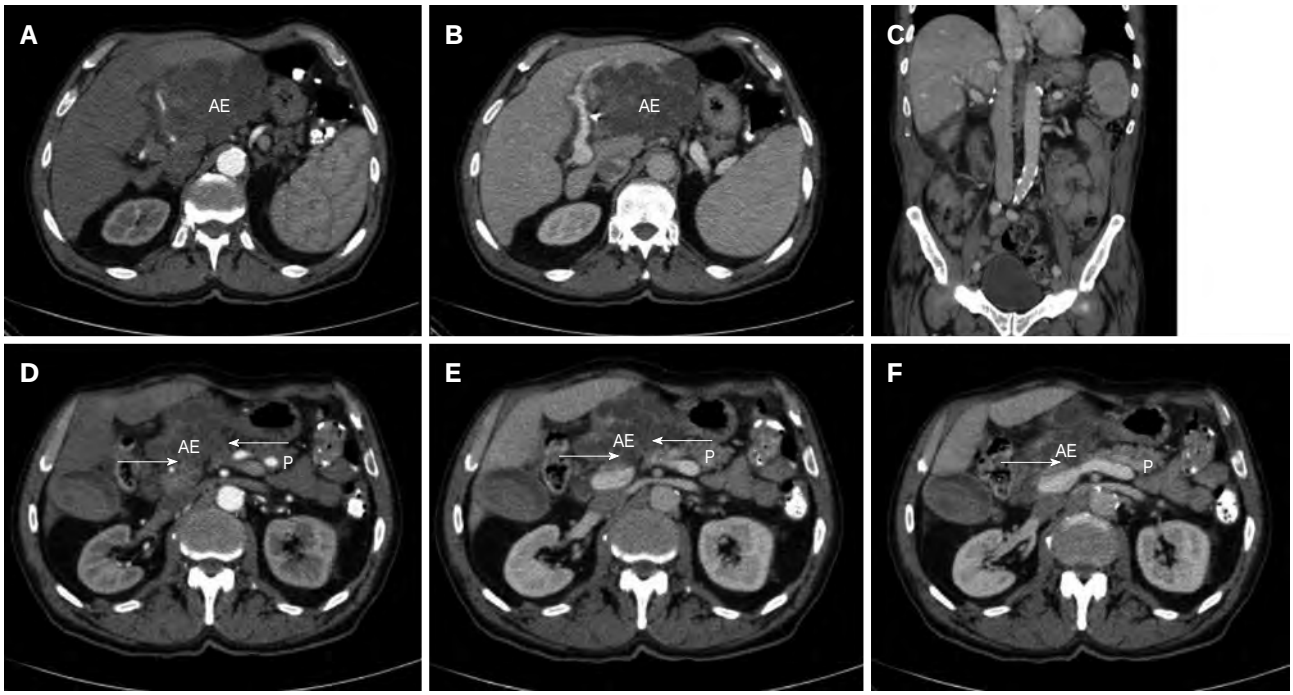

Figure 1 Radiological findings prior and after curative resection. A, B: Computed tomography of the abdomen displaying an extended tumour manifestation prior to resection; C: Computed tomography of the abdomen following extended left hemihepatectomy; D-F: Computed tomography of the abdomen prior to resection. Arrows: possible extensive adhesions to adjacent pancreatic head and corpus. AE: Alveolar echinococcus tumour; P: Pancreas.

area of the diaphragm and cystic infiltration of the pancreatic head and corpus (Figure 1 D-F). The diagnostic evaluation revealed no further extrahepatic manifestations. Hence, additionally to extended left hemihepatectomy, we considered a concomitant resection of the pencreatic head and corpus.

During surgery the tumorous manifestation in the left lobe of the liver was confirmed but, surprisingly, no infiltrative growth to neighboring structures was detectable. Basically, we observed a gross tumor with multiple large cystic structures that varied in size, *i.e.,* a growth pattern that is rather typical for CE. Further exploration revealed no cystic adhesions to the diaphragm. After exploration of the bursa omentalis, no infiltration of the pancreas was notable either. No evidence for further extrahepatic tumorous dissemination or lymph node metastases was found. As the restriction of the tumour to the left liver lobe was confirmed, we affirmed the indication for extended left hemihepatectomy with curative intent. The situs was then suffused with cloths imbued in hypertonic (10%) Sodium chloride (NaCl) solution. Subsequently the isolation of the proper hepatic artery and the selective division of the left hepatic artery followed. After isolation of the main trunk of the portal vein and the left portal vein branch, the latter was divided. Then cholecystectomy was performed. After the left hepatic vein was divided, lobus caudatus (segment 1) was mobilized. Then, a liver resection was completed without any intraoperative complications. Parenchymal transection was performed using an ultrasonic dissection device (cavitron ultrasonic surgical aspirator, CUSA®). The caudate lobe did not appear to be infiltrated but to ensure an additional safety distance it was resected as well. The parenchymal resection was performed along the level of the middle hepatic vein in direction to the gall bladder bed and then caudally to the hepatic hilum. At the hilum the liver dissection diverged to the left and then ended in the parenchymal bridge leading to the caudate lobe. In the region of the hilum the left hepatic duct was isolated and then selectively divided. After removing the left liver lobe and the caudate lobe, the situs was rinsed with hypertonic NaCl solution. A T-Drain was inserted in the main bile duct for decompression and easy access cholangiography. During the postoperative stay in the intensive care unit the patient did not develop any significant complications.

Based on the finding of multiple large cystic formations of the tumorous lesion an additional histological evaluation was performed in a referral centre for pathology. There, the original diagnosis of hepatic AE was confirmed (Figure 2). In the postoperative course no signs of insufficient liver function were notable. The conclusive pathological result showed the parasitic tumour was entirely resected (Figures 1C and 3).

After a total postoperative hospitalization of 30 d the patient was dismissed. The anti-infective drug treatment with albendazole was maintained as long-term therapy. The follow up visit 6 and 12 mo after resection revealed normal liver function and no evidence for recurrent disease.

DISCUSSION

AE, caused by the larval (metacestode) stage of *E. multilocularis*, is found in the northern hemisphere and is a

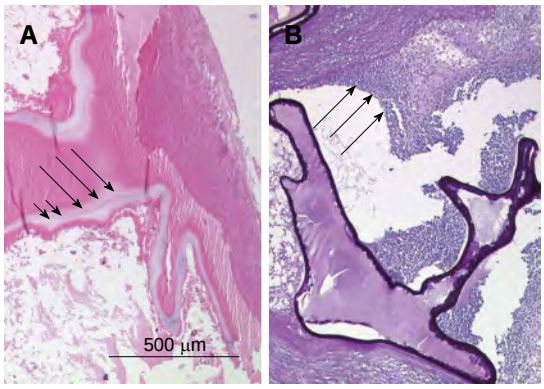

Figure 2 Referral evaluations for diagnosis of alveolar echinococcosis. A: The hematoxylin and eosin stain of paraffin sections displays the laminated layer as a narrow band (long arrows). The germinal layer is marked by short arrows; B: Periodic acid-Schiff (PAS) stain shows a strongly PAS-positive basophilic laminated layer displaying a bizarre narrow structural pattern. The long arrows indicate the typical severe inflammatory process associated with the characteristic tubular growth pattern of the parasite.

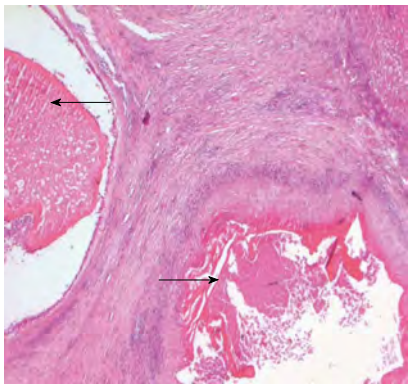

Figure 3 Histological findings after curative resection. Hematoxylin and eosin stain of paraffin sections displaying two daughter cysts containing no vital protoscoleces embedded in a larger lesion. Black arrows indicate avital protoscoleces.

potentially fatal disease. The parasite is transmitted to humans by eggs of the helminth shed into the environment by feces of foxes. Almost exclusively, the liver is affected[19]. Recent studies have shown that the endemic area of *E. multilocularis* is larger than previously known and has regionally expanded from rural to urban areas[20-22]. In addition, increasing fox populations are associated with higher infection risk in humans with a phase lag up to 20 years[15].

The macroscopic appearance of an echinococcal lesion is distinct in regard of its species and developmental stage. While parasitic masses of CE ordinarily display a typical structure of a single or multiple fluid-filled large unilocular cysts that can reach monstrous dimensions, AE preferentially exhibits metastasis and an infiltrative growth to adjacent host tissues with a spongy structure composed of numerous irregular small vesicles of several millimeters. Thus, the surgical therapy for hepatic AE conforms the operative principles established for malignant liver tumours, *i.e., in toto* removal of the tumour with additional safety distance and tumour free resection margins[4].

Chemotherapy with benzimidazoles is the backbone of the comprehensive treatment of AE and long-term anti-infective drug treatment has been established in many centres in Europe as well as in China[9]. In spite of remarkable improvement of long term patient survival after the introduction of anti-helmintic drug treatment, this therapeutic modality proved to be mainly parasitostatic. Therefore, surgical resection represents the therapy of choice for patients with operable lesions of AE.

In the present report we have described the first case of autochthonous infection with *E. multilocularis* in our federal state of Saxony, Germany. The tumour masses affected liver segments 2 and 3 and the patient received a curative extended left hemihepatectomy. Due to a suspected liver malignancy an interventional biopsy of the lesion was preoperatively performed. Usually, such a diagnostic step is considered a contraindication in cases of AE because of the risk of abdominal seeding and anaphylaxis. Fortunately, the postinterventional iatrogenic pseudoaneurysm of the left hepatic artery could be treated with success. Interestingly, the parasitic mass showed a macroscopic pattern that appeared typical for CE which is caused by the larval stage of the dog tapeworm, *Echinococcus granulosus* (*E. granulosus*). The cause of this phenomenon remains for the most part unknown but has been reported occasionally in historic reports. Dual infection with *E. granulosus* and *E. multilocularis* could have also been possible. Indeed, concomitant infections with both echinococcal species have been reported in the literature but in the present case the definite histology, PCR results, serology, and immunohistology for specific structural proteins were all clearly positive for *E. multilocularis* only[23]. Additionally, a major distinguishing factor between *E. granulosus* cysts and *E. multilocularis* is the presence of an adventitial layer around the *E. granulosus* metacestode. The histological evaluations did not detect such a structure in the present case. Furthermore, despite the expected extensive adhesions to the diaphragm and pancreas seen by preoperative imaging, no such condition or infiltration to neighboring structures could be confirmed, showing current limitations of modern imaging techniques. All together this data indicate that in the current era diagnosis as well as assessment of extent of local disease still remain a challenge for clinicians.

Recent investigations suggest that AE remains underreported and human infection can also occur in regions with low overall parasite prevalence. Case reports from regions remote from the areas of high prevalence may be strong hints of new areas at risk[18,24]. In addition to the high prevalence rates for AE in the southern geographic regions of Germany, recent data suggest growing numbers of AE cases in neighboring European countries, such as the Czech Republic. Some of these cases indicate an autochthonous character of the infection[25,26]. Thus, a possible enlargement of fox populations in the last decades as well as migration of infected animals might have

been a possible source for infection in the current case.

In conclusion, we have here described the first autochthonous infection with *E. multilocularis* in Saxony, Germany, providing the first evidence for a new geographical area at risk for the acquisition of AE. Albeit, curatively treated with an extended left hemihepatectomy the disease presented with uncommon findings and had an eventful course, constituting a challenge for clinicians.

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CASE REPORT

A white opaque substance-positive gastric hyperplastic polyp with dysplasia

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Abstract

The endoscopic findings of gastric hyperplastic polyps (HPs) with dysplasia have not been well-defined, and the clinical significance of these lesions, including their malignant potential, is unclear. In this report, we describe a case of a white opaque substance (WOS)-positive gastric HP with dysplasia. A 76-year-old woman was referred to our hospital for endoscopic resection of a gastric HP. Upper endoscopy revealed a 25-mm whitish and reddish polypoid lesion on the greater curvature in the lower third of the stomach. The whitish part was diagnosed as a WOS using conventional and magnifying endoscopy with narrow band imaging. An examination of the biopsy specimen indicated that the lesion was a typical gastric HP. However, because of its color and the presence of a WOS, we suspected that this lesion was an atypical gastric HP. Therefore, we performed a polypectomy. Histopathologically, diffuse low- to high-grade dysplasia was found on the surface of the polyp. We performed immunohistochemical staining using a monoclonal antibody specific for adipophilin as a marker of lipid droplets (LDs). LDs were detected in approximately all of the neoplastic cells, especially in the surface epithelium of the intervening apical parts and were located in the subnuclear cytoplasm of the neoplastic cells. According to endoscopic and histopathological findings, the WOS-positive epithelium indicated dysplasia of the gastrointestinal phenotype, which could absorb lipids. The presence of a WOS in a gastric HP may be considered an endoscopic finding that is predictive of the neoplastic transformation of a gastric HP. We suggest that a WOS-positive gastric HP should be resected endoscopically to investigate its neoplastic transformation.

Key words: Gastric hyperplastic polyp with dysplasia; White opaque substance; Adipophilin; Lipid droplet; Narrow band imaging

Core tip: In this report, we present the first case of a white opaque substance (WOS)-positive gastric hyperplastic polyp (HP) with dysplasia. We performed immunohistochemical staining using a monoclonal antibody specific for adipophilin as a marker of lipid droplets. According to endoscopic and histopathological findings, the WOS-positive epithelium corresponded to the dysplasia in this lesion. The presence of a WOS in a gastric HP may be considered an endoscopic finding that is predictive of the neoplastic transformation of a gastric HP. We suggest that patients with a WOS-positive gastric HP should be treated by endoscopic resection to investigate the neoplastic transformation of the HP.

Ueyama H, Matsumoto K, Nagahara A, Gushima R, Hayashi T, Yao T, Watanabe S. A white opaque substance-positive gastric hyperplastic polyp with dysplasia. *World J Gastroenterol* 2013;

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INTRODUCTION

With the widespread use of digestive endoscopy in recent years, gastric polyps are now diagnosed more frequently and can be easily studied after a biopsy or polypectomy. Gastric hyperplastic polyps (HPs) are among the most common type of benign epithelial gastric polyps[1-6]. Gastric HPs are usually considered to be benign lesions similar to adenomas; however, neoplastic transformation can occur but rarely. Moreover, endoscopic findings of gastric HPs with dysplasia have not been well-defined, and the clinical significance of these lesions, including their malignant potential, is unclear. A white opaque substance (WOS) is a finding from magnifying endoscopy (ME) with narrow band imaging (NBI), which was first reported by Yao *et al*[7-9] to be a substance in the superficial area of gastric neoplasias that obscures the subepithelial microvascular architecture. However, the presence of a WOS in gastric lesions other than adenomas and adenocarcinomas has not been reported. We report a rare case of a WOS-positive gastric HP with dysplasia.

CASE REPORT

A 76-year-old woman was referred to our hospital for further investigation and treatment of a gastric HP. Excluding the existence of the gastric HP, she had no specific symptoms and the results of the physical examination were normal. Her medical history included hyperlipidemia and diabetes mellitus, and there was no family history of gastrointestinal polyposis. She had not undergone proton pump inhibitor therapy. An assessment of Immunoglobulin G antibodies and a histological examination were negative for *Helicobacter pylori* infection. Upper endoscopy revealed a 25-mm polypoid lesion on the greater curvature in the lower third of the stomach (Figure 1A). The entire lesion was reddish with scattered whitish areas. The whitish parts were determined to be a WOS using conventional endoscopy and ME with NBI (Figure 1). The WOS in the lesion was comprised of two morphological types (Figure 1C and D). One type had a symmetrical distribution of a regular dotted pattern (Figure 1C), and the other type had an asymmetrical distribution of an irregular speckled and linear pattern (Figure 1D). An examination of the biopsy specimen revealed findings that were typical of a gastric HP without dysplasia. However, we suspected that this lesion was an atypical gastric HP because of its color and the irregular distribution of the WOS. Therefore, we performed a polypectomy, which was without complications. Histopathologically, the findings for the entire lesion were typical of

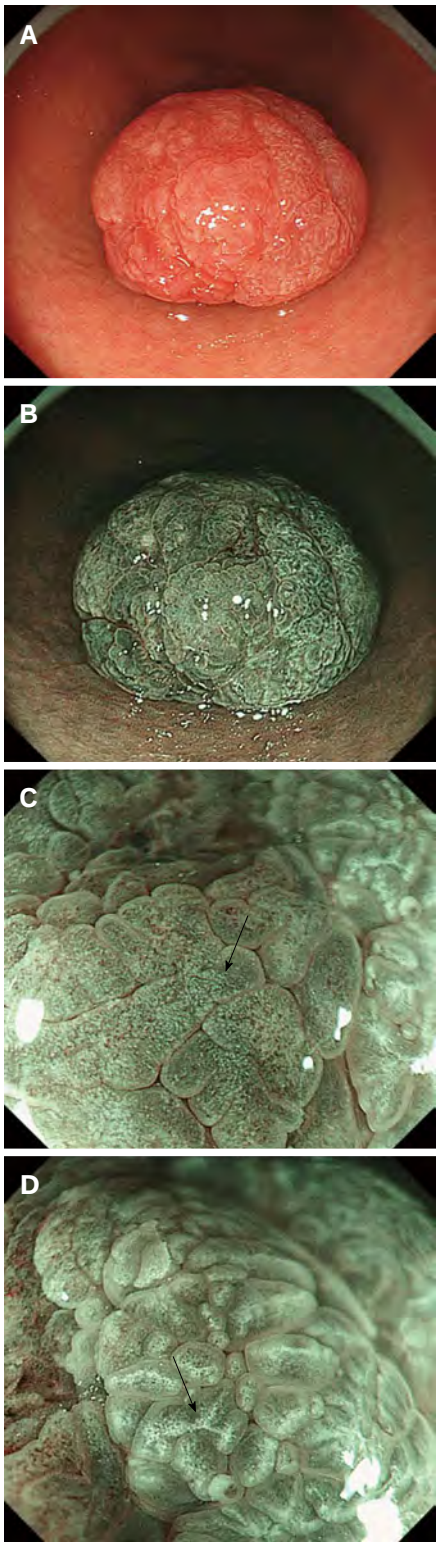

Figure 1 A white opaque substance-positive gastric hyperplastic polyp is shown on upper endoscopy. A: An endoscopic examination with a white light image revealed a 25-mm polypoid lesion on the greater curvature in the lower third of the stomach; B: A white opaque substance (WOS) was visualized on the surface of this lesion using conventional endoscopy and magnifying endoscopy (ME) with narrow band imaging (NBI); C: The ME with NBI findings. A regular dotted pattern of the WOS was distributed symmetrically (arrow); D: The ME with NBI findings. An irregular speckled and linear pattern was distributed asymmetrically (arrow).

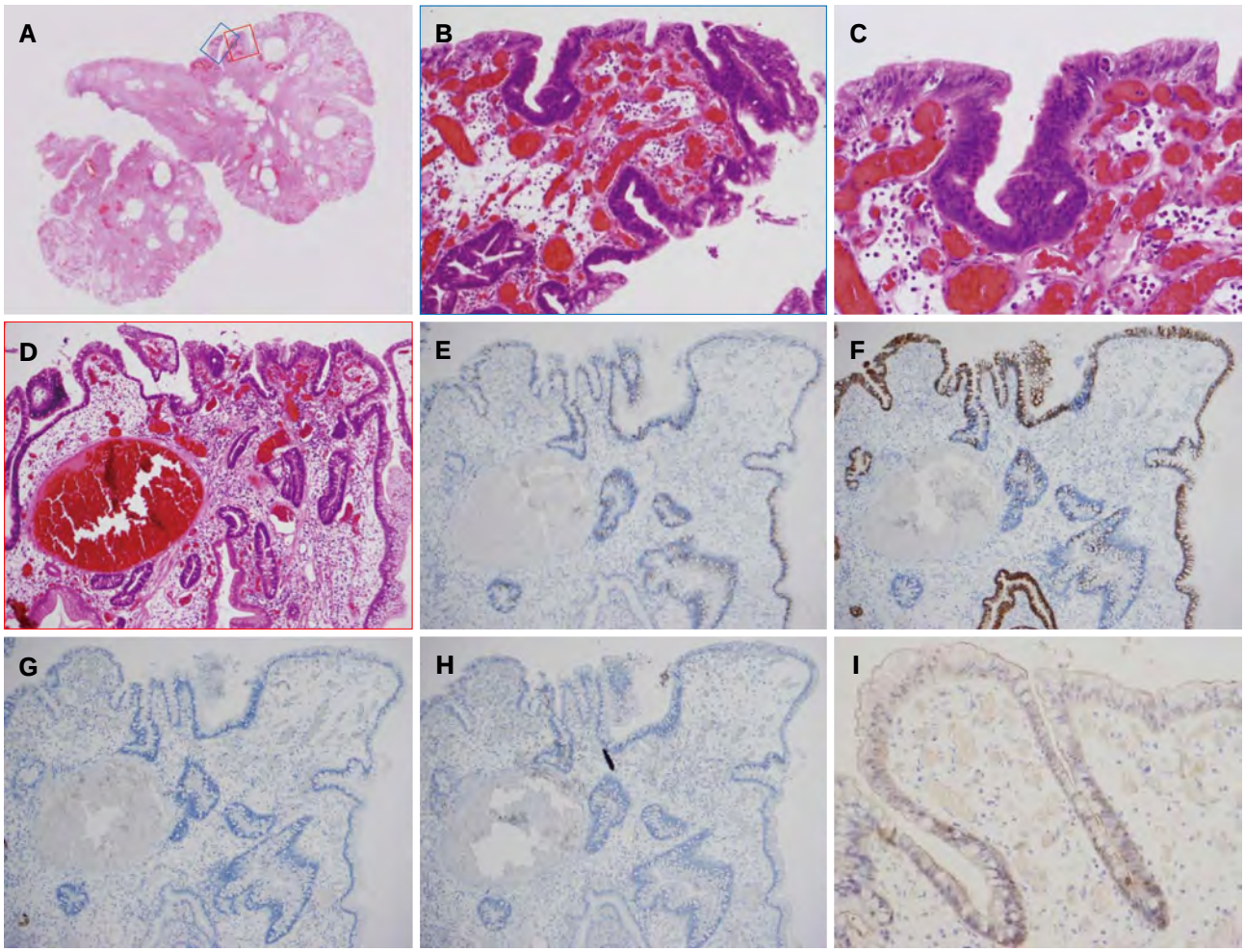

Figure 2 The resected specimen shows a gastric hyperplastic polyp with dysplasia. A-D: The histological examination of the resected specimens (hematoxylin and eosin stain). A: In the low power view, the findings for the entire lesion were typical of a gastric hyperplastic polyp; B, C: High-grade dysplasia was observed on the surface of the lesion; D: Low-grade dysplasia was observed on the surface of the lesion; E-I: The immunohistochemical examination. E: Mucin 2 (MUC2); F: MUC5AC; G: MUC6; H: CD10; I: Villin. The lesion had diffuse positivity for MUC5AC, focal positivity for MUC2 and villin, and negative staining for MUC6 and CD10.

a gastric HP, and diffuse low- to high-grade dysplasia was found on the surface of the lesion (Figure 2A-D). Immunohistochemically, the lesion had diffuse positivity for MUC5AC, focal positivity for mucin 2 (MUC2) and villin, and negative staining for MUC6 and CD10 (Figure 2E-I). This lesion was classified as having the gastrointestinal (GI) phenotype according to combinations of the expression of MUC2, MUC5AC, MUC6, CD10 and villin. The GI phenotype was detected in approximately all of the neoplastic cells, whereas an examination of the other cells indicated a gastric phenotype. The Ki-67 labeling index of dysplasia was slightly higher than that of a typical HP, and the positive cells were irregularly distributed. The overexpression of the p53 protein was not observed. In addition, adipophilin was detected in approximately all of the neoplastic cells, especially in the surface epithelium of the intervening apical parts and was located in the subnuclear cytoplasm of the neoplastic cells (Figure 3). This lesion was finally diagnosed as a WOS-positive gastric hyperplastic polyp with dysplasia. Surveillance endoscopy with biopsy specimens is planned for 6 mo after the endoscopic resection.

DISCUSSION

The endoscopic findings for gastric HP with dysplasia have not been well-defined. Typical HPs are markedly reddish polypoid lesions with a smooth surface, which occasionally has erosions. In this case, the entire lesion was reddish and was scattered with whitish areas, which differs from typical HPs. The whitish areas were determined to be a WOS using conventional and ME with NBI. Histopathologically, low- to high-grade dysplasia was diffusely present on the surface of the gastric HP. Adipophilin was detected in approximately all of the neoplastic cells, especially in the surface epithelium of the intervening apical parts. These findings suggested that the WOS-positive epithelium corresponded to the dysplasia in this lesion. Yao *et al*[10] reported that the hallmark of a WOS is the presence of lipid droplets (LDs) that accumulate in the superficial part of the epithelial neoplasia within the stomach. Using immunohistochemistry and immunoelectron microscopy, Ueo *et al*[11] found that the WOS resulted from an accumulation of LDs with adipophilin. These findings supported a relationship

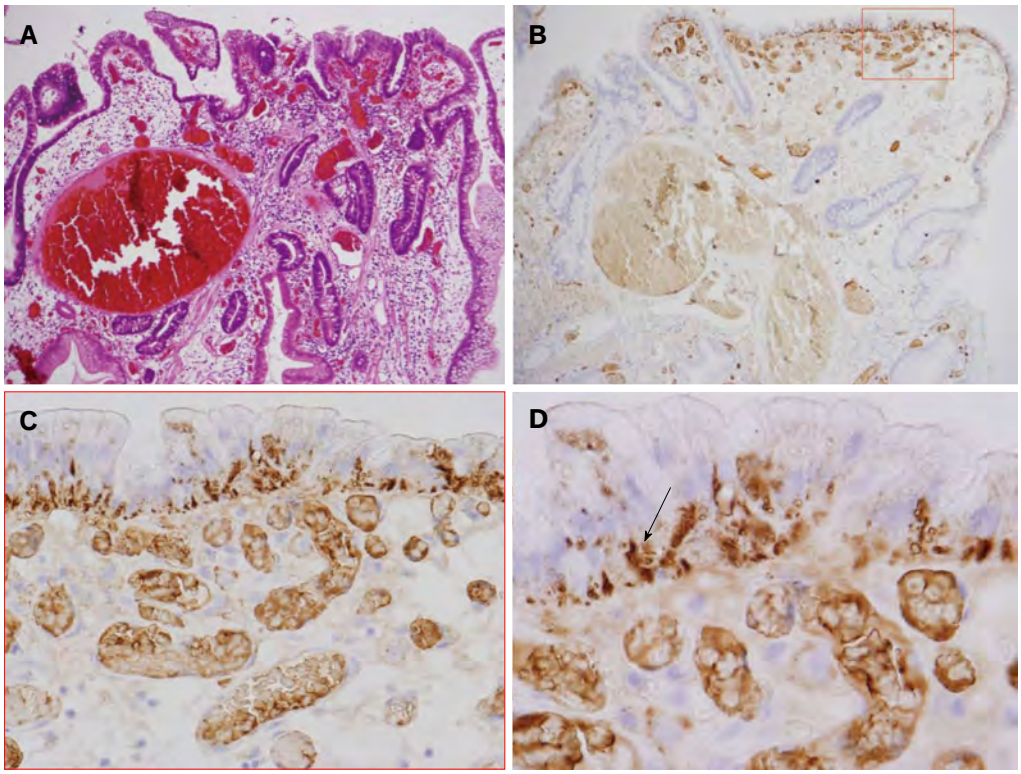

Figure 3 The immunohistochemical analysis indicates that dysplasia is positive for adipophilin. A: Low-grade dysplasia was observed on the surface of the lesion; B-D: The immunohistochemical examination of adipophilin. Adipophilin was detected in approximately all of the neoplastic cells, especially in the surface epithelium of the intervening apical parts and was located in the subnuclear cytoplasm of the neoplastic cells (arrow).

between the WOS and adipophilin in this case. In addition, the WOS in this lesion was comprised of two morphological types: one type with a symmetrical distribution of a regular dotted pattern and the other type with an asymmetrical distribution of an irregular speckled and linear pattern. We could not discriminate between these patterns pathologically. Yao *et al*[7] reported that the WOS in adenomas was regular and homogeneous, whereas the WOS in adenocarcinomas was irregular and speckled. In this case, we speculated that the findings for a WOS may be based on the differences in the shape, the intraepithelial and intracytoplasmic density and the distribution of the LDs between low-grade and high-grade dysplasia. In our case, most of the reddish area indicated low-grade dysplasia. Endoscopically, we determined that these areas were reddish because a slight accumulation of LDs may not allow these areas to be visualized as a WOS. We concluded that a WOS may be visualized only in the dysplastic areas of gastric HPs. The presence of a WOS in a gastric HP may be considered an endoscopic finding that is predictive of the neoplastic transformation of a gastric HP.

The neoplastic transformation of HPs has not been well-defined, and their clinical significance, including their malignant potential, is unclear. Kang *et al*[12] reported that the neoplastic transformation of gastric HPs was significantly associated with the postgastrectomy state and lesions that were 1 cm in diameter, pedunculated, and synchronous neoplastic lesion. Daibo *et al*[13] reported that cancer cells arose from the dysplastic area in HPs rather than directly from nondysplastic hyperplastic epithelium, which is consistent with the histogenesis of the malignant transformation of HPs. Endoscopic resection should be considered for these HPs to avoid the risk of missing HPs with neoplastic potential. In our case, the dysplasia was observed on the surface of the resected HP; however, an examination of the biopsy specimen indicated a typical HP without dysplasia. Regarding this discrepancy, we speculate that when the biopsy specimen was collected, a small sample was unintentionally taken from the part of the lesion that did not exhibit dysplasia. Using the biopsy specimen that was obtained, we could not clearly determine whether the lesion was a typical HP or an HP with low-grade dysplasia. Therefore, in gastric HPs, WOS positivity may be considered an endoscopic finding that indicates endoscopic resection.

The mechanism of the accumulation of LDs in gastric epithelium is unknown. Yao *et al*[10] proposed the following two possible mechanisms: the absorption hypothesis and the production hypothesis. In addition, they reported that the WOS in gastric neoplasms with an intestinal phenotype was caused by the accumulation of lipids and WOS-positive gastric neoplasms may be able to absorb lipids. Matsubara *et al*[14] demonstrated that the expression of adipophilin may be induced during the process of early colorectal carcinogenesis, which supports the production hypothesis that neoplastic cells synthesize LDs. In our case, the neoplastic cells that were

positive for adipophilin were of the GI phenotype. This finding suggests that the neoplastic transformation of gastric epithelium with a phenotypic change to the intestinal phenotype may require the ability to absorb lipids. However, further investigation is needed to elucidate the mechanism of LD accumulation.

In this report, we present the first case of a WOS-positive gastric HP with dysplasia. We suggest that patients with a WOS-positive gastric HP should be treated by endoscopic resection to investigate the neoplastic transformation of the HP.

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CASE REPORT

Unexpected endoscopic full-thickness resection of a duodenal neuroendocrine tumor

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Abstract

A 57-year-old man underwent endoscopy for investigation of a duodenal polyp. Endoscopy revealed a hemispheric submucosal tumor, about 5 mm in diameter, in the anterior wall of the duodenal bulb. Endoscopic biopsy disclosed a neuroendocrine tumor histologically, therefore endoscopic mucosal resection was conducted. The tumor was effectively and evenly elevated after injection of a mixture of 0.2% hyaluronic acid and glycerol at a ratio of 1:1 into the submucosal layer. A small amount of indigo-carmine dye was also added for coloration of injection fluid. The lesion was completely resected *en bloc* with a snare after submucosal fluid injection. Immediately, muscle-fiber-like tissues were identified in the marginal area of the resected defect above the blue-colored layer, which suggested perforation. The defect was completely closed with a total of 9 endoclips, and no symptoms associated with peritonitis appeared thereafter. Histologically, the horizontal and vertical margins of the resected specimen were free of tumor and muscularis propria was also seen in the resected specimen. Generally, endoscopic mucosal resection is considered to be theoretically successful if the mucosal defect is colored blue. The blue layer in this case, however, had been created by unplanned injection into the subserosal rather than the submucosal layer.

Key words: Endoscopic mucosal resection; Submucosal tumor; Neuroendocrine tumor; Hyaluronic acid; Perforation; Duodenum; Endoclip

Core tip: We herein report a case of endoscopic full-thickness resection of a duodenal neuroendocrine tumor after unplanned injection into the subserosal layer. Generally, large perforations require urgent salvage surgery and duodenal perforation is more serious than other sites of the gastrointestinal tract because of bile acid and pancreatic juice. In this case, we found the "mirror target sign" immediately, and repaired the defect endoscopically. Prompt recognition of this sign and rapid closing of the defect is important to minimize injury.

Hatogai K, Oono Y, Fu KI, Odagaki T, Ikematsu H, Kojima T, Yano T, Kaneko K. Unexpected endoscopic full-thickness resection of a duodenal neuroendocrine tumor. *World J Gastroenterol* 2013; 19(26): 4267-4270 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4267.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4267

INTRODUCTION

Endoscopic mucosal resection (EMR) technique has

been developed and widely performed to provide less invasive treatment for gastrointestinal tumors. However, perforation is perhaps one of the most unfavorable complications associated with EMR. The most effective way to avoid perforation is to maintain a sufficiently thick and long-lasting submucosal cushion by endoscopic fluid injection into the submucosal layer. Various solutions such as hypertonic saline, glycerin solution, and hyaluronic acid (HA) have been used, however, among those solutions, HA is preferred because of its higher viscosity[1]. Recently, EMR has been also applied for submucosal tumors, such as neuroendocrine tumors (NETs)[2-4]. We herein report a case of an endoscopic full-thickness resection of a duodenal NET after unplanned fluid injection into the subserosal layer.

CASE REPORT

An asymptomatic 57-year-old man underwent endoscopy for investigation of a duodenal polyp detected in a private full medical checkup at another hospital. Endoscopy revealed a whitish hemispheric submucosal tumor with a smooth surface, about 5 mm in diameter, in the anterior wall of the duodenal bulb (Figure 1). Endoscopic biopsy disclosed a NET histologically. Because endoscopic findings such as size, shape, and mobility of the lesion indicated the tumor existed in the submucosal layer, EMR was conducted for its removal.

The tumor was effectively and evenly elevated after endoscopic submucosal injection of a mixture of 0.2% HA and glycerol at a ratio of 1:1 with a small amount of indigo-carmine dye added for coloration. The lesion was completely resected *en bloc* with a snare after submucosal fluid injection. Immediately, muscle-fiber-like tissues were identified at the margin of the resected defect, which suggested perforation, although a blue-colored layer was detected in the resection defect (Figure 2). The defect was completely closed with a total of 9 endoclips (Figure 3). No pneumoperitoneum was detected during or after EMR and endoscopic closure. The muscle layer was involved in the underside of the resected specimen (Figure 4). Computed tomography performed after the procedure revealed a small amount of free air but no fluid collection in the retroperitoneal space. Though the patient was in the hospital for 5 d longer than planned, he was successfully treated conservatively with intravenous fluids including antibiotics for 4 d without oral intake, and no symptoms associated with peritonitis appeared. Histologically, the tumor was diagnosed as a NET grade (G) 1 limited within the submucosal layer without muscular or lymphovascular invasion. The horizontal and vertical margins of the resected specimen were free of NET and the muscularis propria (MP) was also seen in the resected specimen (Figure 5). The blue layer in the resection defect had been created by fluid injection into the subserosal layer rather than the intended submucosal layer. No local recurrence or metastasis was detected after a follow-up of 30 mo from EMR.

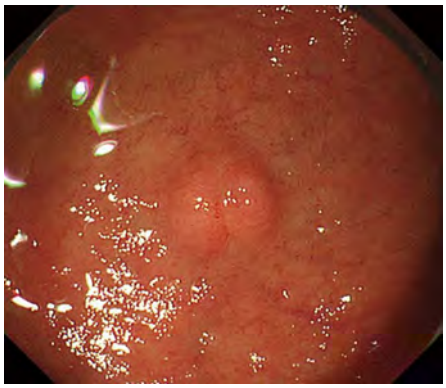

Figure 1 A neuroendocrine tumor, 5 mm in diameter, was detected in the duodenal bulb during endoscopy in an asymptomatic 57-year-old man.

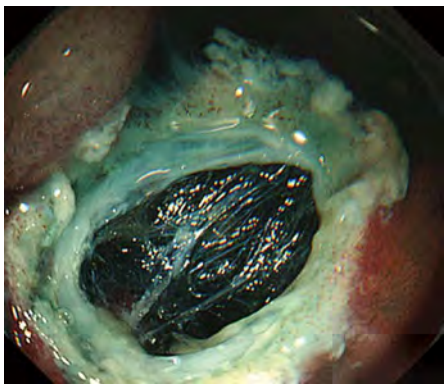

Figure 2 Although a blue-colored layer was identified in the resection defect, a small amount of a whitish layer was detected above the blue layer.

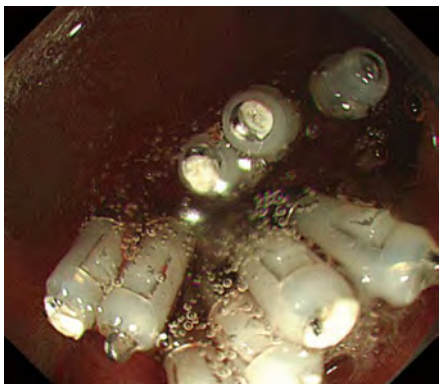

Figure 3 The defect was immediately closed with endoclips.

DISCUSSION

Duodenal NETs account for 2%-15% of gastrointestinal NETs, which are the most common type of NETs[5,6]. Some of them secret bioactive substances such as serotonin, histamine, and prostaglandin, and cause the following typical clinical presentations: cutaneous flushing, sweating, and gut hypermotility with diarrhea[7]. The metastasis rate of duodenal NETs is associated with tumor size. Duodenal NETs, which are located outside the peri-

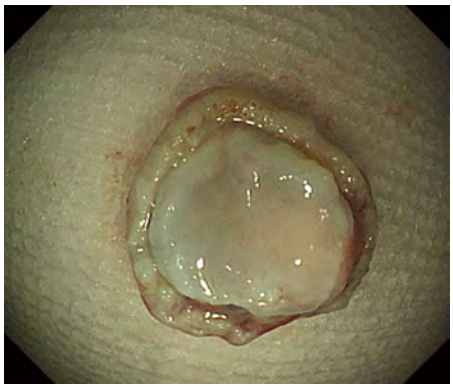

Figure 4 The muscle layer was clearly located on the underside of the resected specimen.

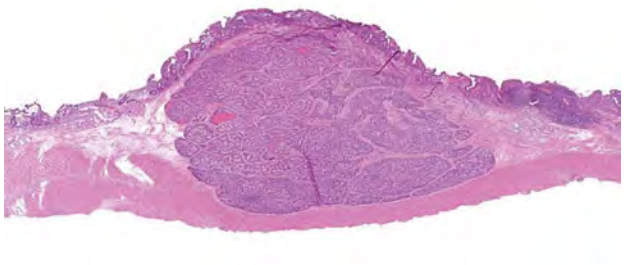

Figure 5 The muscularis propria was detected just beneath the submucosal layer (hematoxylin-eosin staining, loupe image).

ampullary region, 1 cm or smaller in size, and confined to submucosal layer, are considered to be good candidates for endoscopic resection[5,8,9]. Endoscopic resection of duodenal NETs, however, can be associated with a higher risk of perforation, as the bowel wall is thinner in the duodenum and deeper endoscopic resection is necessary because of the tumor localization.

During EMR, a sufficiently thick submucosal elevation created by appropriate fluid injection into submucosal layer is crucial for prevention of perforation, and a small amount of indigo-carmine dye is commonly added for coloration of the injected solution to help determine whether the resection defect is in the submucosal layer or muscular layer (blue or white). In the present case, the tumor was elevated easily, evenly, and sufficiently after fluid injection as if the solution was appropriately injected into the submucosal layer. In addition, as the resection defect was colored blue after resection, we initially thought that EMR was successful. After careful investigation of the defect, however, we detected a few whitish bundles suggesting the presence of MP above the blue layer, a so-called "mirror target sign". Therefore, we could recognize that the blue layer below the MP had been created by unplanned injection of fluid into the subserosal layer rather than the submucosal layer and concluded that our EMR had resulted in an unexpected full-thickness resection.

Both the "target sign" and "mirror target sign" were reported to help in identifying the endoscopic resection of the MP[10]. Surrounded by mucosa and submucosal tissue, the resected MP on the resected surface appears as the "target". In contrast, the resected defect consisted of 2 concentric rings, the inner ring seen as an area of exposed subserosal layer and the outer ring as the commonly encountered submucosal layer, which make the mirror image of the target sign. A similar case has been reported in an EMR during colonoscopy[11]. A detailed examination of the resection defect is important, but endoscopists should remember that the blue layer in the resection defect of EMR is not always the submucosal layer as unexpected subserosal injection can occur. Especially when high viscosity fluid such as HA is used during EMR, unexpected subserosal injection would raise the MP and result in an unexpected full-thickness resection. Retrospectively, the vertical approach of fluid injection in the thin duodenal wall would also explain this unexpected subserosal injection. We suggest that the injection needle could easily be misguided into the subserosal layer, as duodenal submucosal tumors occupy a significant space of the thinner submucosal layer of the duodenum. Furthermore, the thinner duodenal MP could be easily elevated by subserosal injection.

Generally, large perforations require urgent salvage surgery. Duodenal perforation related to endoscopic treatment is reported to require salvage surgery in as many as 43.6% of cases, and these perforations are more serious than those occurring at other sites of the gastrointestinal tract because of bile acid and pancreatic juice[12]. However, selected cases, especially those that can be totally repaired endoscopically, can be managed medically. In this case, prompt recognition of the potential perforation led to the successful endoscopic closure of the defect with endoclips, and moreover, subserosal fluid injection of HA also may have played some important role in sealing the defect. We believe both the clips and the subserosal fluid injection protected this patient against subsequent peritonitis and salvage surgery.

In conclusion, we herein report a case of endoscopic full-thickness resection of a duodenal NET after unplanned fluid injection into the subserosal layer. In this case, we found the "mirror target sign" immediately and repaired the defect endoscopically. A detailed examination of the resection defect regardless of its color, prompt recognition of signs of possible inappropriate resection, and immediate closure of the defect are important to minimize injury.

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Endoscopic band ligation: Beyond prevention and management of gastroesophageal varices

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Abstract

Endoscopic band ligation (EBL) is the preferred endoscopic technique for the endoscopic treatment of acute esophageal variceal bleeding. EBL has also been used to treat nonvariceal bleeding. Recently, Han *et al* demonstrated that EBL can be a feasible and safe alternate technique for the management of iatrogenic gastric perforation especially in cases in which closure with endoclips is difficult. EBL is technically simpler to perform than other methods and provides a good view of the lesions under direct pressure and suction from the transparent ligation cap. EBL can be used even if the diameter of the perforation is greater than 10 mm or if there is a severe tangential angle. In this commentary, we discuss the efficacy and safety of EBL for the closure of iatrogenic gastrointestinal perforation. We also discuss the advantages and disadvantages of EBL for the treatment of nonvariceal bleeding.

Key words: Band ligation; Endoscopy; Intestinal perforation; Gastrointestinal hemorrhage

Core tip: Recently, Endoscopic band ligation (EBL) has been widely used in the treatment of nonvariceal bleeding from angiodysplasia, Dieulafoy's lesion, Mallory-Weiss tears, polypectomy bleeding and colonic diverticular bleeding. In this commentary, we describe EBL may be useful for the endoscopic closure in iatrogenic gastrointestinal perforation in which endoclip closure failed. In addition, the advantages and disadvantages of EBL for the treatment of nonvariceal bleeding are discussed.

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COMMENTARY ON HOT TOPICS

Elastic band ligation was introduced in the United States in 1951[1], and has been used for decades to treat bleeding and/or prolapsed internal hemorrhoids[2]. In the late 1980s, Stiegmann *et al*[3] demonstrated that the results of an initial endoscopic band ligation (EBL) trial were equal to or superior to those obtained with endoscopic sclerotherapy for the treatment of active bleeding, the prevention of recurrences, and survival. Currently, EBL is the most useful and accepted treatment for acute esophageal variceal bleeding[4]. Recently, EBL has been widely used in the treatment of nonvariceal bleeding from angiodysplasia, Dieulafoy's lesion, Mallory-Weiss tears, polypectomy bleeding and colonic diverticular bleeding[5]. This

technique is equally as useful as the standard methods, such as endoclipping, epinephrine injections, and thermal therapy.

In a recent case report series, Han *et al*[6] have shown successful endoscopic closure using band ligation in iatrogenic gastric wall perforations in which primary endoclip closure failed. In this commentary, we describe the clinical efficacy and safety of EBL for the closure of iatrogenic gastrointestinal (GI) perforation. In addition, the advantages and disadvantages of EBL for the treatment of nonvariceal bleeding will be discussed.

Iatrogenic GI perforation

Although iatrogenic perforations occurring during endoscopic procedures are typically managed surgically, the successful endoscopic management of iatrogenic perforation has been increasingly reported. Surgery is required in patients in whom the endoscopic closure of a perforation failed or in whom the recognition of an endoscopic perforation was delayed. The endoscopic devices approved by the United States Food and Drug Administration for the closure of perforations include endoclips, stents, and endoscopic suturing devices[7]. Several case reports or series have described iatrogenic GI perforation managed by endoscopic clip placement[8-11]. Clipping is currently the standard method for closing perforations. Several through-the-scope (TTS) clips have been developed, including the QuickClip2 (Olympus Inc.), the Resolution clip (Boston Scientific Inc.), and the Tri-Clip and Instinct clip (Cook Medical). However, there still are no comparative studies evaluating one type of clip as superior to another for the closure of perforations[7]. The decision to close an iatrogenic perforation with clips is made on the basis of the duration of the procedure; the cause, location, and size of the perforation; the patient's age and general health; and the endoscopist's experience[12]. The most common causes of iatrogenic acute perforation in the GI tract are endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD)[9,10,13]. Perforation can also occur during the process of diagnostic endoscopy. The endoscopic closure of a perforation caused by EMR or ESD is simpler to perform than the endoscopic closure of a perforation caused by a blunt instrument because of the smaller size of the perforation caused by therapeutic endoscopy. Fujishiro *et al*[14] suggested that there are four prerequisites for a successful outcome after closing an iatrogenic perforation with endoscopic clips and using conservative management: (1) the perforation must be small (less than 1 cm); (2) the content of the GI tract must be as clean as possible and the amount of material escaping into the abdomen or mediastinum must be minimized; (3) the closure must be performed by experienced endoscopists'; and (4) there should be no deterioration in the clinical symptoms and laboratory indices, which should be closely monitored by experienced surgeons. The risk factors associated with the need for early surgery within 24 h after endoscopic closure were reported to be a large perforation, leukocytosis, severe abdominal pain, and a large amount of peritoneal free air[15].

The over-the-scope clip (OTSC; Ovesco Endoscopy AG, Tübingen, Germany) has a higher compression force and the capacity to capture a larger volume of tissue than the TTS clip[7]. In a recent prospective multicenter study the CLIPPER study group[16] showed that 32 of 36 consecutive patients with acute iatrogenic perforations (5 esophageal, 6 gastric, 12 duodenal, and 13 colonic perforations) had successful closures without adverse events. Newly developed endoscopic suturing devices for NOTES and antireflux and bariatric surgery may also be suitable for the closure of iatrogenic perforation[17].

Although clipping devices are relatively inexpensive and easy to use, endoscopic clip closure may be difficult in cases that involve large perforations, tangential angles, and/or fibrotic tissue. OTSC and endoscopic suturing devices are not readily available in many countries and require experience in their use for the closure of iatrogenic perforations. Recently several case reports or series of iatrogenic perforations treated by EBL have been described in the literature, involving the stomach, duodenum[18], and colon-rectum[19-21]. Moon *et al*[19] reported the first successful endoluminal closure of a 3 mm rectal perforation with one band following the ESD of a lateral spreading tumor. Two case reports showed that EBL was used to successfully close three iatrogenic colonic perforations in which closure with endoclips initially failed because there were two large perforations and a severe tangential angle[20,21]. Perforations occurring in the process of diagnostic endoscopy, as in these cases, are generally large because of the strong pressure of the endoscope on the wall of the gut over a large area[12]. In these cases, EBL may be applied successfully despite a large perforation. Duodenal perforations are complicated by the leakage of contaminated intestinal fluid and digestive enzymes and require early surgical repair[8]. Even when using a transparent cap on the tip of the endoscope, the application of clips to close perforations is still difficult in the posterior wall of the duodenal bulb because of the limited space and the tangential angle[22]. The deployment of band ligation is simpler than clipping, but displacement of the band poses another problem. Fan *et al*[18] reported the successful repair of a polypectomy-induced duodenal perforation with a combination of hemoclips and band ligation. The perforation was located on the posterior wall of the duodenal bulb and measured 10 mm in diameter. Although the endoscopic closure of large perforations (larger than 20 mm in diameter) is difficult, the successful endoscopic closure of a large duodenal perforation using endoloops and endoclips has been reported[23]. The perforation was located in the duodenal second portion and measured 30 mm in diameter. Two endoloop snare-endoclip sutures achieved complete closure.

In case reports by Han *et al*[6] rescue EBLs were performed in five patients in whom primary endoclip closure either failed or was technically difficult. The common causes of closure failure with clips in four of the

iatrogenic perforations were difficulties in approximating the location of the adjacent gastric mucosa due to wall tension and a tangential angle. In a case of ulcer base perforation, the fibrotic tissue on the base made clipping difficult. Successful endoscopic closure was achieved in all five patients, with no complications occurring. Based on these results, EBL may be a safe and effective alternate therapy for the closure of acute gastric perforations, especially when repairs using endoclips are problematic. Prospective clinical trials are required to demonstrate the efficacy and safety of EBL for the treatment of iatrogenic GI perforations.

Nonvariceal bleeding

The Dieulafoy lesion (DL), a rare vascular abnormality consisting of a large and tortuous artery that is located in the submucosa, can cause potentially life-threatening gastrointestinal bleeding. With advances in endoscopic techniques, endoscopic treatment has become the treatment of choice for many endoscopists because of its effectiveness in the majority of bleeding DLs. Endoscopic treatments include injection, thermal therapy, and mechanical methods such as endoscopic hemoclip placement (EHP) or EBL. These methods have been used with high rates of successful hemostasis[24-26]. Of the endoscopic treatments available, mechanical methods demonstrated good hemostatic efficacy and long-term outcomes, primarily for lesions located in the upper GI tract[27]. In a prospective study, Park *et al*[28] reported that EBL (n = 13) and EHP (n = 13) were equally effective and safe methods for the treatment of bleeding gastric DLs. In a recent large retrospective study that compared EHP with EBL, Ahn *et al*[29] showed that both EHP and EBL were effective for the treatment of bleeding DLs, although recurrent bleeding occurred in 6 of the 66 cases; 5 (15%) and 1 (3%) in the EHP and EBL groups, respectively. This result suggests that EBL may be used as an initial endoscopic treatment for bleeding DLs due to a more favorable clinical outcome than EHP and a shorter procedure time. Although DLs are usually located in the stomach, esophageal, duodenal, and rectal DLs have been successfully treated with EBL[30-32]. However, EBL entails some risks and disadvantages including recurrent bleeding due to ulcer formation, perforation, delay in overtube preparation, and technical difficulties (particularly in retroflexion)[33]. We have previous experience with six patients with acute lower gastrointestinal bleeding due to rectal DL who were treated with thermal therapy, EHP, and EBL[34]. Of the four patients who were treated with EBL, two experienced rebleeding after EBL. Using additional injections and hemoclipping to achieve hemostasis failed, and transanal suture ligations of the vessel were performed. Our cases suggest that residual vessels at the base of a necrotic ulcer may cause delayed bleeding, and it may be difficult to predict this complication on the basis of an endoscopic examination.

Some patients with Mallory-Weiss syndrome (MWS), a vomiting-induced mucosal laceration in the region of the gastroesophageal junction, may require intensive care, especially those with active bleeding, unstable vital signs, and associated comorbid diseases. Although endoscopic injection may be incomplete for a patient with a large and/or long plexuses of vessels, the results of hemostasis using epinephrine injection only are controversial. Chung *et al*[35] reported that rebleeding was more common in hemostasis with hypertonic saline-epinephrine (HSE) injection treatment than in mechanical hemostasis. However, Park *et al*[36] reported that EBL and HSE injections had the same efficacy with a primary hemostasis of 100% *vs* 94%, respectively, without recurrent bleeding or major complications in either group. In addition, Huang *et al*[37] reported that EHP and HSE were equally effective with respect to achieving hemostasis and reducing the rebleeding rate. A prospective, randomized study that compared EBL with EHP in our group showed that the two procedures were equivalent with respect to primary hemostasis (100%) and rebleeding rate (6% *vs* 10%, respectively)[38]. Recently, Lecleire *et al*[39] compared the efficacy of EBL with hemoclips plus epinephrine (H and E) in bleeding MWS. They showed that rebleeding occurred in 0% in the EBL group *vs* 18% in the H and E group and that hemostasis by H and E was an independent predictive factor of early rebleeding. Although a further large prospective study is required, this result suggests that EBL may be the first choice of endoscopic treatments for actively bleeding MWS. Although these studies have shown promising results for the treatment of nonvariceal bleeding, few of these reports were prospective controlled design, and sample sizes of randomized trials were small. Further prospective studies with a large number of patients are necessary to demonstrate the hemostatic efficacy of EBL.

Recently, EBL has been applied to the management of other types of nonvariceal bleeding, including post-polypectomy bleeding[40-43], vascular malformation[44,45], gastric antral vascular ectasia (GAVE)[46-50], blue rubber bleb nevus syndrome[51], and chronic hemorrhagic radiation proctitis[52]. Akahishi *et al*[43] showed that band ligation after endoscopic resection with an HSE injection was effective for the prevention of polypectomy-related bleeding in 20 pedunculated or semipedunculated polyps that were larger than 1 cm. Although the resection margin was histologically affected by the non-neoplastic components in 6 of 20 lesions, all 20 polyps were completely resected. In a prospective study, Junquera *et al*[45] reported that EBL achieved hemostasis in a single endoscopic session in 14 patients with angiodysplasia located in the duodenum (bulb, n = 5; 2nd portion, n = 8; 3rd portion, n = 1), and no patients had further bleeding at 40 days of follow-up. In a recent retrospective study, Sato *et al*[50] demonstrated that the recurrence rate of argon plasma coagulation (68%) was higher than that of EBL (8%) after treatment in patients with GAVE in association with liver disease.

More recently, EBL has been used in the treatment of colonic diverticular bleeding[53-57]. In a large retrospective

study, Ishii *et al*[57] reported that EBL was successful in 27 of 31 cases of colonic diverticular bleeding with the stigmata of a recent hemorrhage, except in 3 diverticula with a small orifice and a large dome and in 1 diverticulum with a large orifice. There were no perforation or penetration complications. Early rebleeding after EBL occurred in 3 cases (11%), which were managed conservatively by a repeat EBL or by a right hemicolectomy. Although the ruptured vasa recta were not identified, the histopathological examination of the surgical specimen showed that the muscularis propria was banded by an O ring. Considering that the direct placement of endoclips is difficult in cases of dome location, massive hemorrhage, or small diverticular orifice, EBL could be an alternative endoscopic treatment for colonic diverticular bleeding.

Endoscopic banding devices

Currently available EBL devices include single-band and multiband ligation devices. While the single-band ligator needs overtube for repeated intubation to place multiple bands, multiband ligator doesn't require use of an overtube. For the treatment of nonvariceal bleeding and GI perforation, single-band ligator has been usually used. For the variceal ligation, the use of multiband device was associated with a significant reduction in sedation requirement, endoscopic time, and patient discomfort compared to single-band ligator[58]. The multiband ligators include the Saeed Multi-band Ligator (Cook Endoscopy, Winston-Salem, North Carolina), the Auto-band Ligator (Scandimed International, Glostrup, Denmark), and the Speedband Superview Super 7 Multiple Band Ligator (Boston Scientific Corp, Natick, Mass)[59]. All EBL devices have a short transparent cylindrical cap that carries 1, 4, 5, 6, 7, or 10 bands, a tripwire that runs from the cap through the accessory channel to the control handle, and a control handle with a retracting spool that is fixed to the biopsy port for attachment and firing of the trip wire[59].

Safety of EBL

Stiegmann *et al*[3] showed that band ligation in the esophagus, when used for variceal bleeding, affected only the mucosa and submucosa. However, the safety of EBL in the anatomically thinner bowel (the colon and small intestine) has not been established. In an *ex vivo* study of EBL for the small bowel and colon using fresh surgical specimens, the histologic evaluation revealed the inclusion by the band ligator of the muscularis propria and serosa on the small bowel, the muscularis propria in the right colon, and the submucosa in the left colon[60]. This result suggests that EBL may not be safe in the small bowel and the right colon but is likely to be safe in the thicker left colon. Recently, Kakutani *et al*[61] showed that the full thickness of the duodenal wall after EBL was captured in the duodenum using *ex vivo* porcine models, and routine EBL is not recommended in the duodenum because of the high risk of perforation. However, Farrell *et al*[54] reported that none of the 11 band ligated colonic diverticula in surgical resected specimens contained either muscularis propria or serosal involvement, and there was no perforation in patients with actively bleeding colonic diverticula controlled by EBL. Further studies are needed to define the appropriate indications and locations for EBL.

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MINIREVIEWS

Endoscopy and polyps-diagnostic and therapeutic advances in management

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Abstract

Despite multiple efforts aimed at early detection through screening, colon cancer remains the third leading cause of cancer-related deaths in the United States, with an estimated 51000 deaths during 2013 alone. The goal remains to identify and remove benign neoplastic polyps prior to becoming invasive cancers. Polypoid lesions of the colon vary widely from hyperplastic, hamartomatous and inflammatory to neoplastic adenomatous growths. Although these lesions are all benign, they are common, with up to one-quarter of patients over 60 years old will develop pre-malignant adenomatous polyps. Colonoscopy is the most effective screening tool to detect polyps and colon cancer, although several studies have demonstrated missed polyp rates from 6%-29%, largely due to variations in polyp size. This number can be as high as 40%, even with advanced (> 1 cm) adenomas. Other factors including sub-optimal bowel preparation, experience of the endoscopist, and patient anatomical variations all affect the detection rate. Additional challenges in decision-making exist when dealing with more advanced, and typically larger, polyps that have traditionally required formal resection. In this brief review, we will explore the recent advances in polyp detection and therapeutic options.

Key words: Polyps; Endoscopy; Colonoscopy; Endoscopic submucosal dissection; Endoscopic mucosal resection; Quality; Combined endoscopic-laparoscopic resection; Combined laparoscopic-endoscopic resection; Combined endoscopic-laparoscopic surgery

Core tip: Changes in polyp detection including chromoendoscopy and narrow band imaging, as well as reliance on quality indicators such as the 6-min withdrawal time, aim to improve adenoma detection rates. Once identified, novel approaches for large and advanced polyps such as endoscopic submucosal dissection and endoscopic mucosal resection, combined laparoscopic-endoscopic resection along with combined endoscopic-laparoscopic resection are available to surgeons that

may obviate the need for formal resection. Although technical expertise and experience is required, physicians caring for these patients should be familiar with each of these alternative procedures.

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INTRODUCTION

Endoscopic technology has undergone dramatic improvements since Philipp Bozzini (1773-1809), a urologist from Frankfurt, Germany, developed the lichtleiter in 1806 - a light-conducting system that featured a candle and system of prisms to inspect the rectum, bladder and esophagus of patients[1]. Since then, multiple different physicians and scientists such as Nitze, Mikulicz, Waye, and Shinya have advanced this technology from a rigid device able to look into the bladder and stomach to a fully flexible endoscope capable of evaluating the entire gastrointestinal tract. Modern endoscopic equipment allows the direct visualization and treatment of many diseases ranging from colorectal polyps, carcinoma, inflammatory bowel disease, intestinal ischemia, diarrhea, diverticular disease, and lower gastrointestinal bleeding. Auxiliary devices ranging from biopsy forceps, snares, injection needles, various knives, baskets and balloon dilators have been developed to expand the ability of surgeons and gastroenterologists alike to manage complex pathology through the use of endoscopes. This update will briefly review some of the emerging advances and evolving parameters as well as their impact on clinical practice.

QUALITY PARAMETERS

Colon cancer remains the third leading cause of cancer-related deaths in the United States when each gender is considered separately and second when combined, with an estimated 50830 deaths in 2013 alone. This is despite multiple efforts aimed at early detection through screening, as well as evidence that routine screening reduces mortality[2-5]. Barriers to screening include patient fear of the exam and results, financial constraints, time off from work, transportation, and (in some regions) access to care. Multiple studies have demonstrated that when compared to flexible sigmoidoscopy and air-contrast barium enemas, colonoscopy is the most effective screening tool to detect colon cancer[6,7]. These dramatic results, in part, prompted Medicare in July 2001 to provide coverage for screening colonoscopy; which, along with technological advances, dramatically increased its overall use in the United States[8]. Despite the success of optical colonoscopy to detect and remove polyps, there remains a substantial rate of undetected polyps. In most major series this rate appears to be low, but has not improved over time, suggesting the need for further advances in the technique. Large studies that include physicians with extensive experience have demonstrated missed polyp rates from 6%-29%, with the variation depending primarily on the size of the lesion[9]. Not surprisingly, missed polyp detection rates have been significantly lower for larger lesions. Pooled analysis of tandem colonoscopies has revealed a failure to detect polyps of any size in as many as 22% of cases (95%CI: 19%-26%). In this systematic review, when further stratified by size, adenoma miss rates were 2.1% lesions for $\geqslant$ 1 cm, 13% for those 5-10 mm, and 26% for polyps 1-5 mm[10]. Others have reported similar results, with miss rates for all polyps at 28%, adenomas (20%), polyps < 5 mm (12%), > 5 mm (9%) and advanced adenomas (11%)[11]. When accounting for other factors such as the concomitant presence of a sub-optimal bowel preparation, these rates have been reported to be higher than 40% for any size polyp, and even up to 36% with advanced adenomas[12]. In order to understand how we may potentially be able to lower this missed adenoma detection rate, we will explore these factors and the data behind each of them.

Time of withdrawal

One factor that has more recently been identified to impact overall polyp detection rates is colonoscopy withdrawal time. In 2002, a United States Multi-Society Task Force on Colorectal Cancer recommended that the withdrawal time for colonoscopies should average 6-10 min. Interestingly, this was based, in part, on a single small series of only 10 consecutive colonoscopies performed by two experienced endoscopists with vastly different withdrawal practices that found different adenoma missed rates[13]. Following confirmatory studies, practice guidelines have since recommended that endoscopists spend a minimum of 6-10 min examining the colonic mucosa during the withdrawal phase of colonoscopy to optimize the diagnostic yield of polyps. In many instances, this has evolved to become a metric that is tracked by hospital administrators to assess the quality of colonoscopies[14]. The response was initially positive, and adherence to this benchmark was supported by findings in a study by Simmons *et al*[15] that included 11000 colonoscopies showing a direct association between longer withdrawal times and higher polyp detection rates (r = 0.76; P < 0.0001). Although this association was overall strong, it dropped significantly as polyp size increased (r = 0.19 for polyps 6-9 mm, r = 0.28 for polyps 10-19 mm, r = 0.02 for polyps $\geqslant$ 20 mm). Small variations on this theme were subsequently reported, with others finding overall procedure time (which included the consent and sedation periods and not just withdrawal time) correlated with increased rates of polyp detection (r = 0.64, OR = 1.4, 95%CI: 1.19-1.64 for polyps > 1 cm; OR = 1.03, 95%CI: 0.74-1.43 for polyps > 2 cm)[16]. In one of the sentinel

papers, Barclay and associates published a study in the *NEJM* with 7882 colonoscopies using 6 min as the minimum length of time to allow for "adequate inspection" during withdrawal[17]. In this study of 12 gastroenterologists, rates of polyp detection ranged widely when measured either by number (0.1-1.05 mean number per patient) or percentage with adenomas (9.4%-32.7%), as well as times of withdrawal (3.1-16.8 min for procedures with no polyps removed). When specifically using a cutoff of 6 min, those with longer withdrawal times had significantly higher rates of detection for any neoplasia (28.3% *vs* 11.8%, $P < 0.001$), as well as advanced lesions (6.4% *vs* 2.6%, $P = 0.005$). Since then, multiple authors have confirmed average withdrawal times of 6 min or longer to be correlated with increased adenoma detection rates, including a quality assurance review of 15955 patients over 49 ambulatory centers, 17 states and 315 gastroenterologists, where longer withdrawals had a 1.8-fold higher rate of polyp detection[18]. In this review, factors that were found to be the strongest predictors of withdrawal time ⩾ 6 min include the presence of carcinoma (OR = 3.7), adenoma (OR = 2), and number of polyps visualized (OR = 1.7). Whether the study is performed in a private practice or academic environment, the relationship between longer withdrawal times and higher rates of overall polyp detection, or adenomas per patient (0.09-0.82), has been consistent[19].

However, the adoption of this quality indicator has not been uniformly supported nor met with complete agreement. Several authors have demonstrated no difference in polyp detection rates, despite improving the frequency of meeting the > 6 min quality metric from 65% to near 100% of the time[14]. Others argue that colonoscopy rarely misses polyps > 1 cm (*i.e.*, the most clinically significant polyps), regardless of the time spent during the withdrawal phase. Still others have agreed that while withdrawal time is associated with higher rates of polyp detection, longer withdrawal times have not been associated with changes in rates of neoplasia discovered at subsequent follow-up colonoscopies, including a recent VA Cooperative Studies Group analysis[20]. Similarly, after a monitoring and feedback program was instituted that focused on withdrawal times and polyp detection rates, there was an increase in mean withdrawal time (6.6-8.1 min, $P < 0.0001$) and overall polyp detection rate (33.1%-38.1%, $P = 0.04$). However, this was again observed to *not* be associated with an increase in neoplasia detection rate from the initial to the post-intervention time periods (19.6%-22.7%, $P = 0.17$)[21].

Despite this, withdrawal time has evolved into a quality metric indicator in many centers for determining the adequacy of colonoscopy. As such, this has led to some changes in clinical practice - both positive and negative. Some authors have reported improved rates of longer withdrawal times to comply with these guidelines, simply knowing that this quality measure was being recorded, but without using that time to perform the corresponding evaluation. To combat this, practices such as vetting through bystander observation and video recording have been attempted, though without a significant increase in polyp detection[22]. Other authors have shifted their focus in an attempt to further clarify the reasons for variations in polyp detection rate. Factors such as number of procedures, mean patient age, percentage of women, and mean procedure time have all been evaluated (in addition to polyp size) with only procedure time being significantly associated with polyp detection rate in a study of 2665 screening colonoscopies[23]. Multiple other patient and physician-related factors have also been identified as causes for higher miss rates including experience of the endoscopist, larger colon folds, morphology of polyp, and polyp location (*i.e.*, blind spots at the flexures)[11,24].

Physician fatigue

Physician fatigue has been considered another variable that affects colonoscopy quality performance and adenoma detection rates. This was first noted in a study demonstrating that afternoon colonoscopies have higher failure rates than morning colonoscopies, with higher overall incompletion rates (6.5% *vs* 4.1%, $P = 0.013$) as well as higher rates of inadequate bowel preparation (15.4% in am *vs* 19.7% in pm)[25]. When using cecal intubation rates as the endpoint, success was again lower in the afternoon (93.5% *vs* 95%, $P = 0.02$), although gender, age and bowel preparation were felt to play a role in these differences as well[26]. Adenoma detection rates have also varied based on the time of day the colonoscopy is performed, with one study reporting rates of 29.3% in the morning *vs* 25.3% in the afternoon ($P = 0.008$), independent of factors such as poor bowel preparation, withdrawal time, or partial evaluation[27]. To further clarify this, authors have compared results of providers that perform a full day of colonoscopy with those limited to half-day blocks. Adenoma detection rates in those only working half days have showed no significant difference between early and late procedures within that time period (27.6% *vs* 26.6%, OR = 1.05, 95%CI: 0.88-1.26, $P = 0.56$), while those in the same practice with full-day blocks reporting higher detection rates in the morning (26.1% *vs* 21%, $P = 0.02$), suggesting that the additional time, and subsequent fatigue, plays a role for this difference[28]. It appears that provider fatigue culminates in lack of focus or acumen in many cases, and translates into lower rates of "successful" colonoscopies as time progresses. Interestingly, polyp detection rates have also been shown to decline as time passes during an endoscopist's schedule, regardless of time of day, or number of prior procedures. Each elapsed hour in their work schedule was associated with a 5.6% reduction in polyp detection ($P = 0.005$), suggesting that physician fatigue can progress more rapidly in certain cases[29].

Training and technique

Regardless of the metric proposed, proper training remains a major factor in becoming and remaining proficient in any endeavor. Historically, intra-procedural quality indicators for colonoscopy have focused primarily on

physician-related factors such as cecal intubation rates, terminal ileal intubation, number of polyps detected, number of polyps retrieved, size of polyps detected, time to reach the cecum, and more recently withdrawal time. Guided by principles such as the United Kingdom Department of Health Global Rating Scale for endoscopy, emphasis has shifted more on defining quality experience through patient-driven metrics including appropriateness of the intervention, proper information/consent, overall safety, patient comfort, and providing timely results[30]. Use of colonoscopy-based virtual-simulator models has been one way to supplement inadequate exposure during residency training, and improve both the trainee experience and end result. Multiple studies have demonstrated that following intervention with 3-D simulators, many of these aforementioned traditional metrics such as cecal intubation rates, overall times, and need for further medication interventions significantly improve[31]. On the other hand, it remains to be seen how these newer quality metrics will be evaluated, reported and enforced.

POLYP CLASSIFICATION

In general terms, a polyp refers to the elevation of tissue above the gastrointestinal epithelium. Colon polyp types range widely from hyperplastic, hamartomatous, and inflammatory varieties to neoplastic adenomatous lesions. Although these lesions are all "benign", up to one-quarter of patients over 60 years old will have "pre-malignant" adenomatous polyps. Traditionally, polyps have been classified most commonly by their histology (*i.e.,* villous, tubular, tubulovillous, *etc.*), location, and physical description - with pedunculated and sessile being the most common descriptive classes. Since their first description in 1985[32], flat adenomas are increasingly more common and represent one of the "high-risk" categories along with adenomas larger than 1 cm, those with high-grade dysplasia, those associated with inflammatory bowel disease, villous or tubulovillous adenomas, and patients with multiple adenomas (typically > 3). Similarly, serrated adenomas represent another high-risk group, and are believed to represent a unique pathway in the adenoma-carcinoma sequence.

Flat polyps and serrated adenomas

While there has been some controversy regarding the impact and importance of flat adenomas in the United States and Europe, they are more widely believed to be significant in Asia. The Japanese Research Society Classification (Kudo classification of adenomas) describes flat lesions as those with a height that is less than one half the diameter[33]; while the Paris classification uses protruding and non-protruding divisions[34]. Increasingly, these lesions are recognized for their role in malignancy as well as difficulty with identification[35]. Serrated polyps represent another type of lesion that has been reported to be more difficult to diagnosis. Originally described following evaluation of hyperplastic polyposis syndrome patients, these lesions have a characteristic serrated architecture and can occur either as a traditional serrated adenoma (classically seen as a polypoid lesion), or as a sessile serrated adenoma (flat, slightly raised, right-sided > left). Though historically often diagnosed as a variant of hyperplastic polyps, these lesions are found in about 7% of all colonoscopies, and are now more properly classified as their own distinct entity. They are also believed to have a higher risk of malignancy that occurs apart from the traditional adenoma to carcinoma sequence[36,37].

The traditional polyp-cancer sequence has been established since Muto *et al*[38] described it in 1975. Adenocarcinoma of the colon can arise *via* multiple different pathways, with the most common described by genetic alterations that result in micro-satellite stable carcinomas[39]. Approximately 1/3rd, however, will arise along the serrated pathway, developed from the precursor lesion known as the sessile serrated adenoma (SSA). This is caused from an extensive methylation at the CpG island promoter site, which may demonstrate microsatellite instability. While controversy exits, it has been reported that SSAs are precursor lesions to micro-satellite unstable carcinomas; though limited data on the rate of progression currently exists[40]. In their pre-malignant state, these polyps show features between those of hyperplastic polyps and adenomas. On a molecular level, they have a high proportion of the *BRAF* mutation and DNA methylation. BRAF, a member of the RAF family of serine/threonine kinases, mediates cellular responses to growth signals, and *BRAF* mutations have been strongly associated with mis-match repair-deficient colorectal cancer[41]. Methylation and inactivation of the DNA repair genes *MLH1* and *MGMT* (06 methylguanine-DNA methyltransferase) similar to that in hereditary non-polyposis colorectal cancer, are felt to be the critical steps that lead to this instability[42]. It has also previously been found that patients with micro-satellite unstable cancers demonstrate an increased serrated polyp to adenoma ratio compared to those with stable cancers. Therefore when encountering patients that have more serrated polyps than adenomas during colonoscopy, subsequent cancers in these patients may demonstrate microsatellite instability and should be considered for appropriate testing[43]. Risk factors for the development of sessile serrated adenomas include greater than 20 pack-year smoking history (OR = 7.31, 95%CI: 3.9-13.6), and, to a smaller extent, diabetes and obesity[44].

Unfortunately, there continues to be inconsistencies in the literature regarding the ultimate prognosis and malignant potential of both flat polyps and serrated adenomas. Even large series comparing flat lesions with polypoid have found that the size of the lesion confers much greater risk than the morphology for the development of malignancy. Furthermore, the incidence of high-grade dysplasia or cancer in flat neoplasms was found to be similar to that of polypoid neoplasms (5.4% *vs* 4.6%, $P = 0.36$)[45]. While still somewhat controversial, what seems increasingly clear is that while further informa-

tion is required to determine the exact malignant risk of these lesions, there is evidence to suggest that they have a higher risk profile and should be followed accordingly.

Laterally spreading tumors

Another subset of high-risk lesions includes laterally spreading tumors (LSTs). These lesions have been increasingly described over the past 20 years and are characterized by their higher likelihood to spread laterally along the lumenal wall rather than vertically[46]. By definition, LSTs applies to lesions > 10 mm in diameter[47]. Okamoto initially described two clinical and histologic subtypes, which are identified as the granular-type (LST-G) and non-granular (LST-NG)[48]. Granular types appear endoscopically as multiple even or uneven nodules with the same color with its surrounding normal mucosa, while the non-granular type (also referred to as flat) appear smooth. These lesions may also be further stratified based on their morphological appearance as LST-G-H (homogenous) and LST-G-M (nodular mixed) type or LST-NG-F (flat elevated) and LST-NG-FD (pseudodepressed)[49].

These lesions have a much higher propensity for being missed *via* standard white light colonoscopy, as well as more advanced techniques such as narrow band imaging (NBI) and chromoendoscopy[50]. More importantly, LSTs have an increased rate of submucosal invasion. Rates of invasion, particularly for the LST-NG subtype are as high as 30%-40%[46], whereas the granular subtype are significantly lower (about 5%-10%)[51]. While the risk of lymph node metastases is low for early invasion[52], the preferred management is still somewhat controversial, but mostly based on clinical and morphological appearance[53,54]. What is clear, however, is that these lesions represent a high-risk group with a substantial rate of concomitant malignancy, and endoscopists need to have an acute awareness of their potential presence and follow-up on them accordingly.

POLYP DETECTION

There is little doubt that colonoscopy is a highly specific and sensitive test for the detection of colonic lesions, and several factors play a role in the adenoma detection rate (Table 1). However, differentiating early colon cancer from polyps can be more difficult. Factors that are associated with the presence of malignancy in a colonic polyp include villous architecture, increasing size, presence of multiple polyps and sessile lesions[55]. To further help in distinguishing benign from cancerous lesions, Kudo *et al*[56] in 1994 reported on differences in mucosal pit patterns of various colorectal polyps. In this classification system, staining patterns that are often seen in hyperplastic polyps or normal mucosa differ from the unstructured surface architecture more commonly identified with malignancy. Pit patterns were classified into seven principal types: (1) normal round pit; (2) small round pit; (3) small asteroid pit; (4) large asteroid pit; (5) oval pit; (6) gyrus-like pit; and (7) non-pit. The authors found that there was a correlation between pit patterns and the histology of the cells in the gland. The authors further went on to categorize these seven principle types into 5 pit patterns: (1) normal round pit; (2) small and large asteroid pits; (3) small round pit; (4) oval pit; (5) gyrus-like pit; and (6) non-pit pattern. By using this schema, types I and Ⅱ are non-neoplastic and Ⅲ, Ⅳ and Ⅴ are neoplastic, with accuracy rates reported as high as 90%[57]. Chromoendoscopy and NBI use these differences in pit pattern to help detect and differentiate polyps.

Table 1 Factors associated with adenoma detection

Variable	Association
Withdrawal Time < 6 min	Worse
Sub-optimal bowel preparation	Worse
Patient anatomy	Variable
Experience of endoscopist	Variable (mostly worse with early)
Afternoon endoscopy	Worse
Flat adenomas	Worse
< 1 cm	Worse
Narrow band imaging	Variable data[1]
Chromoendoscopy	Variable data[1]

[1]Compared to traditional white light colonoscopy.

Chromoendoscopy

In chromoendoscopy, a dye such as indigo carmine can further enhance the surface structure of epithelial lesions with the aid of magnifying endoscopy[58]. Pit patterns become more recognizable, and outlining the borders of polyps is reported to be more accurate. Accuracy rates have been reported as high as 87%-100% and 76%-99.8% in diagnosing non-neoplastic and neoplastic polyps, respectively[59]. Furthermore, this technique has been shown to be beneficial for helping detect small lesions and decreasing the missed polyp rate, with diagnostic accuracies of 95% with magnification chromoendoscopy for lesions < 5 mm compared to 76% with traditional colonoscopy[60,61]. A recent update of the Cochrane review consisting of 5 studies compared chromoendoscopy *vs* conventional endoscopy for detection of polyps, and showed that chromoendoscopy is more apt to identify patients with at least one neoplastic lesion (OR = 1.67, 95%CI: 1.29-2.15), as well as those with ≥ 3 neoplastic lesions (OR = 2.55, 95%CI: 1.49-4.36) over "white" light endoscopy[62]. Although still not widely used, especially in the United States, chromoendoscopy has also been cited to reduce the time, cost and risk with biopsy/polypectomy, once the initial learning curve associated with dye application is complete.

NARROW BAND IMAGING

NBI is an imaging technique that also relies on better definition of capillary pattern to improve the contrast between adenomas and surrounding normal mucosa. Adenomas, like malignancy, have a characteristic angiogenesis that can be detected using various wavelengths

of light that variably penetrate the colon mucosa[63]. The theory behind it's efficacy lies in its ability to contrast the "normal" mucosa from that of adenomatous tissue to a greater degree than standard white light colonoscopy by selecting out specific wavelengths through optical filters that "narrow" the bandwidth of light. Developed by Gono *et al*[63] (and originally described on the vascular pattern and adjacent mucosa of the underside of the human tongue), it uses the reflected light to visualize the superficial structure and enhance the vasculature within the mucosal layers. Unlike chromoendoscopy, which relies on sprays and specialized equipment, NBI is readily available on many colonoscopy systems and does not require additional imaging. The data supporting its use, however, remains somewhat conflicting. In a pilot study by Machida *et al*[64], NBI had a 93.4% diagnostic accuracy, equivalent to chromoendoscopy with magnification, and higher than that of conventional colonoscopy. In one randomized trial during screening colonoscopies, patients randomized to white light (n = 108) and NBI (n = 103) had adenoma detection rates of 58.3 and 57.3 (P = 0.88), respectively. However, when the authors further evaluated only flat adenomas, a lesion believed to be best defined by NBI, the detection rates were 9.3% for traditional colonoscopy and 21.4% for NBI (P = 0.019)[65]. Other randomized data including 1256 patients comparing NBI technology to white light with associated high definition video found no difference in overall adenoma detection rates (33% *vs* 34%), total number of lesions (200 *vs* 216), or any other subgroups of adenomas to include flat lesions[66]. In this study, only hyperplastic polyps were found more commonly in NBI. Several other authors have found NBI did not improve the colorectal neoplasm miss rate compared to traditional methods[67], or even those of small and flat adenomas with the use of high-definition colonoscope[68]. A recent Cochrane review identified 11 randomized trials with 3673 patients comparing NBI to standard white light endoscopy for the detection of colorectal polyps. The authors found similar rates of overall polyp detection (6 trials, n = 2832, RR = 0.97, 95%CI: 0.91-1.04), and adenomas (8 trials, n = 3673, RR = 0.94, 95%CI: 0.87-1.02), even when stratifying by the number of patients with at least one lesion by size [< 5 mm: RR = 0.95, 95%CI: 0.84-1.08, I^2 = 56%; 6-9 mm: RR = 1.06, 95%CI: 0.81-1.39, I^2 = 0%; ≥ 10 mm: RR = 1.06, 95%CI: 0.77-1.45, I^2 = 0%][69].

On the contrary, there are studies that do report improvements in distinguishing neoplastic from non-neoplastic lesions using NBI, with accuracy rates higher than that of colonoscopy and equivalent to chromoendoscopy (80%-82% low magnification NBI; 85% low magnification chromoendoscopy; 87%-90% high magnification NBI; 82%-92% high magnification chromoendoscopy; standard colonoscopy (67%-68%)[70]. Other authors have found sensitivity of 90%-96% and specificity of 85%-89% in differentiation of neoplastic *vs* non-neoplastic lesions, comparable to that of chromoendoscopy. Furthermore, accuracy rates were even higher with the addition of magnifying endoscopy, up to 94% for neoplastic and 89% for non-neoplastic lesions[71,72]. Similar to chromoendoscopy, however, the ultimate role this will have relies on the long-term data, ability to lower costs, and proper training of endoscopists prior to incorporation into everyday and widespread use.

Endoscopic mucosal resection

Endoscopic mucosal resection (EMR) was first described in 1990 by Inoue and Endo in Japan[73], and subsequently followed by Soehendra *et al*[74] in Hamburg, Germany in 1997. In the esophagus and stomach, as well as the colon, EMR allows removal of superficial tumors of the gastrointestinal tract. Unlike polypectomy that removes the tumor at the base of the mucosa, the plane of resection during EMR occurs in the middle or deep submucosal layer. Drawbacks of piecemeal excision include difficulty with proper staging, histological diagnosis, and definitive evaluation of the margins[46,75]. Furthermore, unlike the stomach, the colonic wall is much thinner and haustrated, leading to a technically more difficult procedure. Indications for EMR currently include adenomas or small, well-differentiated carcinomas confined to the mucosa or with minimal invasion into the submucosa, those more than 1/3rd of the lumenal diameter, or flat/depressed polyps. In essence, EMR enables select lesions to be removed endoscopically that would potentially require colectomy[76]. It is important that these early carcinomas do not have lymphovascular invasion, due to the increased risk of lymph node metastases. As this technique is currently performed more commonly in Japan, the Japanese Society for Cancer of the Colon and Rectum's current criteria for curative endoscopic resection are: submucosal invasion of less than 1000 μm, moderate or well-differentiated lesion characteristics, and the absence of vascular invasion[77]. Moss and colleagues have also identified risk factors for submucosal invasion and failure of successful EMR in a prospective, multi-center cohort of 479 patients and 514 lesions[78]. In their collective experience, Paris classification 0-Ⅱa+c morphology, nongranular surface, and Kudo pit pattern type Ⅴ were all risk factors for invasion, with even higher risks (up to 55.5%) when multiple factors were present. EMR was attempted in 464 patients, being successful in 414 (89%), with a prior EMR attempt by the referring endoscopist (OR = 3.75, 95%CI: 1.77-7.94), difficult position (OR = 2.17, 95%CI: 1.14-4.12) and ileocecal valve location (OR = 3.38, 95%CI: 1.20-9.52) all predictors of initial failure.

Local recurrence has been reported in 6.9%-13.4% of cases of EMR, with higher rates reported following piecemeal excision, invasive pathology, and for lesions > 2 cm (rates up to 39%)[79]. Median times for recurrence are typically within the first 6 mo, signifying the importance of follow-up endoscopic evaluation between 3 mo and 1 year[80]. In patients with larger polyps or those with dysplasia or cancer, it is recommended that they undergo more high intensity surveillance[81,82]. Other reported risk factors for recurrence include a granulous appearance of

the lesion and distal rectal lesions. Incomplete (R1) resections and those with deep positive margins should be considered for surgery.

Outcomes for EMR are, in general, very good as most patients are highly selected. When performed by experts, less than 3% of lesions are referred for surgical resection (due to inadequate removal), 44%-60% are performed *en bloc*, and the remaining lesions undergo successful piecemeal removal[83]. In sample series, complications involve procedural (10%-13%) and late (0%-1%) bleeding, post-polypectomy syndrome (2%-3%) and perforations (1%-2%)[84]. In attempt to identify high risk polyps that contain cancer prior to EMR, several authors have shown malignancy rates are higher with sessile polyps and those > 3 cm[83,84]. Although these factors are not absolute contraindications to EMR, it is typically more difficult to remove tumors larger than 2 cm by *en bloc* resection using EMR, with reported rates of about 30%. The decision to perform EMR should be made on an individual basis[85-87].

Endoscopic submucosal dissection

Endoscopic submucosal dissection (ESD) is primarily used to help with resecting larger tumors and aid in achieving higher rates of en-bloc resection of superficial tumors in the gastrointestinal tract than EMR. While EMR is the current standard in most centers outside of Asia, ESD is a technique that should, in general, be reserved for highly selected lesions by specialized endoscopists skilled and experienced in this technique Although still primarily performed in select centers and lacking widespread use, the goals of ESD remain: (1) treating mucosal cancer; (2) achieving an R0 resection; (3) meeting quality standards; and (4) ensuring that procedures are performed by endoscopists trained in this technique and under institutional review board approval[88]. As a general guideline, ESD is more commonly indicated when a snare is unlikely to enable a successful *en bloc* resection with EMR. ESD is also indicated when tumors are diagnosed as carcinomas with intramucosal to shallow submucosal invasion, as well as lesions with submucosal fibrosis that cannot be removed by EMR, even if less than 20 mm in size. Others have proposed that this technique is suitable for all large polyps, early colorectal cancer, and those lesions that cannot be accessed transanally in patients who wish to avoid major resection.

Similar to other new technology, both EMR and ESD have learning curves that play a large role in determining outcomes. Previous reports out of Asia, where experience tends to be much greater, have demonstrated proficiency for larger lesions occurs at about 80 cases, with generalized avoidance of major complications such as perforation at about 30-40 procedures[89,90]. Yet, the learning curve of ESD and its outcome comparison to EMR in centers where endoscopists are not as familiar or experienced is less defined to date. Probst and colleagues evaluated their learning curve in a European study of 82 rectosigmoid lesions, with successful resection using ESD techniques in 76 (93%)[91]. Over the 7-year study period, the authors divided up their experience into three separate phases (1-25, 26-50 and 51-76 years). During this time, both the rates of en-bloc resection (60%, 88% and 96%, respectively) and R0 resection (80%, 86% and 88%) increased, while procedure times significantly decreased (200, 193 and 136 min).

Using different techniques, other authors have reported successful en-bloc resection occurs in up to 85%-89% of cases and piece-meal resection is possible in the remaining 10%-15%[92-96]. Clear lateral and deep margins (*i.e*, complete resections) have been reported in up to 79%-86% of cases[97,98]. As previously stated, because it is difficult to perform en-bloc resection by EMR for lesions larger than 20 mm, ESD may be more suitable for these lesions. The ability to predict depth of invasion in an attempt to decide whether to pursue EMR, ESD or formal resection remains somewhat difficult. Similar to EMR studies, predictors of submucosal *vs* mucosal invasion include poor-differentiation and the absence of background adenoma[83,84].

Briefly the technique of ESD involves an initial bowel preparation to remove residual feces. An endoscope with a single channel is used along with a high-frequency electrosurgical generator. After identification of a lesion, one of several types of solutions (including a mixture of 1% hyaluronic acid solution and 10% glycerin solution) is injected around the lesions to elevate the submucosa[99]. Specialized knives in various shapes and sizes help to perform the dissection and resection. The border of the tumor is initially marked by indigo carmine dye and with approximately 1 cm margins. Following a mucosal incision, and depending on the physician preference, a partial or circumferential incision is made along with injection of hyaluronic acid solution into the submucosa, and the dissection is carried down to the deep submucosa. This process is continued around the tumor until the entire lesion is resected en bloc, when possible.

Perforation using ESD occurs in 1.4%-10.4% of cases, with the majority of series reporting rates < 2%[100]. These rates are classically higher than reported with EMR and are most likely due to the depth of dissection, and in certain cases lesions that are associated with a significant amount of fibrosis[101]. When small perforations occur, endoscopic clips have been utilized to close the site when feasible[102]. In more severe cases or those that cannot be closed endoscopically, more definitive procedures should be performed either by laparoscopy or laparotomy. Cases of delayed perforation occur in < 1% of cases, and are thought to be a result of thermal injury[103]. Postoperative hemorrhage rates are reported between 0%-12%, comparable to that with EMR, and the majority are self-limiting[96]. Another not infrequent complication is the inability to complete the procedure secondary to patient restlessness from abdominal distension and pain (12%-32%), requiring additional conscious sedation or even general anesthesia. Additionally, the use of carbon dioxide has been shown to significantly reduce this pain and bloating when deep sedation compared to traditional

air insufflation[104]. Other more rare complications include obstruction, fever, and pain[105]. Most importantly, residual disease has been reported in 2%-3% with ESD[106]. In one of the few series comparing ESD with EMR, 145 colorectal tumors were treated by ESD and another 228 treated by EMR. ESD was associated with a longer procedure time (108 min *vs* 29 min, $P < 0.0001$), higher en bloc resection rate (84% *vs* 33%, $P < 0.0001$) and larger resected specimen size (37 mm *vs* 28 mm, $P = 0.0006$)[86]. There were three (2%) recurrences in the ESD group and 33 (14%) in the EMR group requiring additional EMR ($P < 0.0001$). Complication rates were similar (perforation 6.2% ESD *vs* 1.3% EMR, delayed bleeding 1.4% ESD *vs* 3.1% EMR; $P > 0.05$). Although both of these techniques are currently only offered in select centers, emerging literature and advances in technology may provide the impetus for more widespread training and utilization.

Combined laparoscopic-endoscopic resection/combined endoscopic-laparoscopic surgery

It is important to note that most lesions can (and should) be approached through traditional techniques. However, for select more advanced lesions, other methods are available. Combined laparoscopic-endoscopic resection (CLER) or combined endoscopic-laparoscopic surgery offers another approach for the removal of these advanced lesions that are not amenable to traditional endoscopic techniques, and would normally go on to formal resection. Lesions that are identified as being larger or more difficult to remove in the endoscopy suite are marked, and the patient is taken for a procedure under general anesthesia. In the operating room, the subcutaneous layer under the polyp is injected to lift the polyp. After laparoscopic ports are placed, the bowel is manipulated from the outside to expose the base of the lesion, and endoscopic polypectomy is performed. This enables direct evaluation for any full thickness injury, as well as the ability to imbricate or close the bowel wall using full thickness sutures should the need arise. Additionally, a sleeve resection can be performed that removes the lesion along with a full-thickness section of the surrounding wall (*i.e.*, in cecal lesions). A leak test can also be performed by submersion of the staple/suture line under water along with CO_2 or air insufflation. Any concerns regarding the applicability of the lesion for this procedure are alleviated by immediate conversion to a standard laparoscopic-assisted oncological resection[107].

Technical success rates have consistently been reported in 77%-97%, with the remaining requiring conversion to resection[108,109]. Common reasons for an inability to perform this procedure include difficult lesion location, poor visualization (which has been aided by CO_2-insufflation), and concerns for malignancy. Post-operative complications have been generally < 10%, with the majority being minor wound infections, bleeding, and ileus. Major complications are rare, with many reports citing a 0%-3% incidence. Recurrence rates are also low, reported in 10%-15% and typically are benign that may be approached *via* similar CLER, standard endoscopy or formal resection[107-109]. Final pathology ultimately will dictate the need for any subsequent segmental resection, and patients should be counseled about this ahead of time. Novel approaches for large and advanced polyps are available to surgeons that may obviate the need for formal resection. Although technical expertise and experience is required, physicians caring for these patients should be familiar with these alternative procedures.

CONCLUSION

Our goal remains to identify and intervene on lesions at the polyp stage, prior to invasion. While colonoscopy is the most effective screening tool to detect pre-cancerous polyps and colon cancer, we must focus on the quality indicators such as withdrawal time and adenoma detection rate to ultimately improve our outcomes. Advances such as NBI, chromoendoscopy, endoscopic mucosal resection, endoscopic submucosal dissection, and CLER are tools that may improve the management of benign and early malignant polyps, and physicians performing endoscopy should be well-versed in their applicability and efficacy.

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ORIGINAL ARTICLE

miR-20b, miR-98, miR-125b-1*, and let-7e* as new potential diagnostic biomarkers in ulcerative colitis

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Abstract

AIM: To use microarray-based miRNA profiling of colonic mucosal biopsies from patients with ulcerative colitis (UC), Crohn's disease (CD), and controls in order to identify new potential miRNA biomarkers in inflammatory bowel disease.

METHODS: Colonic mucosal pinch biopsies from the descending part were obtained endoscopically from patients with active UC or CD, quiescent UC or CD, as well as healthy controls. Total RNA was isolated and miRNA expression assessed using the miRNA microarray Geniom Biochip miRNA *Homo sapiens* (Febit GmbH, Heidelberg, Germany). Data analysis was carried out by principal component analysis and projection to latent structure-discriminant analysis using the SIMCA-P+12 software package (Umetrics, Umea, Sweden). The microarray data were subsequently validated by quantitative real-time polymerase chain reaction (qPCR) performed on colonic tissue samples from active UC patients (n = 20), patients with quiescent UC (n = 19), and healthy controls (n = 20). The qPCR results were analyzed with Mann-Whitney U test. *In silico* prediction analysis were performed to identify potential miRNA target genes and the predicted miRNA targets were then compared with all UC associated susceptibility genes reported in the literature.

RESULTS: The colonic mucosal miRNA transcriptome differs significantly between UC and controls, UC and CD, as well as between UC patients with mucosal inflammation and those without. However, no clear differences in the transcriptome of patients with CD and controls were found. The miRNAs with the strongest differential power were identified (miR-20b, miR-99a, miR-203, miR-26b, and miR-98) and found to be up-regulated more than a 10-fold in active UC as compared to quiescent UC, CD, and controls. Two miRNAs, miR-125b-1* and let-7e*, were up-regulated more than 5-fold in quiescent UC compared to active UC, CD, and controls. Four of the seven miRNAs (miR-20b, miR-98, miR-125b-1*, and let-7e*) were validated by qPCR and found to be specifically upregulated in patients with UC. Using *in silico* analysis we found several predicted pro-inflammatory target genes involved in various pathways, such as mitogen-activated protein kinase and cytokine signaling, which are both key signaling pathways in UC.

CONCLUSION: The present study provides the first evidence that miR-20b, miR-98, miR-125b-1*, and let-7e* are deregulated in patients with UC. The level of these miRNAs may serve as new potential biomarkers for this chronic disease.

Key words: Biomarker; Crohn's disease; Diagnostics; Inflammatory bowel disease; Microarray; MicroRNA; Ulcerative colitis

Core tip: This study contributes to the current knowledge on the putative role of microRNAs in inflammatory bowel disease pathogenesis, and it provides the first evidence that miR-20b, miR-98, miR-125b-1*, and let-7e* are deregulated in patients with ulcerative colitis. The level of these miRNAs may serve as new potential biomarkers for this chronic disease.

Coskun M, Bjerrum JT, Seidelin JB, Troelsen JT, Olsen J, Nielsen OH. miR-20b, miR-98, miR-125b-1*, and let-7e* as new potential diagnostic biomarkers in ulcerative colitis. *World J Gastroenterol* 2013; 19(27): 4289-4299 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i27/4289.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i27.4289

INTRODUCTION

MicroRNAs (miRNAs) are short (about 22 nucleotides in length), endogenous, non-coding single-stranded RNAs that act in concert to regulate expression of their target mRNAs[1-3]. The biogenesis of these small regulatory miRNA is a multistep process occurring in the cell nucleus and cytoplasm. Briefly, miRNAs are transcribed as long primary miRNA transcripts in the nucleus, and are then cleaved into precursor miRNA hairpin (pre-miRNA) by the Drosha-DGCR8 microprocessor complex[4-6]. Next, the pre-miRNAs are exported to the cytoplasm and further cleaved to mature miRNAs by Dicer[7-9]. It is generally believed that the mature miRNA then incorporates into the RNA-induced silencing complex, and guides this complex to the 3'-untranslated region (3'-UTR) of specific target mRNA transcripts to suppress translation or induce their degradation[1,2,10-13]. However, miRNA-binding sites in coding regions as well as in the 5'-UTRs have also been reported[14-19].

It is estimated that nearly one-third of the genes in the human genome might be regulated by the more than 2000 mature miRNAs so far identified[20]. As master regulators of post-transcription in cells, these regulatory miRNAs are involved in key functions in many physiological networks[21-23], and differentially expression miRNAs have been implicated in the pathogenesis of diverse gastrointestinal disorders, such as cancer and inflammatory diseases[24,25]. In facts, loss of intestinal miRNAs in mouse models has been shown to impair differentiation of intestinal cells and epithelial barrier function, resulting in acute inflammation[23].

Recent studies have demonstrated miRNAs to be involved in inflammatory bowel disease (IBD) susceptibility, as polymorphisms in miRNA-binding sites affect the gene expression and thus seem to play a pivotal part in the pathogenesis of this chronic disorder[17,26]. Moreover, it has been found that miRNAs are differentially expressed in ulcerative colitis (UC) and Crohn's disease (CD)[27], the two main forms of IBD. However, the pathogenesis of IBD still remains enigmatic[28], but the identification of differentially expressed miRNAs and subsequent understanding of their molecular mechanisms appear to provide new ways to reveal the pathophysiology, discover new diagnostic biomarkers, and develop new therapeutics[27].

The present study aims to analyze the miRNA expression in colonic mucosal biopsies from IBD patients and healthy individuals in order to identify new potential miRNA biomarkers in IBD using miRNA microarray profiling.

MATERIALS AND METHODS

Patients and tissue samples

Two cohorts, including both IBD individuals and controls, were analyzed in this study. In order to identify potentially deregulated miRNAs, a miRNA microarray analysis was performed on samples from cohort 1 (microarray cohort) consisting of 4 patients with UC (2 with active and 2 with quiescent disease; mean age 34 years, range 34-37 years, 2 females), 4 patients with CD (2 active and 2 quiescent; mean age 40 years, range 25-73 years, 3 females), and 2 controls (mean age 39 years, range 37-41 years, 2 females). A subsequent quantitative real-time polymerase chain reaction (qPCR) validation study was performed on samples from cohort 2 (validation cohort) including 20 patients with active UC, 19 patients with quiescent UC, and 20 healthy controls (Table 1). In both cohorts, the included subjects underwent a routine colonoscopy at the Department of Gastroenterology, Medical Section, Herlev Hospital, Denmark due to their clinical condition. They were included into the study as UC or CD patients with active disease, quiescent disease, or as controls (*i.e.*, an endoscopy was performed due to gastrointestinal symptoms but all clinical and paraclinical investigations subsequently turned out to be normal). All individuals with IBD had their diagnosis established on well-defined criteria[29] and disease activity of all UC patients were before the colonoscopy graded in accordance with the Mayo score[30]: a score $\leqslant 1$ as quiescent UC and > 1 as active UC, and CD patients were graded in accordance with the Harvey-Bradshaw score[31]: a score $\leqslant 4$ as quiescent CD and > 4 as active CD. Exclusion criteria were age above 80 or below 18 years, clinical evidence of infection, recent (within 14 d) use of antibiotics or probiotics, pregnancy, and severe mental illness.

All mucosal pinch biopsies, each of approximately

Table 1 Patients' characteristics (validation cohort)

Characteristics	Control (n = 20)	Inactive UC (n = 19)	Active UC (n = 20)
Gender (male/female)	10/10	6/13	9/11
Age (yr), mean (range)	48 (24-83)	48 (21-69)	38 (16-79)
Age at diagnosis (< 25/> 25 yr)	-	4/15	7/13
Years with disease (< 10/> 10 yr)	-	8/11	15/5
Mayo score, mean (range)	-	0 (0-1)	6 (2-12)
Extension of disease (P/PS/PC/LC/PH)	-		3/3/7/5/2
Smoking/non-smoking	5/15	6/13	3/17
EIM (present/never present)	-	3/16	0/20
Daily medications, *n* (%)			
Systemic mesalazine (1.6-3.2 mg)	-	14 (74)	17 (85)
Topical mesalazine (1000 mg)	-	3 (16)	6 (30)
Systemic glucocorticoids (75 mg)	-	0 (0)	2 (10)
Topical glucocorticoids (100 mg)	-	0 (0)	2 (10)
Azathioprine (100-200 mg)	-	1 (5)	2 (10)
6-mercaptopurine (50-100 mg)	-	0 (0)	1 (5)
Infliximab (5 mg/kg)	-	0 (0)	1 (5)
None	20 (100)	5 (26)	2 (10)

EIM: Extraintestinal manifestations; LC: Left-sided colitis; P: Proctitis; PC: Pancolitis; PH: Proctitis hemorrhagica; PS: Proctosigmoiditis; UC: Ulcerative colitis.

15 mg, were obtained from endoscopically non-inflamed or inflamed areas of the descending colon using routine endoscopic forceps. The descending colon was preferred to avoid any intersegmental variation. Non-inflamed samples originated from CD patients with endoscopically quiescent Crohn's colitis in the descending colon, where no other segments of the colon or ileum were endoscopically inflamed. Inflamed samples originated from CD patients with endoscopically active Crohn's colitis in the descending colon. Endoscopic activity was defined as areas with mucosal oedema, hyperemia, and friability. The endoscopic diagnosis of active or inactive disease was confirmed by histopathology conducted on parallel biopsies taken within an inch of the 1st biopsy. The biopsies were immediately placed in RNA-Later solution (Ambion, Austin, TX, United States), and following 24 h in RNA-Later at 4 ℃ the biopsies were stored at -80 ℃ until RNA extraction.

The study was approved by the Scientific Ethics Committee of the Capital Region of Denmark. All patients gave their informed written consent to participate in the study.

Extraction of total RNA and miRNA microarray profiling

The *mir*Vana™ miRNA isolation kit (Applied Biosystems, Carlsbad, CA, United States) was applied to isolate total RNA according to the manufacturer's protocol, and subsequently analyzed with a Geniom Real Time Analyzer (GRTA) (Febit GmbH, Heidelberg, Germany) using the Geniom Biochip miRNA *Homo sapiens*. The total RNA quality was assayed on an Agilent BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, United States). The quantity of total RNA was measured using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, United States). The 260/280 nm absorbance values were consistently above 1.9.

The array contained ten replicates of each human miRNA and miRNA star (*) sequences as annotated in the Sanger miRBase v.11.0[32]. Sample labelling with biotin was carried out using the *mir*Vana™ miRNA labeling kit (Ambion). Following hybridization for 16 h at 42 ℃ the biochip was washed as indicated by the supplier and signal enhancement was processed with the GRTA. For each array, signal intensities were calculated using the Geniom Wizard Software (Febit GmbH).

qPCR

Total RNA (10 ng) was reverse transcribed into cDNA using miRNA-specific primers (Applied Biosystems) and the TaqMan® miRNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. MiRNA expression levels of seven selected miRNAs identified by microarray were measured using commercially available pre-designed miRNA-specific TaqMan® miRNA assays (Applied Biosystems) according to the manufacturer's recommendations. All PCR reactions were performed using a Mx3000P thermocycler (Stratagene, La Jolla, CA, United States), and cycles were as follows: 95 ℃ for 10 min, 45 cycles of 95 ℃ for 15 s, and 60 ℃ for 1 min. The expression levels of each miRNA were normalized to endogenous RNU6B expression - a widely used internal control - and analyzed using the $2^{-\Delta\Delta CT}$ method.

miRNA target gene predictions

Target genes of miRNAs were predicted using the miRWalk database (http://www.ma.uniheidelberg.de/apps/zmf/mirwalk/) that allows simultaneous searches of several databases[33]. Four additional programs were selected: TargetScan, miRanda, miRDB, and RNA22 as target prediction programs. The search was performed on the 3'-UTR regions of target mRNAs with a P value of 0.05 defining the probability distribution of random matches set in the software with a minimum miRNA seed length of 7. When at least three programs co-identified a specific transcript, then the target(s) were selected for our list of potential targets. In addition, due to the limited ability of all algorithms to predict targets of miRNA complementary strands (*), the miRNA* targets were identified using miRWalk and miRanda and only those targets predicted by both programs were examined more closely.

Statistical analysis

miRNA data analysis: The raw microarray-data were background corrected and the ten replicate intensity values of each miRNA were summarized by their median value.

In order to reduce data complexity the unsupervised multivariate data analysis tool principal component analysis (PCA) was applied to determine if any intrinsic clustering existed within the dataset. If intrinsic clustering was found, the supervised multivariate data analysis tool projection to latent structure-discriminant analysis (PLS-DA) was employed. PLS-DA, like PCA, involves reduction of

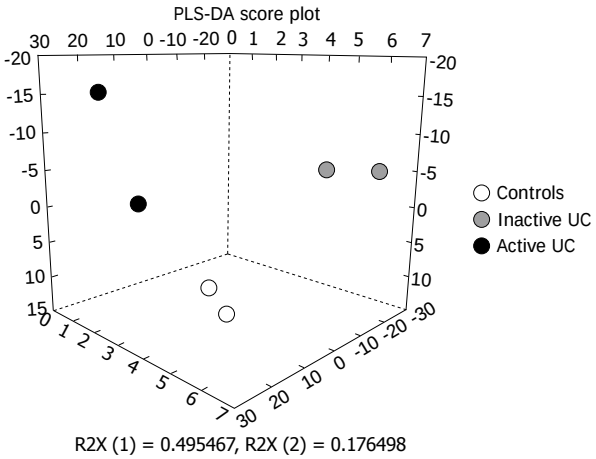

Figure 1 Projection to latent structure-discriminant analysis score-plot of the miRNA microarray expression profiles from mucosal colonic biopsies of controls, active ulcerative colitis, and inactive ulcerative colitis reveals a clear separation of these three groups. All patient with active ulcerative colitis (UC) are positioned in the left part of the space, and all patients with inactive UC are placed in the right space, whereas all control subjects are found in the middle. PLS-DA: Projection to latent structure-discriminant analysis.

data complexity and is commonly used where quantitative or qualitative relationships are sought between a matrix, X, in this case miRNA expression profiles, and another matrix, Y, in this case the class belonging of the samples. Such PLS-DA models offer the opportunity to create lists of miRNAs with the highest regression coefficients for each class, thus making it possible to identify the miRNA expression profiles responsible for the differentiation between the classes and subsequently the unique miRNAs with the strongest differential power. The multivariate data analysis was performed using SIMCA-P+ 12.0 (Umetrics, Umea, Sweden).

qPCR data analysis: Groups were compared using the Mann-Whitney *U* test, and *P* values less than 0.05 were considered significant.

RESULTS

Identification of differentially expressed miRNAs by miRNA microarray profiling

We have previously demonstrated that gene expression profiles using microarray studies can differentiate between active UC, inactive UC, and control samples[34,35]. Thus, in an initial attempt to identify new miRNAs that are differentially expressed in patients with IBD, we performed miRNA microarray profiling of colonic tissue samples from cohort 1. The PCA score-plot indicated a 3-way separation of the samples; controls, active CD, and inactive CD in one cluster, and active UC and inactive UC in two separate clusters (data not shown). The PCA model was described by 2 components explaining a total of 61% (R2X) of the variation in the dataset. The clustering of controls, active CD, and inactive CD in the PCA model indicates a similar miRNA expression profile in all three groups making further comparisons questionable. Thus, the subsequent PLS-DA model only contained the following three groups: active UC, quiescent UC, and controls. As seen in Figure 1, the PLS-DA score-plot resulted in a clear separation of these three groups. This PLS-DA model contained 2 components explaining a total of 67% (R2X) of the variation in the dataset and with a cross-validation parameter Q2Y (cum) of 0.89, indicating the predictability of the model. In order to substantiate the initial interpretation of the PCA score-plot, *i.e.*, clustering of active CD, inactive CD, and controls, an identical statistical procedure (creation of a PLS-DA model and comparison of regression coefficient lists) was performed with respect to these three groups. As expected, this resulted in an extensive number of miRNA duplicates in the regression coefficient lists, and the few miRNAs that were not duplicates had fold changes below 2 (results not shown).

In order to identify the miRNA expression profiles responsible for the differentiation between UC, CD, and controls, a list of 50 miRNA with the highest regression coefficients was generated for each group. When comparing the lists, no miRNA duplicates were found. Several of the differentially expressed miRNAs (such as miR-23a, miR-155, miR-16, miR-150, miR-346, and miR-126) reported to be associated with UC in previous studies[36-38] were also identified within our analyses (Tables 2 and 3). However, in addition to these previously reported miRNAs, we additionally identified five potential miRNAs, miR-20b, miR-99a, miR-203, miR-26b, and miR-98 to be differentially up-regulated more than a 10-fold in active UC compared to inactive UC, active CD, inactive CD, and controls (Table 2). Two miRNAs, miR-125b-1* and let-7e*, were differentially up-regulated more than a 5-fold in inactive UC compared to active UC, active CD, inactive CD, and controls (Table 3). These miRNAs have not previously been reported to be involved in IBD[27]. Thus, in the subsequent study, we focused on these seven new candidate miRNAs.

Verification of differences by qPCR

The expression levels of the seven candidate miRNAs (miR-20b, -99a, -203, -26b, -98, -125b-1*, and let-7e*) were tested in an independent validation cohort on samples from patients with active UC (n = 20), inactive UC (n = 19), and controls (n = 20) using individual miRNA-specific primers. In accordance with the microarray data, qPCR results showed significantly increased miR-20b ($P < 0.05$) expression in active UC *vs* controls. Moreover, miR-20b expression was significantly higher ($P < 0.05$) in inactive UC than in controls (Figure 2). Additionally, the qPCR analysis confirmed a significant ($P < 0.05$) higher expression of let-7e* in inactive UC *vs* controls (Figure 2).

In contrast to microarray results, miR-98 was significantly over-expressed in inactive UC ($P < 0.05$), when compared to both active UC and controls (Figure 2). Similarly, miR-125b-1* was not significantly up-regulated in inactive UC, as predicted by the microarray profiling,

Table 2 Top fifty differentially expressed miRNAs (active ulcerative colitis *vs* inactive ulcerative colitis, active Crohn's disease, inactive Crohn's disease, and controls) and their respective fold-changes from miRNA microarray expression profiling

MicroRNAs	Active UC/ active CD	Active UC/ controls	Active UC/ inactive CD	Active UC/ inactive UC
hsa-miR-15a	64	7	19	24
hsa-miR-199b-3p	55	6	17	61
hsa-miR-20b	**53**	**12**	**20**	**35**
hsa-miR-20a	51	9	14	47
hsa-miR-106b	43	4	7	40
hsa-miR-27b	36	6	6	15
hsa-miR-99a	**33**	**33**	**17**	**31**
hsa-miR-222	32	7	12	26
hsa-miR-151-5p	19	4	18	20
hsa-miR-203	**19**	**11**	**17**	**24**
hsa-miR-30a	14	9	14	7
hsa-miR-25	14	6	9	11
hsa-miR-26b	**12**	**15**	**10**	**13**
hsa-miR-646	12	19	13	8
hsa-miR-100	10	4	3	10
hsa-miR-125b	10	15	8	4
hsa-miR-98	**10**	**10**	**10**	**12**
hsa-miR-411*	8	3	8	5
hsa-miR-28-5p	8	3	3	6
hsa-miR-768-3p	7	5	4	17
hsa-miR-195	7	10	35	22
hsa-miR-99b	7	4	4	3
hsa-miR-23a	7	8	18	49
hsa-miR-18a	7	11	7	8
hsa-miR-17	7	5	6	44
hsa-miR-155	6	7	11	19
hsa-miR-23b	6	7	10	18
hsa-miR-1201	6	6	6	8
hsa-miR-130a	6	6	11	26
hsa-miR-199a-3p	6	5	15	37
hsa-miR-93	5	3	4	8
hsa-miR-199a-5p	5	3	3	7
hsa-miR-16	5	8	17	175
hsa-miR-146a	5	3	3	6
hsa-miR-103	5	3	6	30
hsa-miR-126	5	16	39	65
hsa-miR-107	5	3	7	18
hsa-miR-106a	5	5	6	21
hsa-miR-1248	4	5	8	93
hsa-miR-27a	4	6	6	25
hsa-miR-222*	4	4	4	6
hsa-miR-24	3	3	4	17
hsa-miR-182	3	3	3	5
hsa-miR-193a-3p	3	7	3	7
hsa-let-7e	3	2	4	2
hsa-miR-548a-3p	2	2	2	6
hsa-miR-99a*	1	1	1	3
hsa-miR-758	1	1	1	2
hsa-miR-568	1	5	6	7

Differentially up-regulated (≥ 10-fold) miRNAs are indicated in bold in the lists. *miRNA complementary strands. CD: Crohn's disease; UC: Ulcerative colitis.

Table 3 Top fifty differentially expressed miRNAs (inactive ulcerative colitis *vs* active ulcerative colitis, active Crohn's disease, inactive Crohn's disease, and controls) and their respective fold-changes from miRNA microarray expression profiling

MicroRNAs	Active UC/ inactive UC	Inactive UC/ controls	Active CD/ inactive UC	Inactive UC/ inactive CD
hsa-miR-506	13	4	5	5
hsa-miR-125b-1*	**12**	**6**	**27**	**5**
hsa-let-7e*	**6**	**6**	**6**	**6**
hsa-miR-512-5p	5	5	5	4
hsa-miR-637	5	3	4	4
hsa-miR-1288	4	4	4	5
hsa-miR-330-3p	4	5	9	4
hsa-miR-623	4	6	6	6
hsa-miR-34b	4	9	9	7
hsa-miR-138-1*	3	6	6	5
hsa-miR-154	3	7	7	7
hsa-miR-760	3	3	17	3
hsa-miR-1296	3	5	11	4
hsa-miR-523	3	3	3	3
hsa-miR-149	3	5	10	5
hsa-miR-509-3-5p	3	3	3	3
hsa-miR-1276	3	3	19	4
hsa-miR-1178	3	4	2	5
hsa-miR-885-5p	3	3	3	3
hsa-miR-1264	2	2	2	2
hsa-miR-521	2	6	6	5
hsa-miR-218-2*	2	3	2	2
hsa-miR-551a	2	8	13	4
hsa-miR-505*	2	2	3	2
hsa-miR-1226	2	3	15	4
hsa-miR-495	2	4	9	3
hsa-miR-220c	2	3	3	4
hsa-miR-550*	2	4	2	3
hsa-miR-371-3p	2	3	3	3
hsa-miR-596	2	4	4	3
hsa-miR-216a	2	6	9	4
hsa-miR-1293	2	3	15	3
hsa-miR-1247	2	3	47	2
hsa-miR-34c-3p	2	4	12	5
hsa-miR-1233	2	3	6	3
hsa-miR-346	2	3	4	3
hsa-miR-211	2	8	9	11
hsa-miR-302c*	2	5	11	6
hsa-miR-520a-3p	2	2	2	2
hsa-miR-485-3p	2	5	14	3
hsa-miR-92a-2*	2	2	4	3
hsa-miR-328	1	2	2	2
hsa-miR-661	1	3	7	4
hsa-miR-453	1	7	8	5
hsa-miR-30c	1	5	7	7
hsa-miR-520h	1	2	2	2
hsa-miR-193b	1	4	10	4
hsa-miR-483-3p	1	2	4	2
hsa-miR-150	1	3	6	2

Differentially up-regulated (≥ 5-fold) miRNAs are indicated in bold in the lists. *miRNA complementary strands. CD: Crohn's disease; UC: Ulcerative colitis.

but was significantly different ($P < 0.01$) between active UC and controls (Figure 2). The expression levels of the other predicted miRNAs (miR-99a, -203, and -26b) did, however, not reach a statistical significance (Figure 3).

Prediction of miRNA target genes associated with UC

Having identified significant changes in the four miRNA expression profiles (miR-20b, miR-98, miR-125b-1*, and let-7e*) between UC and controls, we next examined the biological relevance of these miRNAs by identifying their target genes. *In silico* analysis using miRWalk, which combines the output of multiple prediction algorithms, was used to identify putative targets. Among the large number of predictive targets identified by this approach,

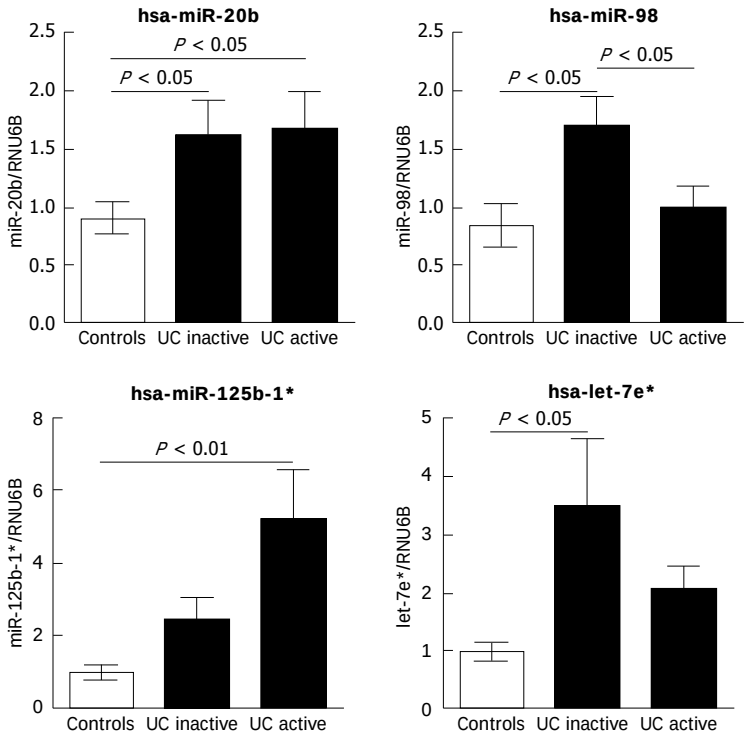

Figure 2 **Expression levels of four miRNAs in mucosal colonic biopsies were significantly up-regulated in ulcerative colitis.** Expression differences of miRNA in active ulcerative colitis (UC) (n = 20), inactive UC (n = 19), and controls (n = 20). Levels were determined using quantitative real-time polymerase chain reaction. Relative expression differences of each miRNA were normalized to endogenous RNU6B expression and calculated using the $2^{-\Delta\Delta CT}$ method. P value was calculated by Mann-Whitney U test and data are represented as medians with interquartile ranges.

several putative targets well known to be associated with the inflammatory response were revealed. For example, predicted target genes of miR-20b included interleukin-6 receptor (*IL-6R*), *IL-8, IL-10, REL* [a member of the nuclear factor (NF)-κB family], autophagy related 16-like 1 (*ATG16L1*), NOD-LRRs containing pyrin domain 3 (*NLRP3*), CASP8 and FADD-like apoptosis regulator (*CFLAR*), extracellular signal-regulated kinase 2 (*ERK2*), *p38-α* (MAP kinase), MAP kinase kinase kinase 1 (*MEKK1*), and signal transducer and activator of transcription 3 (*STAT3*). Putative target genes of miR-98 included *IL-8*, *IL-10*, toll-like receptor 4 (*TLR4*), *MEKK1, ATG16L1, CD95,* claudin-1 (*CLDN1*) and *STAT3*. Finally, miR-125b-1* and let-7e* were predicted to regulate genes such as, hepatocyte nuclear factor-1α (*HNF1α*) and *p38-α*, respectively.

The progress in gene discovery in complex disease genetics has increased with the genome-wide association studies (GWAS). Until recently, 99 IBD susceptibility loci were reported: 71 associated with CD, 47 with UC, and 28 with both CD and UC[39,40]. However, new IBD susceptibility loci were recently added bringing the total number of IBD loci to 163, most of which are associated with both CD and UC[41]. Thus, in order to identify which of the predicted miRNA targets that are actually reported as UC associated susceptibility genes, we used the web application BioVenn[42] (http://www.cmbi.ru.nl/cdd/biovenn/) to compare the list of the miRNA predicted target genes and UC associated susceptibility genes reported in the literature[40,41,43-45]. The UC associated predicted miRNA target genes have been illustrated in a Venn diagram (Figure 4). The four circles represent predicted miRNA targets found in the list of reported UC susceptibility genes. Interestingly, 25 of the miR-20b predicted targets were identified as UC associated susceptibility genes, and 14 miR-98 targets were found to be associated with UC, with 6 common target genes (Figure 4). Additionally, we found four UC associated target genes for miR-125b-1*, however, only one let-7e* target gene were predicted to be associated with UC susceptibility (Figure 4).

DISCUSSION

In recent years, miRNA profiling studies using tissue or blood samples from IBD patients have provided us with new ways to understand this otherwise enigmatic disease, and identifying differentially expressed miRNAs is a first step in the development of miRNA profile-based diagnostic tools.

Thus, in this study, using miRNA microarray profiling followed by qPCR analysis, we identified new miRNAs that were altered in patients with UC *vs* controls. Previous studies have demonstrated different mRNA expression profiles of mucosal colonic biopsies from IBD patients and healthy individuals[34,35,46,47]. Furthermore, Olsen *et al*[34] demonstrated that active and inactive UC could be distinguished from CD patients and controls. However, these studies could not differentiate between inactive CD and controls[34,48]. Similarly, in the PCA and PLS-DA scoreplots of miRNA expression profiles, we observed a clear separation of active and inactive UC patients from CD patients and controls. These results suggest that miRNA and mRNA profiles might follow the same pattern in UC patients and could be combined to differentiate between UC *vs* CD and controls in order to identify potential diagnostic biomarker panels. However, Wu *et al*[49] claimed to be able to discriminate active CD from controls using

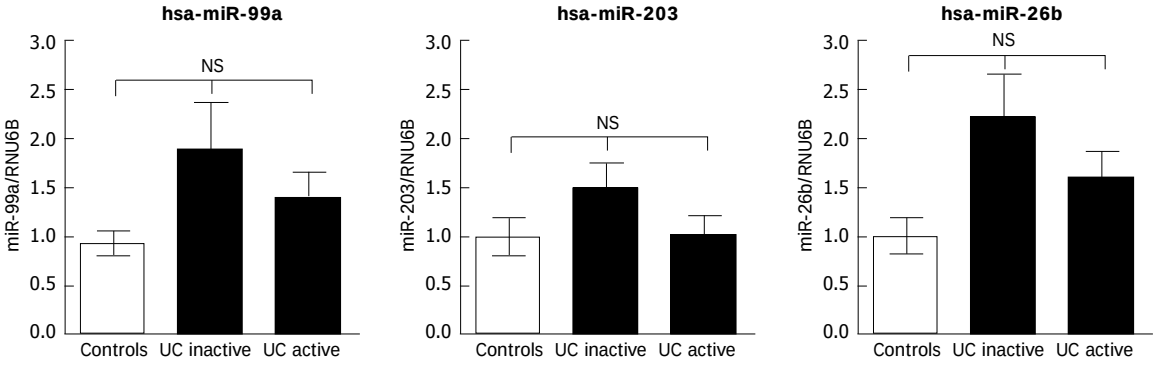

Figure 3 Expression of other miRNAs identified by microarray profiling. Expression differences of miRNA in active ulcerative colitis (UC) (n = 20), inactive UC (n = 19), and controls (n = 20). Levels were determined using quantitative real-time polymerase chain reaction. Relative expression differences of each miRNA were normalized to endogenous RNU6B expression and calculated using the $2^{-\Delta\Delta CT}$ method. NS: Not significant.

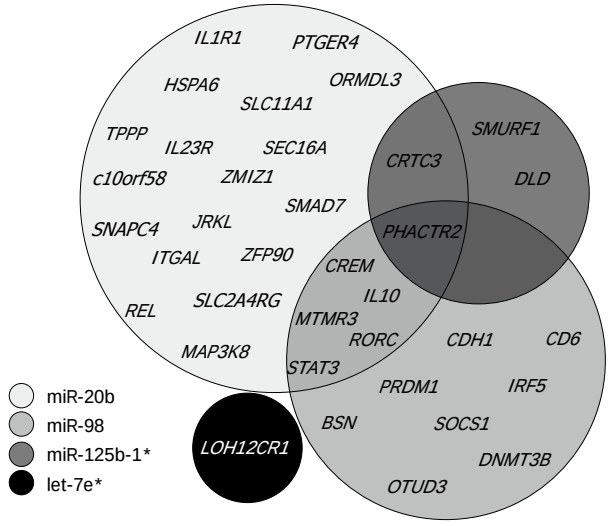

Figure 4 Venn diagram illustrating the miRNA-specific and overlapping ulcerative colitis associated target genes. The four circles represent predicted miRNA targets found in the list of reported ulcerative colitis susceptibility genes.

microarray analysis of intestinal biopsies, whereas other studies have mainly examined the expression of miRNAs by qRT-PCR analysis[27], which is inherently a much more sensitive technique than microarray[50]. When comparing our study with the study by Wu *et al*[49] there is, however, a striking difference in the miRNA microarray platforms used; Wu *et al*[49] used a miRNA microarray platform from NCode (Invitrogen, La Jolla, CA, United States) detecting about 470 unique human miRNAs, while we used a platform from Geniom Biochip miRNA (Febit) detecting almost 850 unique human miRNAs. Thus, the overall miRNA expression panel may possibly not follow the same pattern due to a dissimilar number of detected miRNAs in different arrays. Furthermore, the stringent significance and fold change criteria set by the current study might very well exclude subtle differences present in the expression profiles of patients with CD and controls. However, it cannot be excluded that the limited size of the microarray cohort as well as the medical therapy that each patient received also contributed to the inability to discriminate between groups.

Among the miRNAs with the strongest differential power from the microarray expression data we identified several differentially expressed miRNAs (such as miR-23a, miR-155, miR-16, miR-150, miR-346, and miR-126) previously reported to be associated with UC[36-38]. However, we also identified seven miRNAs (miR-20b, miR-99a, miR-203, miR-26b, miR-98, miR-125b-1*, and let-7e*) that were not previously reported to be involved in IBD[27]. Therefore, we focused on these seven new candidate miRNAs. The four significantly up-regulated human miRNAs (miR-20b, miR-98, miR-125b-1*, and let-7e*) identified in UC patients in the present paper contributes to the current knowledge of the roles of miRNAs in IBD. In particular, we show that miR-20b expression is increased in both active and quiescent UC as compared to controls. This could make miR-20b a potential biomarker to differentiate between controls and UC as it is not dependent on disease activity. Increased levels of miR-20b have been reported in human cancers including lung cancer, gastric cancer and leukemias where miR-20b facilitates cellular adaption to normoxia and hypoxia *in vitro* by regulating the transcription factor hypoxia-inducible factor 1-alpha[51]. Therefore, it is possible that miR-20b might be involved in the pathophysiology of colitis-associated colorectal cancer[52].

We also found miR-125b-1* to be significantly up-regulated in active UC. The miR-125 family has 2 mature isoforms: miR-125a and miR-125b (encoded by miR-125b-1 and miR-125b-2). Deregulated expression of miR-125 family members in various cancers has been reported[53-55]. Tili *et al*[56] has earlier demonstrated that miR-125b is of importance for the innate immune response, as lipopolysaccharide (LPS) stimulation of a murine macrophage cell line caused suppressed miR-125b levels. LPS is the principal component of bacteria in terms of pro-inflammatory properties as it activates the innate immune system through toll-like receptors to produce pro-inflammatory cytokines, including interferon-γ or tumor necrosis factor-α[57].

In the present study, we additionally found two let-7 miRNA family members, miR-98 and let-7e*, significantly altered in UC. MiR-98 was significantly up-regulated in

inactive UC compared to controls and active UC. Moreover, miR-98 has been reported to negatively regulate the anti-inflammatory cytokine, IL-10, production in macrophages - an important factor in the immune response that protects the host from excessive inflammation[58]. Additionally, a recent study has revealed that miR-98 targets the Fas-receptor mRNA, and decreases Fas-mediated apoptosis[59]. The increased expression of miR-98 could explain the attenuated Fas-mediated cell death response of lamina propria T-cells in IBD[60]. This resistance of mucosal T-cells to Fas-mediated apoptosis might explain the sustained mucosal inflammation seen in IBD[61].

In accordance with the microarray data, we found significantly increased let-7e* levels in inactive UC when compared to healthy individuals. The let-7 family of miRNAs is highly conserved across diverse animal species from worms to humans, and plays important roles in the regulation of cell proliferation and differentiation[62]. Humans have ten mature let-7-family sequences that are produced from 13 precursor sequences[63]. Most of the human *let-7* genes map to regions altered or deleted in human tumours[64], and are reported to be down-regulated in various cancerous conditions[65,66]. In addition to cancer, an altered expression of let-7 has been reported in inflammation. In allergic airway inflammation, administration of let-7 mimic to mice represses IL-13 production and reduces the inflammation[67]. Furthermore, other let-7 family members than let-7e* described in this study have previously been linked to IBD. Wu *et al*[36] found let-7f differentially expressed in the intestinal tissue of active UC patients when compared to controls, and Zahm *et al*[68] found higher concentrations of let-7b miRNA in sera of pediatric CD patients *vs* controls. It has been reported that there is a regulatory interaction between NF-κB activation and expression of let-7 members[69-72], which suggests a significant importance for the pathogenesis of IBD. Let-7 miRNAs has also been demonstrated to be repressed in inflammation, which result in increased expression of pro-inflammatory cytokines and enhanced inflammatory responses[72].

The miRNAs described and analyzed in the present study were initially selected based on the microarray results. The subsequent validation procedure using qPCR resulted in partial discrepancy between the microarray and qPCR results. This was, however, to be expected as correlation coefficients between microarray and qPCR assays have been found to be as low as 0.4[73], and just substantiates the microarray technology as a hypothesis generating tool and illustrates the importance of verifying microarray expression data.

Another challenge in miRNA research is the identification of genes regulated by miRNAs. One common method to address this challenge is by predicting targets by computer algorithms. Through *in silico* predictions we identified multiple potential miRNA targets in the inflammatory response well-known to be associated with UC. Interestingly, the most target genes were associated with the mitogen-activated protein kinase pathway and cytokine signaling, which are important key signaling pathways in IBD[74]. These findings further suggest that the miRNA molecules found in this study may have a pivotal influence on the clinical course of IBD.

In conclusion, this study contributes to the current knowledge on the putative role of miRNAs in IBD pathogenesis, and it provides the first evidence that miR-20b, miR-98, miR-125b-1*, and let-7e* are deregulated in patients with UC. The level of these miRNAs may serve as new potential biomarkers for this chronic disease. However, this area of research is in its infancy, and studies in the field of IBD need to identify all of the miRNAs that are consistently deregulated in IBD.

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COMMENTS

Background

MicroRNAs (miRNAs) are small, non-coding single-stranded RNA molecules that regulate the expression of target genes and are involved in many cellular and physiological mechanisms. The discovery of miRNAs in inflammatory bowel disease (IBD), particularly their role in cell signaling, offers a novel way of understanding this chronic disease and gives rise to new potential diagnostic tools and therapeutic strategies. In this paper, the authors identified new potential miRNA biomarkers in ulcerative colitis (UC).

Research frontiers

The pathogenesis of IBD remains largely unknown, but involves a complex interaction between genetic, environmental, and immunological factors. However, to date, there are still no ideal methods to asses the severity of inflammation and to differentiate between UC and Crohn's disease (CD). In this context, research on miRNAs is a promising new research, providing novel insights into the pathogenesis of IBD, biomarker identification, and treatment. Thus, there is great promise that miRNAs will aid in the early diagnosis of IBD, and in the development of more targeted, personalized therapies.

Innovations and breakthroughs

Recent reports have highlighted the importance of using miRNAs in IBD pathogenesis, diagnostics and therapeutics. Through microarray-based miRNA profiling of colonic mucosal biopsies from patients with UC, CD, and controls, the authors found that the expression of miR-20b, miR-98, miR-125b-1*, and let-7e* was upregulated in patients with UC. This is the first study to report that these four miRNAs may serve as potential biomarkers for UC.

Applications

The present study suggest that miR-20b, miR-98, miR-125b-1*, and let-7e* levels are deregulated in patients with UC. The level of these miRNAs may serve as new potential biomarkers for this chronic disease.

Peer review

The authors analyzed the miRNA expression in IBD patients and healthy individuals in order to identify new potential miRNA biomarkers in IBD using miRNA microarray profiling of colonic mucosal biopsies. Among the most differentially expressed miRNAs, the authors found that the levels of miR-20b, miR-98, miR-125b-1*, and let-7e* were specifically upregulated in patients with UC. These data are quite important and may help to clarify the complex pathogenesis of UC and further suggest that the level of these miRNAs may serve as new potential biomarkers for this disease.

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ORIGINAL ARTICLE

Pink-color sign in esophageal squamous neoplasia, and speculation regarding the underlying mechanism

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Abstract

AIM: To investigate the reasons for the occurrence of the pink-color sign of iodine-unstained lesions.

METHODS: In chromoendoscopy, the pink-color sign of iodine-unstained lesions is recognized as useful for the diagnosis of esophageal squamous cell carcinoma. Patients with superficial esophageal neoplasms treated by endoscopic resection were included in the study. Areas of mucosa with and without the pink-color sign were evaluated histologically. The following histologic features that were possibly associated with the pink-color sign were evaluated. The keratinous layer and basal cell layer were classified as present or absent. Cellular atypia was classified as high grade, moderate grade or low grade, based on nuclear irregularity, mitotic figures, loss of polarity, chromatin pattern and nuclear/cytoplasmic ratio. Vascular change was assessed based on dilatation, tortuosity, caliber change and variability in shape. Vessels with these four findings were classified as positive for vascular change. Endoscopic images of the lesions were captured immediately after iodine staining, 2-3 min after iodine staining and after complete fading of iodine staining. Quantitative analysis of color changes after iodine staining was also performed.

RESULTS: A total of 61 superficial esophageal neoplasms in 54 patients were included in the study. The lesions were located in the cervical esophagus in one case, the upper thoracic esophagus in 10 cases, the mid-thoracic esophagus in 33 cases, and the lower thoracic esophagus in 17 cases. The median diameter of the lesions was 20 mm (range: 2-74 mm). Of the 61 lesions, 28 were classified as pink-color sign positive and 33 as pink-color sign negative. The histologic diagnosis was high-grade intraepithelial neoplasia (HGIN) or cancer invading into the lamina propria in 26 of the 28 pink-color sign positive lesions. There was a significant association between pink-color sign positive epithelium and HGIN or invasive cancer (P = 0.0001). Univariate analyses found that absence of the keratinous layer and cellular atypia were significantly associated with the pink-color sign. After Bonferroni correction, there were no significant associations between the pink-color sign and presence of the basal membrane or vascular change. Multivariate analyses found that only absence of the keratinous layer was independently associated with the pink-color sign (OR = 58.8, 95%CI: 5.5-632).

Quantitative analysis was performed on 10 superficial esophageal neoplasms with both pink-color sign positive and negative areas in 10 patients. Pink-color sign positive mucosa had a lower mean color value in the late phase (pinkish color) than in the early phase (yellowish color), and had similar mean color values in the late and final phases. These findings suggest that pink-color positive mucosa underwent color fading from the color of the iodine (yellow) to the color of the mucosa (pink) within 2-3 min after iodine staining. Pink-color sign negative mucosa had similar mean color values in the late and early phases (yellowish color), and had a lower mean color value in the final phase (pinkish color) than in the late phase. These findings suggest that pink-color sign negative mucosa did not undergo color fading during the 2-3 min after iodine staining, and underwent color fading only after spraying of sodium thiosulfate.

CONCLUSION: The pink-color sign was associated with absence of the keratinous layer. This sign may be caused by early fading of iodine staining.

Key words: Chromoendoscopy; Esophageal cancer; Esophageal squamous neoplasia; Iodine staining; Pink-color sign

Core tip: The pink-color sign of iodine-unstained lesions is useful for the diagnosis of esophageal squamous cell carcinoma. We investigated histologic findings of esophageal neoplasms, and found that absence of the keratinous layer because of neoplastic cell proliferation may be responsible for the pink-color sign. Quantitative analysis of color showed that pink-color sign positive mucosa underwent early color fading from the color of the iodine (yellow) to the color of the mucosa (pink) within 2-3 min. Based on these results, we speculated on the mechanism underlying the pink-color sign. These findings may improve our understanding of the characteristics of esophageal neoplasms.

Ishihara R, Kanzaki H, Iishi H, Nagai K, Matsui F, Yamashina T, Matsuura N, Ito T, Fujii M, Yamamoto S, Hanaoka N, Takeuchi Y, Higashino K, Uedo N, Tatsuta M, Tomita Y, Ishiguro S. Pink-color sign in esophageal squamous neoplasia, and speculation regarding the underlying mechanism. *World J Gastroenterol* 2013; 19(27): 4300-4308 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i27/4300.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i27.4300

INTRODUCTION

Esophageal cancer is the sixth most common cause of cancer-related mortality worldwide[1]. The overall survival of patients with esophageal cancer remains poor, regardless of histologic type. However, a favorable prognosis can be expected after treatment with esophagectomy[2,3], chemoradiotherapy[4,5] or endoscopic resection[6-9] if the cancer is detected at an early stage.

Conventional endoscopy has limited usefulness for the treatment of esophageal cancer, because it is not easy to identify early neoplastic changes[10,11]. Chromoendoscopy with iodine staining is reported to be more sensitive for the early diagnosis of esophageal squamous cell carcinoma[12,13], but has low specificity and requires multiple biopsy specimens[10,14]. A dramatic color change after iodine staining, from the initial yellow color to a pink color 2-3 min later, is known as the pink-color sign, and is useful for identifying cancerous lesions[15,16]. This sign has been reported to dramatically improve specificity for esophageal high-grade intraepithelial neoplasia (HGIN) and invasive cancer[15,16]. Choosing adequate biopsy sites is sometimes difficult, especially in patients with scattered-type staining of the esophagus, which is characterized by multiple Lugol-voiding lesions[17]. Some of these iodine-unstained lesions may indicate inflammation or low-grade intraepithelial neoplasia (LGIN), and in such cases a lack of iodine staining is not a good indication for taking a biopsy specimen. Because of its high specificity, the pink-color sign is a good indicator for choosing adequate biopsy sites in patients with scattered-type staining. However, the mechanism underlying the occurrence of the pink-color sign has not been fully investigated. Improved understanding of this mechanism may improve our understanding of the characteristics of the relevant lesions, and increase the likelihood of accurate diagnosis. This study therefore aimed to clarify the histologic changes responsible for the occurrence of the pink-color sign in esophageal squamous neoplasia.

MATERIALS AND METHODS

Endoscopic examination and resection

The current clinical investigation was conducted during routine endoscopic procedures for resection of esophageal squamous lesions. Patients with superficial esophageal neoplasia confirmed by histologic examination were included in the study. Superficial esophageal neoplasia was defined as a lesion limited to the submucosa. Typical endoscopic findings were superficial protruding type, superficial flat type and superficial excavated type. If a lesion had a large broad-based protrusion, crater and stiffened wall, it was diagnosed as advanced cancer. Patients were excluded if they had previously undergone surgery, chemotherapy or radiotherapy for esophageal cancer. The endoscopic procedures were performed using a high-resolution magnifying upper-gastrointestinal endoscope (GIF-Q240Z or GIF-H260Z; Olympus, Tokyo, Japan). The structure enhancement function of the video processor was set at level B8 for narrow-band imaging (NBI). A black soft hood (MB-162 for GIF-Q240Z or MB-46 for GIF-H260Z; Olympus) was mounted on the tip of the endoscope to maintain an adequate distance between the tip of the endoscopic zoom lens and the mucosal

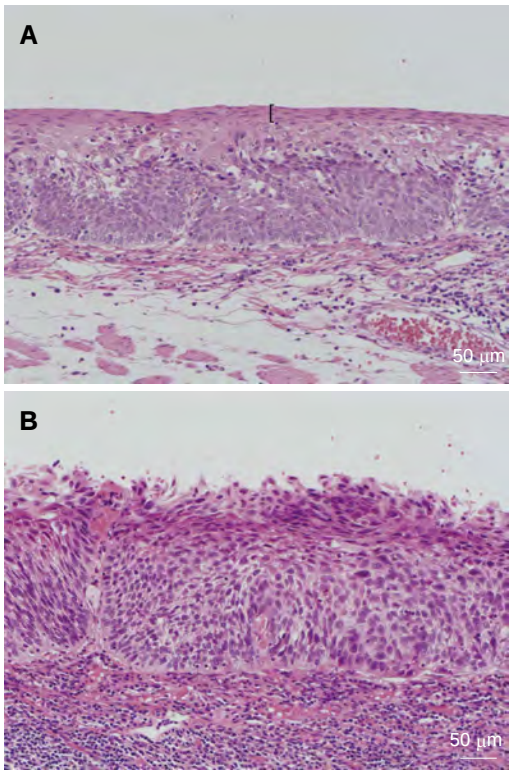

Figure 1 Histologic findings of esophageal lesions. A: Esophageal lesion with keratinous layers, shown by black parentheses; B: Esophageal lesion without keratinous layers.

surface during observations. The endoscopic procedure was performed under intravenous sedation with midazolam (Dormicam; Yamanouchi Pharma, Tokyo, Japan) and pentazocine (Pentazin; Sankyo Pharmaceuticals, Tokyo, Japan).

The esophageal mucosa was initially examined using white-light imaging or NBI. A catheter was then used to spray 20-40 mL of 0.6%-1.2% iodine solution until the esophageal mucosa was evenly stained, and the subsequent color changes were observed. Immediately after spraying of the iodine solution, the normal mucosa was dark brown, whereas abnormal mucosa suspicious of dysplasia or cancer was yellow. The iodine-unstained (yellow) areas were classified as pink-color sign positive or pink-color sign negative. In pink-color sign positive mucosa, the yellow areas changed to pink within 2-3 min of spraying, and in pink-color sign negative mucosa, these areas remained yellow after 3 min. Representative areas (2-5 mm diameter) were marked with marker dots, and the lesions were resected by endoscopic submucosal dissection or endoscopic mucosal resection. Iodine staining and marking of pink-color sign positive and negative areas were performed immediately before resection. Considering the potential disadvantages of intraoperative endoscopy and iodine staining, patients requiring surgical resection were excluded. To ensure that the study protocol was strictly followed, marking before endoscopic resection and confirmation of marking after endoscopic resection were performed by one of two endoscopists (Ishihara R or Kanzaki H). Procedures that were performed without these two endoscopists in attendance were excluded from the study. Written informed consent was obtained from all patients before endoscopic examination and resection of lesions. Institutional review board approval was granted for a retrospective chart review and analysis of the data.

Histologic evaluation

All specimens were cut into 2-mm slices and embedded in paraffin. Sections were cut from the paraffin blocks and stained with hematoxylin and eosin. The pink-color sign positive and negative lesions marked by the marker dots were examined histologically. The depth of cancer involvement was classified according to the Japanese Classification of Esophageal Carcinoma[18], and intraepithelial neoplasms were classified as LGIN or HGIN, according to the World Health Organization classification[19]. Intraepithelial cancer was included in HGIN.

The following histologic features that were possibly associated with the pink-color sign were evaluated. The keratinous layer (Figure 1) and basal cell layer were classified as present or absent. Cellular atypia was classified as high grade, moderate grade or low grade, based on nuclear irregularity, mitotic figures, loss of polarity, chromatin pattern and nuclear/cytoplasmic ratio. Vascular change was assessed based on dilatation, tortuosity, caliber change and variability in shape. Vessels with these four findings were classified as positive for vascular change[20]. All histologic assessments were performed by the same pathologist (SI), who was blinded to the endoscopic and clinical findings.

Quantitative analysis of color

This study included esophageal cancers those have both pink-color sign positive and pink-color sign negative areas. The esophageal mucosa was initially examined with white-light imaging or NBI. A catheter was used to spray 20-40 mL of 1.2% iodine solution until the normal esophageal mucosa was evenly stained, and the subsequent color changes were examined. Sodium thiosulfate solution was then sprayed to relieve symptoms caused by the iodine, which also accelerated the color fading by reduction of the iodine.

The digital images were used to perform quantitative analysis of the color changes after iodine staining. Endoscopic images of the lesions were captured immediately after iodine staining, 2-3 min after iodine staining and after complete fading of iodine staining, taking care to ensure that all images were obtained from a similar direction and a similar distance, to enable accurate analysis (Figure 2). All the images were captured under the instruction of one endoscopist (Ishihara R). The images were stored in bitmap format (.bmp) with a resolution of 640 × 480 pixels. A small region of interest was chosen in both the pink-color sign positive and pink-color sign negative areas. These regions of interest were carefully chosen to be of similar size in each area (Figure 3). The red-green-blue components of each region of interest were calculated

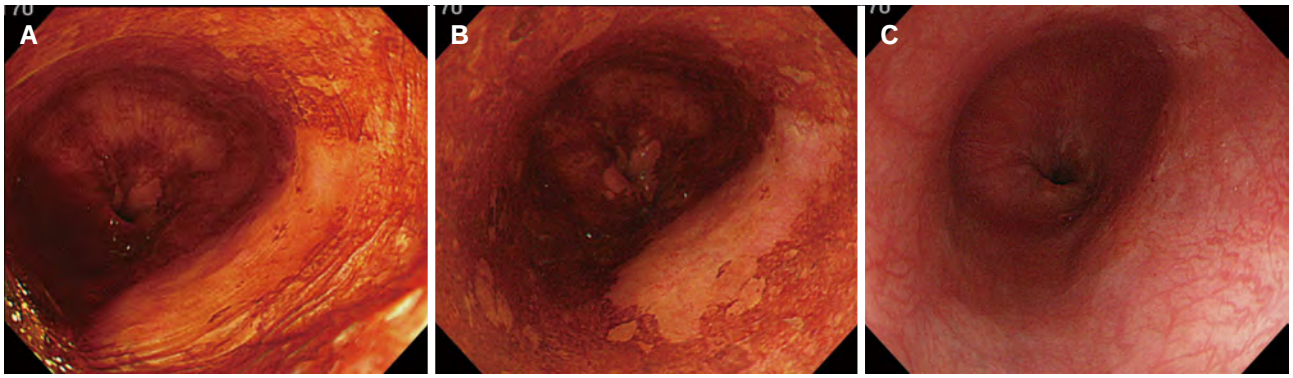

Figure 2 Endoscopic image of type IIc esophageal cancer. A: Endoscopic image of type IIc esophageal cancer immediately after iodine staining; B: Endoscopic image of type IIc esophageal cancer 2 min after iodine staining. The pink-color sign is observed on the right side of the lesion; C: Endoscopic image of type IIc esophageal cancer 3 min after sodium thiosulfate spraying.

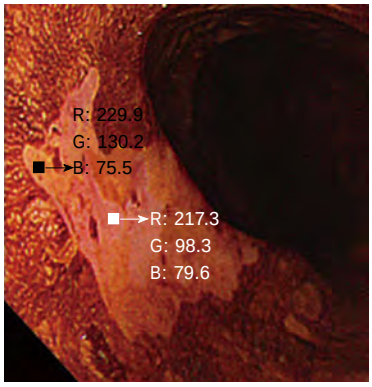

Figure 3 A small region of interest was chosen in both the pink-color sign positive and pink-color sign negative areas. These regions of interest were carefully chosen to be of similar size in each area. The red-green-blue components of each region of interest were calculated using Image J software.

using Image J software (National Institutes of Health, Bethesda, MD, United States), and color diagrams were created using the graphing function of Microsoft Excel (Microsoft Corp., Redmond, WA, United States).

The LU'V' color system is a uniform color space that was adopted by the Commission Internationale de l'E' clairage in 1976 and is used to systematically represent the different colors[21,22]. A color system is a model for representing colors in terms of intensity values. Generally, colors are described using color systems with three or four dimensions (red-green-blue or cyan-magenta-yellow-black). The diagram of the LU'V' system has U' and V' coordinates that represent the chromaticity values of each color. This color space is designed to be perceptually uniform, meaning that a given change in value roughly corresponds to the same perceptual difference over any part of the space. Using this system, color can be quantified and evaluated on a two-dimensional plane. However, determining the range of color is challenging because there is no clear border between colors.

Statistical analysis

The relationships between the pink-color sign and each histologic finding were analyzed. If more than one lesion was resected from a patient, each lesion was considered separately for the purposes of statistical analysis. Univariate analyses of the relationships between the pink-color sign and the histologic findings were performed using the χ^2 test with Yates' correction. Factors independently associated with the pink-color sign were identified using multivariate logistic regression analysis. The model fit was assessed using the Hosmer-Lemeshow test. A two-sided P value of < 0.05 was considered statistically significant. A Bonferroni-adjusted P-value was used for multiple comparisons to control for experimental errors due to multiple testing. All analyses were performed using SPSS software version 11.0 (SPSS Inc, Chicago, IL, United States).

RESULTS

Histologic evaluation of pink-color sign positive areas

A total of 97 patients with superficial esophageal cancer were treated by endoscopic resection at the Osaka Medical Center for Cancer and Cardiovascular Diseases from May 31, 2011 to March 1, 2012. Of these, 10 patients were excluded because of previous radiation, 29 were excluded because endoscopic resection was performed without the attendance of the two endoscopists (Ishihara R or Kanzaki H) and 4 were excluded because no images were captured at 2-3 min after iodine staining. A total of 61 superficial esophageal neoplasms in 54 patients were included in the study. The lesions were located in the cervical esophagus in one case, the upper thoracic esophagus in 10 cases, the mid-thoracic esophagus in 33 cases, and the lower thoracic esophagus in 17 cases. The median diameter of the lesions was 20 mm (range: 2-74 mm) (Table 1). Of the 61 lesions, 28 were classified as pink-color sign positive and 33 as pink-color sign negative. The histologic diagnosis was HGIN or cancer invading into the lamina propria in 26 of the 28 pink-color sign positive lesions. Two lesions that were classified as pink-color sign positive were diagnosed as LGIN. One of these lesions had a thin keratinous layer and mild cellular atypia. This lesion showed an obscured pink-color sign, which was classified as positive in this study. In retrospect, it is possible that this lesion should have been classified as pink-color sign negative.

Table 1 Characteristics of patients and lesions

Characteristics	*n*
Gender	
Male	50
Female	4
Age (yr)	
Median (range)	67 (45-82)
Lesion location	
Cervical esophagus	1
Upper thoracic esophagus	10
Middle thoracic esophagus	33
Lower thoracic esophagus	17
Lesion size (mm)	
Median (range)	20 (2-74)
Histological diagnosis of the marked area	
LGIN	21
HGIN	37
LPM	3

LGIN: Low-grade intraepithelial neoplasia; HGIN: High-grade intraepithelial neoplasia; LPM: Cancer invading into the lamina propria.

Table 2 Associations between histologic diagnosis, keratinous layer and pink-color sign

	HGIN or invasive cancer	LGIN	*P* value
Pink-color-sign			0.0001
Positive	26	2	
Negative	14	19	
Keratinous layer			0.0007
Present	19	20	
Absent	21	1	

HGIN: High-grade intraepithelial neoplasia; LGIN: Low-grade intraepithelial neoplasia.

The other lesion did not have a keratinous layer and had moderate cellular atypia. However, this lesion showed surface differentiation and was diagnosed as LGIN because obvious cytological abnormalities were confined to the lower half of the squamous epithelium. There was a significant association between pink-color sign positive epithelium and HGIN or invasive cancer ($P = 0.0001$) (Table 2). There was also a significant association between the presence of a keratinous layer and HGIN or invasive cancer ($P = 0.0007$).

Univariate analyses (Table 3) found significant associations between the pink-color sign and absence of the keratinous layer or cellular atypia. After Bonferroni correction, there were no significant associations between the pink-color sign and presence of the basal membrane or vascular change. Multivariate analyses (Table 4) showed that absence of the keratinous layer was independently associated with the pink-color sign (OR = 58.8, 95%CI: 5.5-632). Hosmer-Lemeshow testing indicated that the model achieved a sufficient goodness-of-fit ($P = 0.678$).

Quantitative analysis of the pink-color sign

A total of 1373 patients underwent esophagogastroduodenoscopy at the Osaka Medical Center for Cancer and Cardiovascular Diseases from September 21, 2012

Table 3 Associations between pink-color sign and histologic findings (univariate analysis)

	Pink-color-sign positive	Pink-color-sign negative	*P* value
Keratinous layer			< 0.0001
Present	7	32	
Absent	21	1	
Cellular atypia[1]			0.004
Mild	1	13	
Moderate	20	19	
Severe	7	1	
Presence of basal membrane			0.018
Yes	14	27	
No	14	6	
Vascular change			0.070
Severe	20	15	
Mild	8	18	

[1]Cellular atypia was classified based on nuclear irregularity, mitotic figures, loss of polarity, chromatin pattern and nuclear/cytoplasmic ratio.

Table 4 Associations between pink-color sign and histologic findings (multivariate analysis)

	OR (95%CI)	*P* value
Keratinous layer		0.001
Present	1	
Absent	58.8 (5.5-632)	
Cellular atypia[1]		0.580
Mild	1	
Moderate	3.5 (0.3-35.5)	
Severe	3.4 (0.07-165)	
Presence of basal membrane		
Yes	1	0.610
No	1.6 (0.3-9.3)	
Vascular change		
Mild	1	0.770
Severe	1.3 (0.3-6.3)	

[1]Cellular atypia was classified based on nuclear irregularity, mitotic figures, loss of polarity, chromatin pattern and nuclear/cytoplasmic ratio.

to December 5, 2012. Of these, 29 were diagnosed with esophageal squamous cell carcinoma. Fourteen of these 29 patients were excluded for the following reasons: history of chemoradiotherapy (8 patients), the entire lesion was pink-color sign positive (4 patients), or the pink-color sign was negative (2 patients). Ten of the 15 patients that had lesions with both pink-color sign positive and pink-color sign negative areas were examined under the instruction of the endoscopist (Ishihara R) and were included in the analysis. Endoscopic images of the lesions immediately after iodine staining (early phase), 2-3 min after iodine staining (late phase) and after complete fading of iodine staining (final phase) were analyzed (Figure 4). The mean U' and V' values of the pink-color sign positive and negative areas are shown in Figure 5. Pink-color sign positive mucosa had a lower mean V' value in the late phase (pinkish color) than in the early phase (yellowish color), and had similar mean U' and V' values in the late and final phases (Figure 5A). These findings suggest that pink-color positive mucosa underwent color fading from

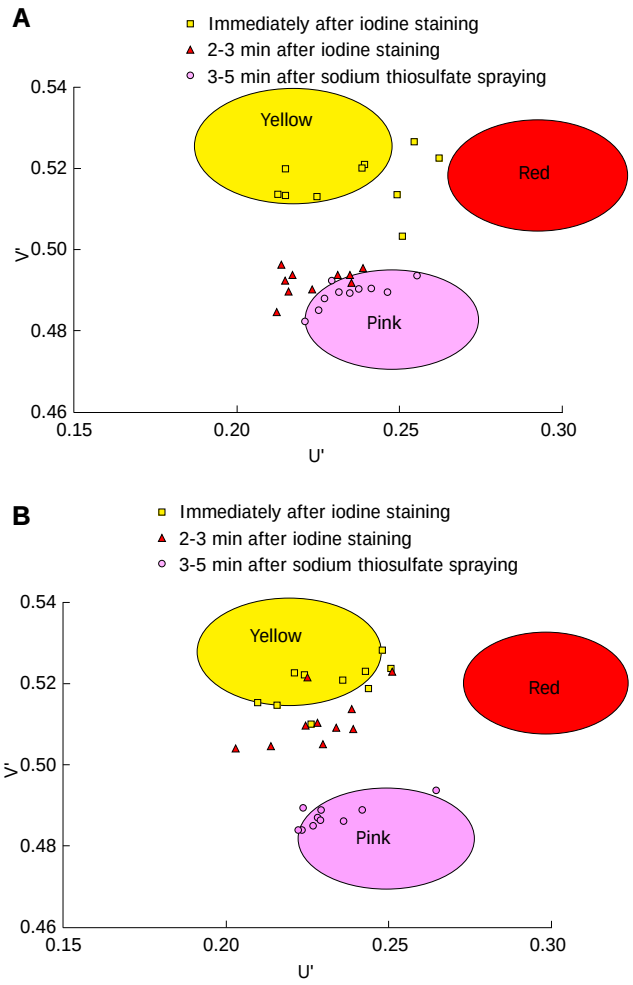

Figure 4 The U' and V' values of the pink-color sign positive mucosa and negative mucosa in the early, late and final phases were plotted on a color diagram. A: Pink-color sign positive mucosa; B: Pink-color sign negative mucosa.

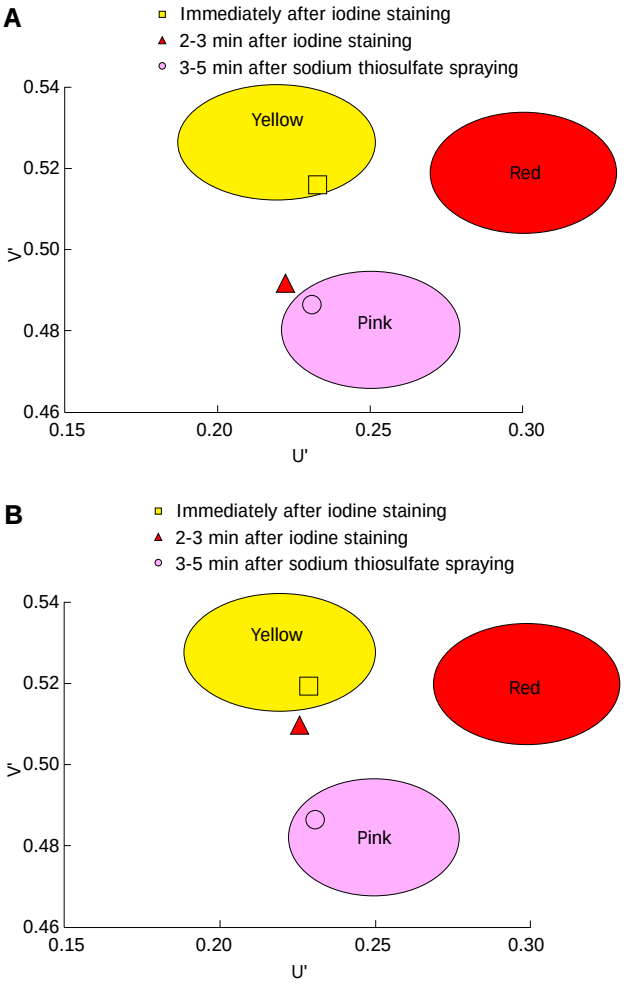

Figure 5 Mean U' and V' values of the mucosa in the early, late and final phases were plotted on a color diagram. A: Pink-color sign positive mucosa had a lower mean V' value in the late phase (pinkish color) than in the early phase (yellowish color), suggesting that the pink-color sign positive mucosa underwent a color change from yellow to pink. The mucosa had similar mean U' and V' values in the late and final phases, suggesting that the color of pink-color sign positive mucosa in the late stage was similar to the color of mucosa after complete fading of iodine staining; B: Pink-color sign negative mucosa had similar mean U' and V' values in the early and late phases (yellowish color), suggesting that pink-color sign negative mucosa did not change in color during this time period.

the color of the iodine (yellow) to the color of the mucosa (pink) within 2-3 min after iodine staining. Pink-color sign negative mucosa had similar mean U' and V' values in the late and early phases (yellowish color), and had a lower mean V' value in the final phase (pinkish color) than in the late phase (Figure 5B). These findings suggest that pink-color sign negative mucosa did not undergo color fading during the 2-3 min after iodine staining, and underwent color fading only after spraying of sodium thiosulfate.

DISCUSSION

Analysis of the endoscopic and histologic findings of this study found that absence of the keratinous layer was independently associated with the pink-color sign. Quantitative analysis of color changes found that pink-color sign positive mucosa changed from yellowish to pinkish within 2-3 min after iodine staining, suggesting that the mucosa underwent early color fading from the color of the iodine (yellow) to the color of the mucosa (pink).

This study did not include all patients who met our inclusion criteria, because we wanted to ensure that all procedures were of high quality to obtain accurate results. Marking before endoscopic resection and confirmation of marking after endoscopic resection were performed by one of two endoscopists (Ishihara R or Kanzaki H) to accurately identify the region of interest. All endoscopic images were captured under the instruction of one endoscopist (Ishihara R) to ensure that they were captured under similar conditions. This may have caused some selection bias, but it was felt necessary to limit the number of endoscopists involved for this detailed analysis.

Figure 6 shows our speculated mechanism for the occurrence of the pink-color sign. Locally administered iodine is usually absorbed into the epithelium by passive diffusion[23]. In normal esophageal epithelium, absorbed iodine combines with glycogen in the micro-granules of the prickle cells[24]. The resulting glycogen-iodine complex

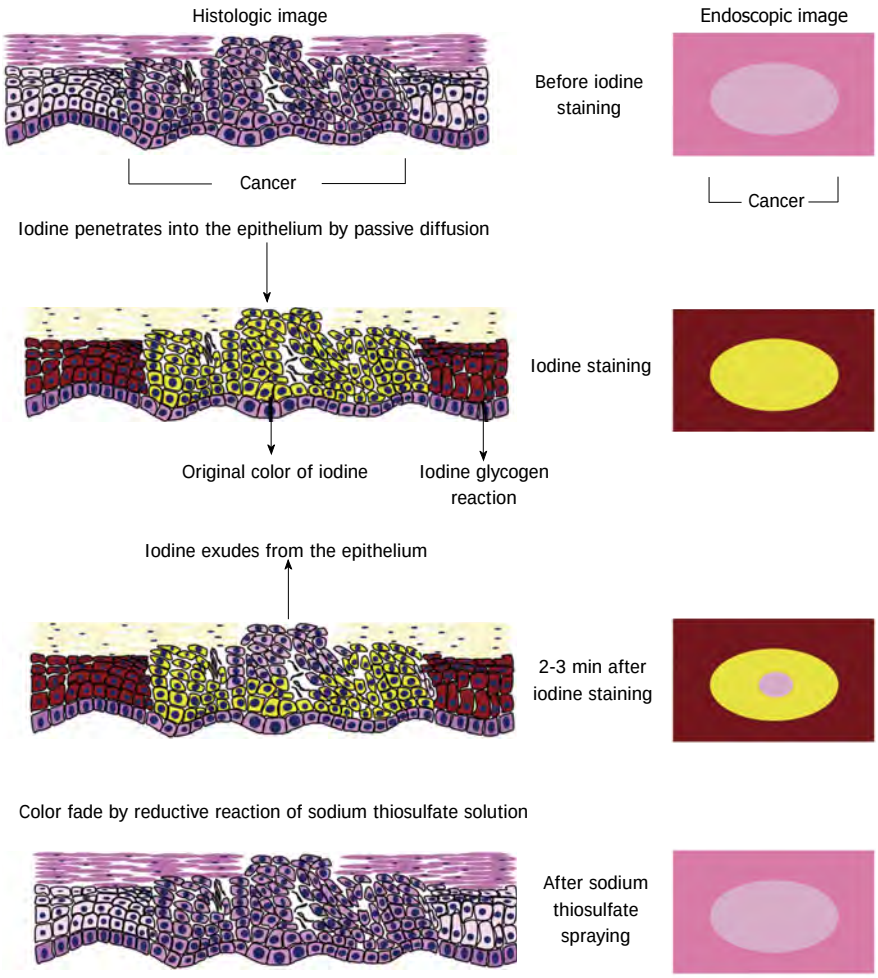

Figure 6 Speculated mechanism of the pink-color sign.

gives the epithelium a brown color. In the epithelium of neoplastic lesions, the prickle-cell layer is usually replaced by neoplastic cells, and no glycogen-iodine complex is formed. The yellow iodine solution therefore gives the epithelium a yellow color.

In the epithelium of neoplastic lesions, the yellow color may fade because of reduction of the iodine, absorption of iodine into the bloodstream, or leakage of iodine into the esophageal lumen. Application of sodium thiosulfate solution reduces the adverse effects of iodine staining and accelerates color fading by reduction of the iodine[25]. However, iodine is a strong oxidizing agent[26], and reduction of iodine requires a strong reducing agent. Absorption of iodine by the blood stream may occur in the esophagus[27]. However, iodine is mainly absorbed in the small intestine[28] and the amount of iodine absorbed by the esophagus is not large. Leakage of iodine into the esophageal lumen may therefore be the main cause of color fading, rather than reduction of iodine or absorption of iodine into the bloodstream.

Epithelium plays an important role in regulating the permeability of mucosa, and serves as a barrier between the outside world and the internal milieu of the organism[29,30]. The barrier function of epithelium is determined by its microstructure, and varies among different types of epithelium. The keratin layer of squamous epithelium has a barrier function[29,30], and may play an important role in preventing leakage of iodine from the epithelium. Disruption of the normal epithelial structure, especially of the keratin layer, may increase early leakage of iodine and early color fading. Considering the association between the absence of the keratinous layer and the pink-color sign, this may be the mechanism underlying the occurrence of the sign.

Neoplastic lesions show less staining when exposed to iodine solution than normal mucosa. However, both cancer and LGIN result in iodine-unstained areas. Assessment of iodine staining without assessment of the pink-color sign is therefore not very accurate for the diagnosis of cancer[10,14]. The keratinous layer was found to be absent in areas where most of the epithelium was replaced by neoplastic cells. Most areas that were pink-color sign positive had a proliferation of neoplastic cells in the upper half of the epithelium. The pink-color sign was associated with HGIN or cancer, those are both characterized by abnormal cells in the upper half of the epithelium. Accurate endoscopic diagnosis of esophageal lesions is therefore possible by assessment of the pink-color sign after iodine staining.

Differentiating HGIN or cancer from LGIN is im-

portant when deciding on a treatment strategy, because resection of the lesion is required for HGIN and cancer[19]. Histologic diagnosis of biopsy specimens may result in misdiagnosis if the correct area was not biopsied. Areas that are pink-color sign positive should always be biopsied, because this finding is closely associated with HGIN and cancer. Moreover, the keratinous layer is usually absent in areas that are pink-color sign positive, and it may therefore be relatively easy to biopsy neoplastic cells from these areas. However, if further studies confirm that the accuracy of endoscopic diagnosis is similar to that of biopsy diagnosis, cancer could eventually be diagnosed based on the pink-color sign without a need for biopsy.

In conclusion, the pink-color sign was closely associated with absence of the keratinous layer. The pink-color sign may be caused by early leakage of iodine into the esophageal lumen because of impaired barrier function of the epithelium.

COMMENTS

Background

A dramatic color change after iodine staining, from the initial yellow color to a pink color 2-3 min later, is known as the pink-color sign, and is useful for identifying cancerous lesions of the esophagus. This sign has been reported to dramatically improve specificity for esophageal squamous high-grade intraepithelial neoplasia and invasive cancer.

Research frontiers

The mechanism underlying the occurrence of the pink-color sign has not been fully investigated. Improved understanding of this mechanism may improve our understanding of the characteristics of the relevant lesions, and increase the likelihood of accurate diagnosis.

Innovations and breakthroughs

This study clarified the histologic changes responsible for the occurrence of the pink-color sign in esophageal squamous neoplasia as follows. Analysis of the endoscopic and histologic findings of this study found that absence of the keratinous layer was independently associated with the pink-color sign. Quantitative analysis of color changes found that pink-color sign positive mucosa changed from yellowish to pinkish within 2-3 min after iodine staining, suggesting that the mucosa underwent early color fading from the color of the iodine (yellow) to the color of the mucosa (pink). The pink-color sign may be caused by early leakage of iodine into the esophageal lumen because of impaired barrier function of the epithelium.

Applications

The pink-color sign is a good indicator for choosing adequate biopsy sites, because of its high specificity. Improved understanding of this mechanism may improve their understanding of the characteristics of the relevant lesions, and increase the likelihood of accurate diagnosis.

Terminology

Pink-color sign: A dramatic color change after iodine staining, from the initial yellow color to a pink color 2-3 min later. Keratinous layer: The outer layer of the squamous epithelium, which contain a tough, fibrous protein. This layer acts as a protective barrier against outside elements.

Peer review

This study investigated the detailed histologic findings of esophageal neoplasms, and found that absence of the keratinous layer because of neoplastic cell proliferation may be responsible for the pink-color sign. Quantitative analysis of color changes showed that pink-color sign positive mucosa changed from yellowish to pinkish within 2-3 min after iodine staining, suggesting that the mucosa underwent early color fading from the color of the iodine (yellow) to the color of the mucosa (pink). Based on these results, the authors speculated on the mechanism underlying the pink-color sign. These findings may improve understanding of the characteristics of these lesions, and increase the accuracy of diagnosis of esophageal neoplasms.

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BRIEF ARTICLE

Krüppel-like factor 8 overexpression is correlated with angiogenesis and poor prognosis in gastric cancer

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Abstract

AIM: To investigate Krüppel-like factor 8 (KLF8) expression in gastric cancer and its relationship with angiogenesis and prognosis of gastric cancer.

METHODS: One hundred and fifty-four patients with gastric cancer who underwent successful curative resection were retrospectively enrolled in the study. Fifty tumor-adjacent healthy gastric tissues (≥ 5 cm from the tumor margin) obtained during the original resection were randomly selected for comparative analysis. *In situ* expression of KLF8 and CD34 proteins were examined by immunohistochemistry. The intratumoral microvessel density (MVD) was determined by manually counting the immunostained CD34-positive endothelial cells in three consecutive high-magnification fields (× 200). The relationship between differential KLF8 expression and MVD was assessed using Spearman's correlation coefficient test. χ^2 test was performed to evaluate the effects of differential KLF8 expression on clinicopathologic factors. Kaplan-Meier and multivariate Cox survival analyses were used to assess the prognostic value of differential KLF8 expression in gastric cancer.

RESULTS: Significantly higher levels of KLF8 protein were detected in gastric cancer tissues than in the adjacent non-cancerous tissues (54.5% *vs* 34.0%, $P < 0.05$). KLF8 expression was associated with tumor size ($P < 0.001$), local invasion ($P = 0.005$), regional lymph node metastasis ($P = 0.029$), distant metastasis ($P = 0.023$), and tumor node metastasis (TNM) stage ($P = 0.002$), as well as the MVD ($r = 0.392$, $P < 0.001$). Patients with KLF8 positive expression had poorer overall survival ($P < 0.001$) and cancer-specific survival ($P < 0.001$) than those with negative expression. Multivariate analysis demonstrated that KLF8 expression independently affected both overall and cancer-specific survival of gastric cancer patients ($P = 0.035$ and 0.042, respectively).

CONCLUSION: KLF8 is closely associated with gastric tumor progression, angiogenesis and poor prognosis, suggesting it may represent a novel prognostic biomarker and therapeutic target for gastric cancer.

Key words: Gastric cancer; Krüppel-like factor 8; Angiogenesis; Prognosis

Core tip: In this study, we found that the expression of Krüppel-like factor 8 (KLF8) was up-regulated in resected gastric cancer tissues. The differential KLF8

expression was significantly associated with enhanced malignant potential, including tumor size, local invasion, regional lymph node metastasis, distant metastasis, and tumor node metastasis stage, as well as the intratumoral microvessel density. Multivariate analysis indicated that KLF8 expression independently affected both overall and cancer-specific survival of gastric cancer patients. Collectively, our findings suggest that KLF8 may be a predictive marker of clinical outcome and represent a novel target for anti-angiogenic therapy of gastric cancer patients.

Wang WF, Li J, Du LT, Wang LL, Yang YM, Liu YM, Liu H, Zhang X, Dong ZG, Zheng GX, Wang CX. Krüppel-like factor 8 overexpression is correlated with angiogenesis and poor prognosis in gastric cancer. *World J Gastroenterol* 2013; 19(27): 4309-4315 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i27/4309.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i27.4309

INTRODUCTION

Gastric cancer is one of the most common malignant tumors worldwide, accounting for the second highest rate of cancer-related death[1]. Despite the decline in gastric cancer mortality has occurred over the past few decades, this disease remains a significant burden in China, with approximately 400000 newly-diagnosed cases being reported each year[2]. Patients with gastric cancer are often asymptomatic or display nonspecific symptoms in the early stages, and by the time symptoms appear the disease has generally reached an advanced stage, when treatment options are limited and the prognosis for long-term survival is poor. Therefore, there remains a critical need for discovery of novel biomarkers that will reflect not only gastric cancer onset but also its progression and risk of mortality; such diagnostic and prognostic indicators may also represent novel therapeutic targets for treating gastric cancer patients at various stages.

Krüppel-like factor 8 (KLF8) was initially identified as a ubiquitously expressed transcriptional repressor[3]. Like other members of the KLF transcription factor family, KLF8 harbors three conserved C2H2 zinc finger DNA-binding domains in its C-terminus, but also has a unique sequence in its N-terminus that is believed to mediate its functional specificity through interactions with other protein binding partners[4,5]. KLF8 activity has been implicated as crucial to a wide range of cellular processes, such as cell cycle progression[6-9], oncogenic transformation[10], epithelial-to-mesenchymal transition, migration and invasion[11,12]. Moreover, overexpression of KLF8 has been found in several human malignant tumors, including those of ovarian, renal and breast origin[13-15]. *In vitro* studies using gastric cancer cell lines have revealed that lentivirus-mediated knockdown of KLF8 inhibits cell growth and invasion[16,17]. Yet, the clinical significance of KLF8 overexpression in relation to clinicopathologic features of gastric cancer and patient prognosis remains unknown.

Angiogenesis, the formation of new capillary blood vessels, is essential for the growth, progression and metastasis of malignant tumors[18,19]. The microvessel density (MVD) is now widely used to evaluate the degree of tumor angiogenesis and has been proven to be associated with the metastasis and prognosis of a wide range of human tumors[20-22]. One previous study has shown that transfection with KLF8-RNAi lentivirus significantly decreases tumor vessels in a mouse model of liver cancer[11], suggesting that KLF8 may play potentially interesting roles in tumor angiogenesis. However, the angiogenic property of KLF8 protein in primary gastric cancer remains unclear.

In the present study, we detected the expression status of KLF8 by immunohistochemistry in surgically resected gastric cancer, and further explored its potential correlations with clinicopathologic parameters, tumor angiogenesis and prognosis of the patients with gastric cancer.

MATERIALS AND METHODS

Patients and follow-up

Between July 2004 and October 2006, a total of 161 gastric cancer patients underwent curative resection in the Department of General Surgery at Qilu Hospital of Shandong University (Jinan, China). Of these 161 patients, seven cases were lost to follow-up, so a total of 154 patients were eventually enrolled in this study. Retrospective review of the patients' medical records and direct communication with the patients provided complete diagnosis, treatment and follow-up data for all cases. The resected tumor specimens had been immediately formalin-fixed and paraffin-embedded. Each paraffin block was cut into serial sections at 4 μm intervals for future analysis. In addition, we randomly selected 50 normal gastric tissues (at least 5 cm from the margin of the tumor) as normal controls. The postoperative pathological TNM staging was evaluated based on the 2009 criteria of the International Union Against Cancer (UICC). This study was approved by the Ethics Committee of Qilu Hospital, and written informed consent was obtained from each patient.

None of the patients had received preoperative adjuvant therapy. All patients were followed-up at the outpatient clinic at 3-6 mo intervals, with the follow-up durations ranging from 5-77 mo (median, 45 mo). The follow-up examinations included laboratory testing, physical examination, ultrasound scan, and, if necessary, fibergastroscopy.

Immunohistochemical staining for KLF8 and CD34

Paraffin-embedded tissue sections were baked at 60 ℃, dewaxed by soaking in xylene, and rehydrated by passing through an alcohol gradient series. Antigen retrieval was carried out by boiling samples (*via* microwave) in EDTA

Table 1 Krüppel-like factor 8 expression and clinicopathologic characteristics in gastric cancer

Variables	Cases (*n*)	KLF8 expression		*P* value
		Negative	Positive	
Sex				0.856
Male	122	55	67	
Female	32	15	17	
Age (yr)				0.136
≤ 60	89	45	44	
> 60	65	25	40	
Tumor (cm)				< 0.001
≤ 5	92	53	39	
> 5	62	17	45	
Tumor differentiation				0.608
Well	14	8	6	
Moderate	43	18	25	
Poor	97	44	53	
Local invasion				0.005
T1-T2	31	21	10	
T3-T4	123	49	74	
Regional lymph node metastasis				0.029
N0	45	26	19	
N1	37	20	17	
N2	33	9	24	
N3	39	15	24	
Distant metastasis				0.023
No	141	68	73	
Yes	13	2	11	
TNM stage				0.002
I	20	14	6	
II	54	30	24	
III	67	24	43	
IV	13	2	11	

TNM: Tumor node metastasis; KLF8: Krüppel-like factor 8.

(pH 9.0). After cooling to room temperature, the sections were immersed in 3% hydrogen peroxide for 15 min to inhibit endogenous peroxidase activity, followed by blocking in 10% goat serum to reduce nonspecific binding. An overnight incubation at 4 ℃ was then carried out with anti-KLF8 or anti-CD34 primary antibodies (1:100 in phosphate-buffered saline (PBS); both from Santa Cruz Biotechnology, Santa Cruz, CA), which was followed by incubation with the corresponding horseradish peroxidase-conjugated secondary antibody (Zhongshan Golden Bridge Biotechnology, Beijing, China). The peroxidase reactivity was visualized using 3,3'-diaminobenzidine as substrate. Finally, the sections were counterstained with hematoxylin, dehydrated, and mounted with neutral balsam. Negative controls were generated by the same procedure, except with the primary antibody being replaced by PBS alone.

Evaluation of KLF8 expression and MVD

Microscopic evaluation of the immunostained sections was carried out by two pathologists working independently, who were not associated with this study and who were blinded to the patients' clinicopathologic factors and outcomes. In cases of discrepancy, the slides were re-assessed jointly by both pathologists using a multi-head microscope to establish a consensus result.

KLF8 protein expression was semi-quantitatively assessed by scoring both the immunoreactive staining intensity (0: no staining; 1: weak staining; 2: moderate staining; 3, strong staining) and the proportion of positively-stained cells (0: 0%-9%; 1: 10%-25%; 2: 26%-50%; 3: 51%-75%; 4: > 75%). The overall staining score was then calculated as (percentage score × intensity score), with final scores of 0 indicating negative expression, of 1-5 indicating weakly positive expression, and of ≥ 6 indicating strongly positive expression[11].

MVD was assessed by immunohistochemical staining of CD34, according to the international consensus on the methodology and criteria of evaluation of angiogenesis quantification in solid human tumors[23]. The entire sections were scanned at low magnification (× 100) initially to select regions with the highest vascularity ("hot spots") for focused investigation using high magnification (× 200) to manually count the stained microvessels present in three consecutive fields. A single, countable microvessel was defined as any brown-stained endothelial cell (or cluster) clearly separated from the adjacent microvessels. The average microvessel counts in the three fields under high magnification (× 200) were recorded as the final value of MVD.

Statistical analysis

All statistical analyses were performed using the SPSS software suite, version 17.0 (SPSS, Chicago, IL, United States). χ^2 test was used to assess the correlations between KLF8 protein expression and clinicopathologic factors. The relationship between KLF8 protein expression and MVD was determined by Spearman's correlation coefficient test. Survival curves were calculated by the Kaplan-Meier method, and the significance of intergroup differences in survival was determined by log-rank test. Multivariate survival analysis based on the Cox proportional hazard model was carried out to identify the significant independent prognostic factors. A P value < 0.05 was considered statistically significant.

RESULTS

Correlation of KLF8 expression with clinicopathologic factors

Immunohistochemical analyses of the 154 gastric cancer tissues and 50 adjacent non-cancerous tissues showed KLF8 protein staining of various intensities in both the nuclear and cytoplasmic compartments. Overall, 54.5% (84/154) of the gastric cancer tissues showed KLF8-positivity, including 25.3% which were strongly positive and 29.2% which were weakly positive, and only 34.0% (17/50) of the adjacent non-cancerous tissues showed KLF8-positivity. The difference in KLF8 staining between the gastric cancer and adjacent normal tissues was statistically significant ($P < 0.05$). Figure 1 shows representative cases with different expression levels of KLF8 protein. Moreover, as shown in Table 1, KLF8-positivity was significantly related to tumor size, local invasion,

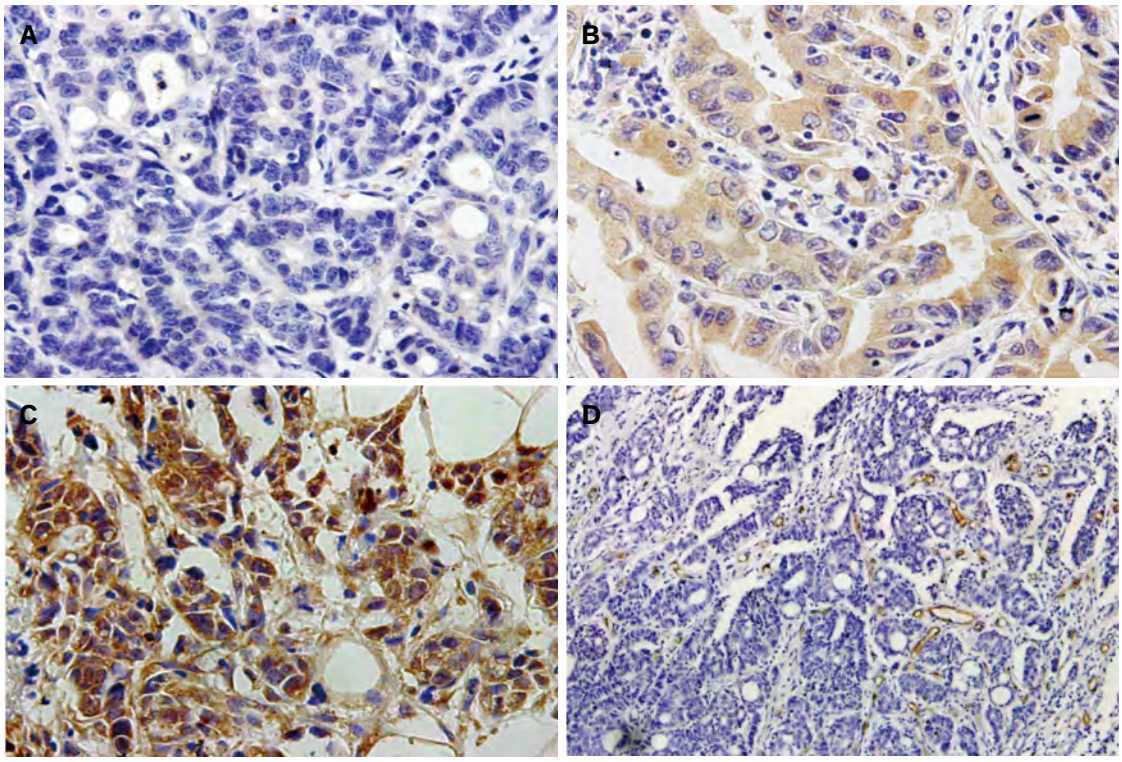

Figure 1 Immunohistochemical staining of Krüppel-like factor 8 and CD34 in gastric cancer specimens. A: Negative expression of Krüppel-like factor 8 (KLF8) in cancer cells; B: Weakly positive expression of KLF8 in cancer cells; C: Strongly positive expression of KLF8 in cancer cells; Magnification: × 400; D: Intratumoral microvessels detected by anti-CD34 antibody (brown) in gastric cancer tissues; Magnification: × 200.

regional lymph node metastasis, distant metastasis, and TNM stage (all $P < 0.05$); however, no significant associations were detected for KLF8 expression with patient sex, age, or degree of tumor differentiation (all $P > 0.05$).

Correlation of KLF8 expression with tumor angiogenesis

As shown in Figure 1D, the intratumoral MVD was determined by CD34 immunohistochemical staining. The MVD values of the 154 cancer specimens ranged from 14 to 62 (mean, 32.0 ± 8.8). When this mean value was applied as a cut-off point, 86 cases (55.8%) were categorized as low MVD (< 32) and 68 cases (44.2%) were categorized as high MVD (≥ 32). KLF8 expression was found to be significantly positively correlated with MVD ($r = 0.392$, $P < 0.001$; Table 2).

Prognostic significance of KLF8 expression in gastric cancer

Of the 154 patients, 101 (65.6%) cases died during the follow-up period. Among them, the majority ($n = 97$) died from cancer-related causes, and four cases died from other causes. The 5-year overall and cancer-specific survival rates were 34.4% and 35.3%, respectively.

Kaplan-Meier analysis indicated that KLF8 expression was significantly associated to the survival of gastric cancer patients. The overall 5-year survival rate of patients with KLF8-positive expression was significantly shorter than those with KLF8-negative expression ($P < 0.001$; Figure 2A). Consistent with this trend, the patients with KLF8-positive expression also had a poorer 5-year cancer-specific survival ($P < 0.001$; Figure 2B). In addition, we compared the prognostic value of KLF8 strongly positive expression to that of weakly positive expression, and found that there was no significant difference between the two groups for either overall survival ($P = 0.116$) or cancer-specific survival ($P = 0.224$). Multivariate analysis using Cox regression indicated that KLF8-positive expression in gastric cancer was a significant independent prognostic factor for both overall survival (95%CI: 1.035-2.539, $P = 0.035$) and cancer-specific survival (95%CI: 1.017-2.554, $P = 0.042$) (Table 3).

DISCUSSION

Surgical resection is the most effective treatment for gastric cancer patients with curative potential, yet the clinical outcomes of these patients are generally poor, largely due to a high ratio of postoperative metastasis or recurrence[24]. In China, the overall 5-year survival rate of gastric cancer patients is only about 40%[25]. Identification of novel biomarkers that can be used as prognostic predictors or therapeutic targets will likely benefit these patients and help to ease the burden of this disease. Aberrant expression of KLF8 has been detected in several types of human malignant tumors and closely associated with oncogenic transformation and tumor progression[10-15]. To the best of our knowledge, however, the study described herein is the first to evaluate the clinicopathologic and prognostic significance of KLF8 in gastric cancer patients.

Our findings confirmed the remarkable up-regulation

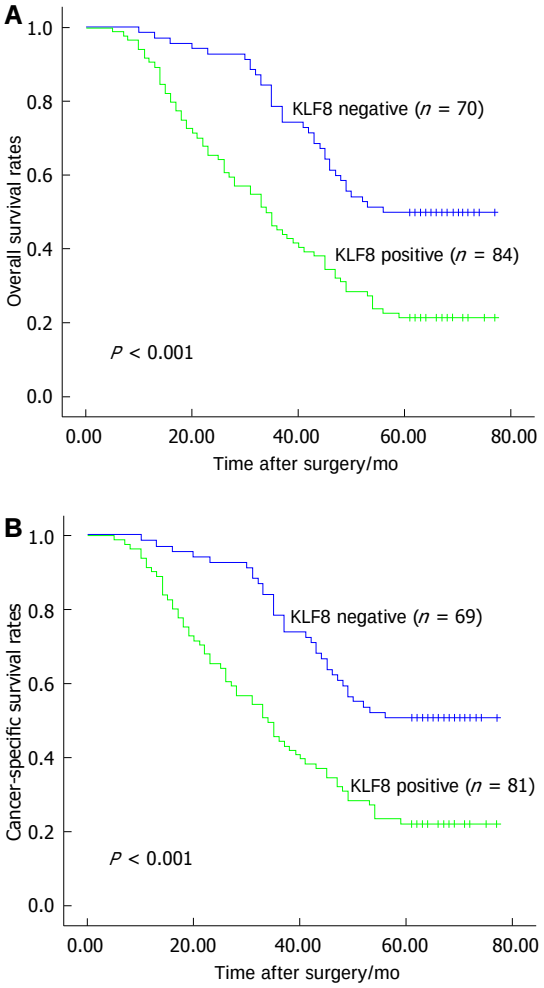

Figure 2 Kaplan-Meier survival curves of overall and cancer-specific survival in gastric cancer patients based on Krüppel-like factor 8 expression. Patients with Krüppel-like factor 8 (KLF8)-positive (weakly and strongly) expression had poorer overall (A) and cancer-specific (B) survival as compared to those with KLF8-negative expression.

of KLF8 in gastric cancer tissues and indicated significant associations for KLF8 expression with prognosis-related features, including tumor size, local invasion, regional lymph node metastasis, distant metastasis, and TNM stage. Thus, it is likely that KLF8 plays important roles in gastric cancer progression. Consistent with our results, previous study of renal cell carcinoma has shown that overexpression of KLF8 is strongly associated with larger tumor size and higher clinical stage[14]. However, future studies are still needed to investigate the detailed mechanism of KLF8-mediated gastric cancer progression.

Predicting the clinical outcomes of patients with malignant tumors may provide valuable information for better treatment stratification and personalized therapeutic regimens. Previous study demonstrated that KLF8 overexpression was significantly related with early tumor recurrence and poor prognosis in hepatocellular carcinoma[11]. However, no clinical data has indicated the potential benefit of KLF8 as a biomarker for predicting prognosis of gastric cancer patients. In the current study, multivariate survival analysis based on the Cox proportional hazard model revealed that, among all the factors analyzed, KLF8 expression was a significant independent prognostic factor for both overall and cancer-specific survival of gastric cancer patients following curative resection. These findings clearly demonstrate that aberrant expression of KLF8 may be closely associated with gastric cancer progression in our patient cohort, and suggest its potential as a prognostic biomarker of gastric cancer outcome.

Table 2 Correlations between Krüppel-like factor 8 expression and the microvessel density in gastric cancer

MVD	KLF8 expression		*P* value	*r*
	Negative	Positive		
Low	54	32	< 0.001	0.392
High	16	52		

KLF8: Krüppel-like factor 8; MVD: Microvessel density.

It is well known that tumors are endowed with angiogenic capability, and their growth, invasion, and metastasis are angiogenesis-dependent[26]. The survival rate of patients with highly-vascularized gastric cancer is significantly lower than those with less-vascularized gastric cancer[27]. The findings from the current study indicated a close correlation between KLF8-positivity in tumor specimens and the extent of intratumoral MVD (as determined by CD34 staining), suggesting that KLF8 may facilitate tumor progression *via* induction of angiogenesis. Therefore, KLF8 may represent a potential novel target of anti-angiogenic therapy for gastric cancer patients. It is important to note, however, that tumor angiogenesis is a complex process involving dynamic interplay of various factors[28], and further studies are needed to elucidate the detailed molecular mechanisms underlying KLF8-mediated tumor angiogenesis before the precise molecular targets of KLF8 are identified and used to develop an optimal treatment for gastric cancer patients.

When interpreting the results presented herein, several inherent limitations to the study design must be considered. First, only gastric cancer patients who underwent curative resection were enrolled in the present study, which restricted the total study population to a relatively low number. Second, the retrospective design provided only previously resected human gastric cancer tissues that were not originally collected with the intent of detecting the prognostic value of KLF8. To address this limitation, multicenter, randomized studies should be conducted to confirm whether KLF8 can be used as an accurate prognostic maker for gastric cancer. In addition, the impact of postoperative adjuvant therapy on prognosis was not evaluated in this study, due to the fact that some gastric cancer patients received different therapeutic regimens (*i.e.*, agents, doses, and cycles of treatment), and not all patients completed the adjuvant therapy because of severe side effects or economic-related restrictions.

In conclusion, this study confirmed the up-regulated expression of KLF8 in gastric cancer tissues and, for

Table 3 Multivariate analysis of prognostic factors in patients with gastric cancer

Variables	Multivariate analysis					
	Overall survival			Cancer-specific survival		
	RR	95%CI	*P* value	RR	95%CI	*P* value
Sex	1.132	0.678-1.890	0.636	1.071	0.622-1.844	0.803
Age	0.785	0.521-1.182	0.247	0.775	0.509-1.180	0.235
Tumor size	1.219	0.794-1.872	0.365	1.220	0.790-1.886	0.370
Tumor differentiation	0.841	0.591-1.196	0.334	0.830	0.582-1.184	0.304
Local invasion	1.194	0.799-1.785	0.387	1.197	0.791-1.811	0.395
Regional lymph node metastasis	1.025	0.782-1.343	0.858	1.029	0.783-1.352	0.839
Distant metastasis	6.384	1.943-20.975	0.002	7.091	2.104-23.901	0.002
TNM stage	2.401	1.182-4.878	0.015	2.323	1.128-4.783	0.022
KLF8 expression	1.621	1.035-2.539	0.035	1.612	1.017-2.554	0.042
MVD	2.801	1.790-4.381	0.000	2.782	1.751-4.419	0.000

KLF8: Krüppel-like factor 8; MVD: Microvessel density; TNM: Tumor node metastasis.

the first time, demonstrated its clinicopathologic and prognostic significance. Specifically, positive expression of KLF8 was shown to be significantly correlated with enhanced malignant potential, tumor angiogenesis, and poor prognosis. Furthermore, Cox regression model analysis showed that KLF8-positivity in resected gastric tumors was a meaningful independent prognostic factor for both overall and cancer-specific survival. Collectively, these findings demonstrate that KLF8 may have clinical potential not only as a prognostic marker, by which individuals with risk of poor clinical outcome may be identified, but also as a novel target for anti-angiogenic therapy of gastric cancer patients.

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COMMENTS

Background

Gastric cancer remains one of the most common malignancies worldwide, and accounts for the second highest rate of cancer-related death. Despite recent advances in comprehensive therapeutic strategies, the clinical outcome of gastric cancer patients remains poor, mainly due to delayed clinical presentation and a high ratio of postoperative metastasis or recurrence. Therefore, it is necessary to identify novel biomarkers that can be used as prognostic indicators or therapeutic targets for gastric cancer patients.

Research frontiers

Aberrant expression of Krüppel-like factor 8 (KLF8) has been detected in several types of human malignant tumors, including those of ovarian, renal and breast origin. Considerable evidence has indicated that KLF8 expression is closely associated with oncogenic transformation and tumor progression. One previous study showed that lentivirus-mediated interference of KLF8 expression significantly decreased tumor vessels in a mouse model of liver cancer. However, the angiogenic property and prognostic significance of KLF8 in gastric cancer has not been fully elucidated.

Innovations and breakthroughs

In this study, KLF8 expression and microvessel density (MVD) were assessed by immunohistochemical analysis of resected tumors from patients with gastric cancer. Results indicated that the level of KLF8 was significantly higher in gastric cancer tissues than in adjacent non-cancerous tissues. Moreover, the high KLF8 expression was found to be closely associated with prognosis-related features, including tumor size, local invasion, regional lymph node metastasis, distant metastasis, and TNM stage. KLF8-positive expression in tumor specimens was significantly positively correlated with MVD. Patients with KLF8-positive expression had poorer overall and cancer-specific survival than those with KLF8-negative expression. Multivariate analysis demonstrated that the KLF8 expression in tumors independently affected both overall and cancer-specific survival of gastric cancer patients.

Applications

Collectively, the findings from this study demonstrate that KLF8 may have clinical potential not only as a prognostic biomarker, by which individuals with risk of poor clinical outcome may be identified, but also as a novel target for anti-angiogenic therapy of gastric cancer patients.

Terminology

KLF8, which is located on Xp11.21, was initially identified as a ubiquitously expressed transcriptional repressor belonging to the KLF family of transcription factors. Like other members of the KLF family, KLF8 shares three conserved C2H2 zinc finger DNA-binding domains in its C-terminus, but harbors a unique sequence in the N-terminus that is thought to mediate its functional specificity through interactions with other proteins.

Peer review

The authors demonstrated that KLF8 was significantly up-regulated in clinical specimens of gastric cancer, and evaluated its angiogenic property and prognostic significance. The results suggest that KLF8 may represent a novel prognostic biomarker and therapeutic target for gastric cancer.

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BRIEF ARTICLE

Endoscopic papillectomy: Data of a prospective observational study

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Abstract

AIM: To investigate the clinical value of endoscopic papillectomy indicated by feasibility and safety of the procedure in various diseases of the papilla in a representative number of patients in a setting of daily clinical and endoscopic practice and care by means of a systematic prospective observational study.

METHODS: Through a defined time period, all consecutive patients with tumor-like lesions of the papilla, who were considered for papillectomy, were enrolled in this systematic bicenter prospective observational study, and subdivided into 4 groups according to endoscopic and endoscopic ultrasonography (EUS) findings as well as histopathological diagnosis: adenoma; carcinoma/neuroendocrine tumor (NET)/lymphoma; papilla into which catheter can not be introduced; adenomyomatosis, respectively. Treatment results and outcome were characterized by R0 resection, complication, recurrence rates and tumor-free survival.

RESULTS: Over a 7-year period, 58 patients underwent endoscopic papillectomy. Main symptoms prompting to diagnostic measures were unclear abdominal pain in 50% and cholestasis with and without pain in 44%. Overall, 54/58 patients [inclusion rate, 93.1%; sex ratio, males/females = 25/29 (1:1.16); mean age, 65 (range, 22-88) years] were enrolled in the study. Prior to papillectomy, EUS was performed in 79.6% (*n* = 43/54). Group 1 (adenoma, *n* = 24/54; 44.4%): 91.6% (*n* = 22/24) with R0 resection; tumor-free survival after a mean of 18.5 mo, 86.4% (*n* = 19/22); recurrence, 13.6% (*n* = 3/22); minor complications, 12.5% (*n* = 3/24). Group 2 (carcinoma/NET/lymphoma, *n* = 18/54; 33.3%): 75.0% (*n* = 10/18) with R0 resection; tumor-free survival after a mean of 18.5 (range, 1-84) mo, 88.9% (*n* = 8/9); recurrence, 11.1% (*n* = 1/9). Group 3 (adenomyomatosis, *n* = 4/54; 7.4%). Group 4 (primarily no introducible catheter into the papilla, *n* = 8; 14.8%). The overall complication rate was 18.5% (*n* = 10/54; 1 subject with 2 complications): Bleeding, *n* = 3; pancreatitis, *n* = 7; perforation, *n* = 1 (intervention-related mortality, 0%). In summary, EUS is a sufficient diagnostic tool to preoperatively clarify diseases of the papilla including suspicious tumor stage in conjunction with postinterventional histopathological investigation of a specimen. Endoscopic papillectomy with curative intention is a feasible and safe approach to treat adenomas of the papilla. In high-risk patients with carcinoma of the papilla with no hints of deep infiltrating tumor growth, endoscopic papillectomy can be considered a reasonable treatment option with low risk and an approximately 80% probability of no recurrence if an R0 resection can be achieved. In patients with jaundice and in case the catheter can not be introduced into the papilla, papillectomy may help to get access to the bile duct.

CONCLUSION: Endoscopic papillectomy is a challenging interventional approach but a suitable patient- and local finding-adapted diagnostic and therapeutic tool with adequate risk-benefit ratio in experienced hands.

Key words: Papilla of Vater; Papillectomy; Endoscopic ultrasonography; Adenoma; Carcinoma; Carcinoid-like tumor; Adenomyomatosis

Core tip: Taken together, endoscopic ultrasonography is an essential and sufficient diagnostic tool and plays an eminent role in the diagnostic spectrum to preoperatively clarify lesions and diseases of the papilla in conjunction with the competent postinterventional histopathological investigation of a specimen. Endoscopic papillectomy with curative intention is a feasible and safe approach to treat adenomas of the papilla, *i.e.*, it is only reasonable if there is no infiltrating tumor growth. In high-risk patients with carcinoma of the papilla but no hints of deep infiltrating tumor growth, endoscopic papillectomy can be considered a reasonable treatment option with reduced risk and an approximately 80% probability of no recurrence if an R0 resection can be achieved. In patients with jaundice and in case the catheter can not be introduced into the papilla, papillectomy may help to get access to the bile duct to avoid more traumatic surgery. Endoscopic papillectomy is therefore not only used for therapeutic but also for diagnostic purpose. There is a high clinical value of endoscopic papillectomy for well defined indications not only for adenoma but also for carcinoma/neuroendocrine tumor/lymphoma (uT1 and high-risk patient), and adenomyomatosis. Follow-up investigations according to a defined schedule appear to be reasonable including macroscopic assessment, taking a representative biopsy and subsequent histopathological investigation. In addition, continuous systematic investigation of endoscopic papillectomy in daily clinical practice is indicated for the purpose of quality assurance.

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INTRODUCTION

The adequate management of diseases of the papilla of Vater (papilla) is challenging. There are several morphological changes and lesions of the papilla such as functional dysfunction, inflammation, stenosis or malignant tumor growth. For instance, a stenosis of the papilla is subdivided based on an autopsy registry as follows: Benign lesions, 42.7% (frequency); adenoma (premalignant lesion), 19.6%; carcinoma, 37.7%[1].

The incidence of malignant tumor lesions of the papilla has been reported to be 0.5/100000. Based on the concept of an anticipated adenoma-carcinoma sequence even at the papilla[2,3], adenoma is considered a premalignant tumor lesion[4-7], *e.g.*, adenomatous portions can be found in 35% to 91% of the histologically detected carcinomas. In this context, the incidence of an occurring carcinoma in papillary adenoma is 1 over 15.5 patient years.

Diagnostic measures of pathological changes at the papilla are a complex challenge since a differential and stage-adapted treatment depends on the early set-up of the correct diagnosis. Combination of clinical exam, laboratory parameters and abdominal ultrasound provides a sensitivity of up to 100% to diagnose cholestasis. However, the accuracy in characterizing the cause of cholestasis is considerably lower.

Endoscopic ultrasonography (EUS) may solve the dilemma since it allows to clarify etiopathogenesis and actual diagnosis in the vast majority of cases[1]. In addition, this diagnostic measure provides a sensitivity of up to 100% in detecting tumor-like lesions at the papilla or within the peripapillary region and, in addition, it enables the investigator to characterize tumor infiltration status and possible involvement of lymphatic tumor growth according to TNM staging[8]. Furthermore, taking a biopsy becomes possible by an adequate imaging.

Whether in case of a tumor lesion of the papilla, endoscopic intervention such as papillectomy or surgical intervention is used depends on tumor entity, tumor stage and individual characteristics of the patient[9-11].

Today, the spectrum of indications for endoscopic papillectomy comprises adenoma, carcinoma of stage uT1N0[12-18], neuroendocrine tumor (NET)[8,19,20], non-introducible catheter into the papilla[21], cholestasis and diagnostic purpose. Ponchon *et al*[22] reported on endoscopic papillectomy and Binmoeller *et al*[9] for the first time. Further therapeutic options in adenoma are kephal pancreaticoduodenectomy and local surgical resection (ampullectomy) *via* a transduodenal approach. But in case of resectable carcinoma of the papilla, surgical intervention is the treatment of choice since there is a probability of approximately 20%-40% of manifest lymph node metastases if there is a submucosal infiltration.

The aim of the study was to investigate the clinical value of endoscopic papillectomy indicated by feasibility[16] and safety of the procedure in various diseases of the papilla in a representative number of patients in a setting of daily clinical and endoscopic practice and care by means of a systematic prospective observational study, to balance advantages and disadvantages of the endoscopic approach, as well as, in particular, to elucidate: (1) Which were the main and propper indications? (2) What results of resections could be achieved? or (3) What was the long-term outcome in various diseases of the papilla?

MATERIALS AND METHODS

Through a defined time period, all consecutive patients with tumor-like lesions of the papilla who were selected for an endoscopic approach, were enrolled in this systematic bicenter prospective observational study (design). In addition to physical exam and laboratory analysis, the patients underwent upper gastrointestinal (GI) endoscopy including EUS. Endoscopic papillectomy (modified

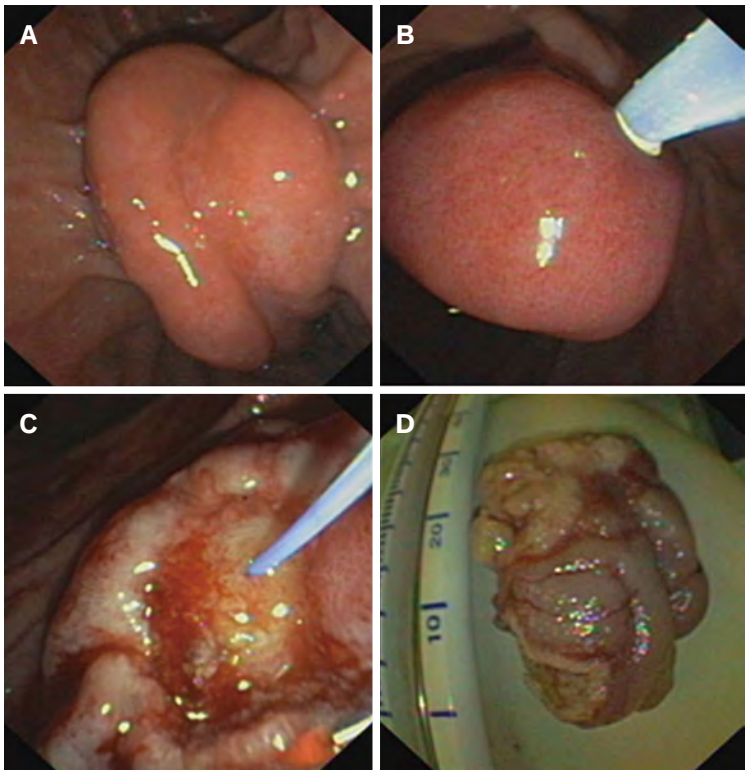

Figure 1 Steps of endoscopic papillectomy. A: Endoscopic view onto the local tumor site (adenomatous papilla of Vater); B: Insertion of a tube through the papilla of Vater for cholangiography; C: Postinterventional endoscopic view onto the papillary region after endoscopic papillectomy; D: Tumor specimen *ex situ*.

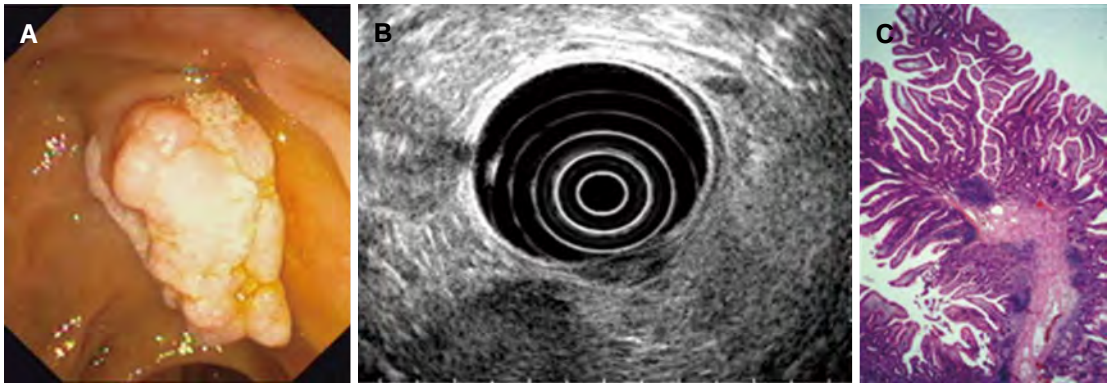

Figure 2 Diagnostic workup of a representative case (tubulovillous adenoma of the papilla). A: Initial endoscopic view onto the tumor-like lesion of papillary region; B: Endoscopic ultrasonography-based imaging indicating echo-poor tumor lesion but no infiltrating tumor growth; C: Histopathological picture (HE staining; magnification, × 40).

procedure according to Han and Kim[23]) was chosen in case of promising potential for R0 resection, in uT1 lesions with no hints of deep infiltrating tumor growth and/or in high-risk patients (balancing risk-benefit ratio of open surgery) after appropriate diagnostics (imaging and/or biopsy) and additional decision-making in the institutional multi-disciplinary GI tumor board. It was performed by only 2 experienced interventional endoscopists as follows, in brief: Patients underwent papillectomy during upper GI endoscopy after signing informed consent the day before intervention, in particular, containing information on risk, complication profile and major complications such as acute pancreatitis, perforation and bleeding as well as necessary follow up and prognosis of each specific procedure as appropriate, "npo" for 12 h, and premedication with 5-10 mg Midazolam (Midazolam Ratioph®, Ratiopharm GmbH, Ulm, Germany) under antibiotic prophylaxis with ceftriaxone (Rocephin®, 2 g; Hoffmann-La Roche AG, Grenzach-Wyhlen, Germany) and cardiopulmonal monitoring.

Using a duodenoscope [Olympus Optical Co. (Europe) GmbH, Hamburg, Germany], peripapillary region was endoscopically inspected (Figures 1 and 2) eventually completed by diagnostic EUS (Hitachi Medical Systems, Lübbecke, Germany) (Figure 2B), and cholangiography was performed if possible (catheter which can be introduced into the papilla). Papillectomy was executed using high frequency diathermia loop (MTW Endoskopie, Wesel, Germany) (Figure 1C and D). If required, sphincterotomy using papillotome [Olympus Optical Co. (Europe) GmbH, Hamburg, Germany] to prevent postinterventional stenosis; stent implantation into the pancreatic duct

Table 1 Spectrum and frequency of preinterventional endoscopic investigations in the whole group of patients with following endoscopic papillectomy (n = 54)

Investigation	Gastroscopy		EUS		ERCP	
	w/Hx	w/o Hx	w/Hx	w/o Hx	w/Hx	w/o Hx
Case n (%)	20 (37.0)	8 (14.8)	4 (7.4)	39 (72.2)	9 (16.6)	15 (27.8)
In total (%)	51.80%		79.60%		44.40%	

Hx: Histopathological finding; EUS: Endoscopic ultrasonography; ERCP: Endoscopic retrograde cholangiopancreatography.

(5-French plastic endoprosthesis; GIP Medizintechnik GmbH, Achenmühle, Germany) for 4-5 d to drain the pancreas sufficiently because of possible postinterventional swelling of the papilla and peripapillary region[24]; and/or APC (Erbe APC, Medika, Hof, Germany), in particular, to encrust tumor residuals with electrocoagulation were combined. Bleedings were immediately tried to be controlled as appropriate using adrenalin injection (dilution, 1:10000), fibrin glue application (Baxter Deutschland GmbH, Heidelberg, Germany) and/or placement of hemoclips [Olympus Optical Co. (Europe) GmbH, Hamburg, Germany]. Specimens were immediately transferred to routine histopathological investigation (Figure 2C) and followed by specific stainings and/or immunohistochemistry if necessary.

Data such as clinicopathological features (age, sex, gender, symptomatology leading to initiation of diagnostic, diagnostic profile and findings, spectrum of diagnoses, tumor size, TNM stage and occurrence of metastases in case of malignancy, profile of indications for papillectomy) were prospectively collected, documented using a computer-based registry and retrospectively evaluated using SPSS for Windows (version 13.0, Chicago, IL, United States).

The patients were subdivided into 4 groups according to endoscopic and EUS findings as well as the histopathological diagnosis: Group 1: Adenoma; Group 2: Carcinoma/NET/lymphoma; Group 3: Papilla into which catheter can not be introduced; Group 4: Adenomyomatosis.

Treatment results were characterized by R0 resection and complication rate, the latter one further specified by periinterventional morbidity and intervention-related mortality. Outcome was assessed by recurrence rate as well as general and tumor-free survival after a long-term period of follow-up investigations which were performed using clinical exam, abdominal ultrasound and endoscopy with biopsy as well as EUS (if required) every 3-6 mo for 2 years followed by time intervals of 6 mo in cases of adenomas and malignant tumor growth (but immediately if required and indicated by suspicuous symptomatology).

Study was performed according to the guidelines of the Declaration of Helsinki for Biomedical Research from 1964 and the standards of the Institutional Review Board.

Statistical analysis

Data were evaluated by descriptive statistics and further analyzed using SPSS for Windows (version 13.0, Chicago, IL, United States) to proof validity of the preinterventional histopathological diagnosis by means of binary diagnostic tests such as 2 × 2 square panel, which allows to determine sensitivity, specificity, negative and positive predictive values as appropriate.

RESULTS

Through a 7-year study period, endoscopic papillectomy was performed in 58 patients at the Departments of Gastroenterology of the University Hospital in Jena (Germany) and the Municipal Hospital in Gera (Germany). Overall, 54 patients were evaluated (inclusion rate, 91.6%; 25 males (M), 29 females (F); sex ratio, M/F = 1:1.16). The mean age was 64.4 years in males, in females 67 years (range, 22-88 years).

The main clinical symptoms were predominated by abdominal pain in 50% of cases followed by cholestasis in 33%. The combination of both was found in 11% (others, 6%).

All tumors were detectable, imaged and characterized with regard to the locoregional tumor growth using various diagnostic tools (detection rate, 100%). Tumor size ranged between 1 and 4.5 cm. The spectrum and frequency of endoscopic investigations to proof the indication of endoscopic papillectomy is shown in Table 1. It clearly shows that EUS was the most frequent endoscopic procedure in more than half of cases (79.6%). Endoscopic retrograde cholangiopancreatography (ERCP) was only used in less than 50% of patients (44.8%; gastroscopy, 51.8%). Interestingly, gastroscopy contributed to achieve a histopathological finding by taking a biopsy in the majority of cases.

Histopathological diagnosis was determined in 32 patients in whom preinterventional histopathological investigation was performed which was distributed as indicated in Table 2 whereas slightly different, there was a profile and frequency of indications, which led to endoscopic papillectomy as a result of preinterventional diagnostic measures (n = 54; Table 2), and finally endoscopic papillectomy with subsequent histopathological investigation resulted in the distribution of definitive histopathological diagnoses as listed in Table 2 (n = 54).

This resulted in a cumulative sensitivity and specificity for preinterventional histopathological findings such as adenoma and carcinoma/NET/lymphoma of 64.2% and 55.0%, respectively. The negative and positive predictive values were 65.5% and 56.5%, respectively. Interestingly and in combining the diagnoses adenoma and carcinoma/NET/lymphoma in the preinterventional diagnostic measures, there were a sensitivity of 64.2%, a specificity of 65.5%, a negative predictive value of 65.5% and a positive predictve value of 56.5%, respectively, for the preinterventional diagnostic measures (Table 3).

In adenomas (patient group 1, Table 4), there was a tumor recurrence rate of 13.6% (n = 3/22). Interestingly, there were two cases in whom no R0 resection was achieved but aspects of recurrent adenomatous tumor

Table 2 Absolute and relative frequency and spectrum of various findings by preinterventional histopathological investigation and diagnostic measures as well as definitive postinterventional diagnoses *n* (%)

Category of single finding	Preinterventional histopathological finding (after taking a biopsy; *n* = 32)	Indications leading to endoscopic papillectomy (result of preinterventional diagnostic measures; *n* = 54)	Definitive histopathological findings of the specimen (after endoscopic papillectomy; *n* = 54)
Adenoma			
Tumor mass with no malignancy	63	35 (64.8)	24 (45.0)
Adenocarcinoma	19	10 (18.5)	18 (33.0)
NET	3		
Lymphoma	3		
Mucosal specimen	9	/	/
Papilla into which catheter can not be introduced	/	8 (14.8)	8 (15.0)
Diagnostic papillectomy	/	1 (1.8)	/
Adenomyomatosis	/	/	4 (7.0)

NET: Neuroendocrine tumor.

Table 3 Various parameters characterizing value of preinterventional histopathological investigation (after taking a biopsy) and diagnostic measures

Parameter	Pre-interventional	
	Histopathological investigation (after taking a biopsy)	Diagnostic measures
Sensitivity	64.20%	64.20%
Specificity	55.00%	65.50%
Predictive value		
Negative	65.5%	65.5%
Positive	56.5%	56.5%

Table 4 Characteristics of adenoma patients after endoscopic papillectomy *n* (%)

	Adenoma					
	In total	Resectiom status				
		R0	Rx	R0 + Rx	R1	R2
Case	24	9 (37.5)	13 (54.2)	22 (91.6)	1 (4.2)	1 (4.2)
Recurrence		1 (11.1)	2 (15.4)	3 (13.6)		

Rx, resection status could not be defined because of loss of specimen or no detectable adenoma cells in the resected specimen despite adenoma finding in the histopathological investigation of the preinterventional biopsy. Recurrence, only related to "R0 + Rx" according to the definition of recurrent tumor growth, namely, tumor-free resection area.

Table 5 Characteristics of patients with a malignant tumor lesion (adenocarcinoma, neuroendocrine tumor and lymphoma) after endocopic papillectomy *n* (%)

	In total	Resection status				
		R0	Rx	R0 + Rx	R1	R2
All stages						
Case	18	8 (44.4)	2 (11.1)	10 (55.5)	1 (5.5)	7 (38.8)
Recurrency		1 (12.5)	1 (50.0)	2 (20.0)		
T1 stage only						
Case	12	8 (66.7)	1 (8.3)	9 (75.0)	1 (8.3)	2 (16.6)
Recurrence		1 (12.5)	0 (0.0)	1 (11.1)		

Rx, resection status could not be defined because of loss of specimen or no detectable adenoma cells in the resected specimen despite adenoma finding in the histopathological investigation of the preinterventional biopsy. Recurrence, only related to "R0 + Rx" according to the definition of recurrent tumor growth, namely, tumor-free resection area.

growth have not been observed yet during the follow-up investigation period. Recurrent adenomas were re-approached using endoscopic papillectomy with good success.

Considering all patients with malignant tumor growth, *i.e.*, all tumor lesions and stages (Table 5; patient group 2; *n* = 18:13 patients with adenocarcinoma, 4 individuals with NET, and one subject with a lymphoma), there was a recurrence rate of 20.0% [*n* = 2/10 (R0 + Rx)] after a mean follow-up investigation period of 18.5 (range, 1-84) mo. The two cases out of ten with recurrent carcinoma were transfered to abdominal surgery with favorable outcome. According to the results listed in Table 5, 12 patients with the more relevant uT1 carcinoma for a minimally invasive, endoscopic approach underwent endoscopic papillectomy with curative intention, in whom R0 resection status was achieved in 9 patients (75%) while no tumor recurrence was found in 88.9% of patients (*n* = 8/9). The one patient with R1 and the seven patients with R2 resection status were re-approached using Argon beamer with a good long-term result (no recurrent tumor growth within the reported follow-up investigation period). Rescue surgical intervention did not become necessary in case of R1/R2 resection since all of these patients had been classified of high perioperative risk.

In cases of a papilla into which catheter can not be introduced (*n* = 8; patient group 3) for which there are no data from the literature, the catheter placement was achieved in 87.5% (*n* = 7/8) of cases after endoscopic papillectomy. A re-intervention because of a stent occlusion became necessary in 2 patients (no table shown).

If an adenomyomatosis (*n* = 4) was diagnosed (patient group 4), there was a successful papillectomy in 100% of cases with no necessary reinterventions (again, no table shown).

Overall, there was a successful endoscopic papillectomy with regard to R0/Rx resection and/or placement of a catheter into the papilla in case of former not introducible catheter in 87.5% (*n* = 42/48) according to technical success rate, but related to no tumor recurrence

Table 6 Number and percentage of complications depending on the tumor entity and/or indication for endoscopic papillectomy *n* (%)

	Case (*n*)	Complications
Adenoma	24	3 (12.5)
Carcinoma/NET/lymphoma	18	3 (16.6)
Papilla into which catheter can not be introduced	8	2 (25.0)
Adenomyomatosis	4	2 (50.0)
In total	54	10 (18.5)
Major complication		1 (1.9)

NET: Neuroendocrine tumor.

and/or placement of a catheter into the papilla in 79% (n = 38/48) according to clinical success rate. The basis for this calculation was the number of 48 patients since there were 6 patients with a tumor stage T > 1Nx (Table 5; n = 18 min n_{uT1} = 12).

As shown in Table 6, complications occured in 18.5% (n = 10/54), in particular, bleeding (n = 3); pancreatitis (n = 7) and perforation (n = 1; the only case with need for rescue surgical intervention) (Table 6), in 12.5% (n = 3/24) of adenoma patients (Table 6) but no postinterventional stenosis of the orifice at the papilla was observed (major complication rate, 1.9%; n = 1/54; Table 6). There was no intervention-related death (mortality, 0%).

DISCUSSION

In addition to clinical exam, laboratory analysis, and abdominal ultrasound, EUS[8] and ERCP[25,26] are the appropriate diagnostic measures for suspected tumor (-like) lesions of the papilla. Furthermore, magnetic resonance cholangiopancreatography, magnetic resonance imaging and/orcomputed tomography can be performed to further assess local tumor growth including possible tumor infiltration to the neighboring organs and to detect distant metastases (suggested institutional diagnostic algorithm including therapeutic measures, Figure 3). However, EUS appears to be the best diagnostic tool, which is able to reliably predict the correct diagnosis with a high percentage prior to papillectomy[1].

In a suspected local tumor lesion, a papillectomy can be helpful to clarify the diagnosis, in particular, in getting access to the obstructed bile duct; to remove a representative specimen; or to even provide adequate resection[9,10,24] since in non-clear benign or malignant tumor growth, an early, correct and reliable diagnosis-finding allows an appropriate subsequent therapeutic decision-making according to differential diagnosis and stage of disease[2].

Interestingly, a curative resection is possible by the means of minimally invasive endoscopic intervention under certain circumstances such as adenoma of smaller size[5-7,9,10,24] and uT1 carcinoma[12] with no hints of deep infiltrating tumor growth if R0 resection status can be achieved or in high-risk patients[6] though carcinoma is usually an indication for surgical intervention[2].

Also in case of a catheter which can not be introduced into the papilla (into the minor papilla[27] if there is a suspected "pancreas divisum" or into the major papilla after previous gastric resection [Billroth Ⅱ] or because of a carcinoma of the papilla[12]) and even if an adenomyomatosis is detected, there is need for endoscopic papillectomy since, on one hand, access to the pancreaticobiliary system is needed and, on the other hand, adenomyomatosis can not be macroscopically distinguished from an adenoma.

If an R0 resection was achieved, recommended follow-up investigation periods are every 6 mo for 2 years followed by further endoscopic control in case of suspicious symptoms[2]. In addition, a colonoscopy is basicly recommended. If there is an incomplete resection of an adenoma, the case needs to be controlled within 2-3 mo.

Interestingly, complication rate was similar or lower than those reported in the literature[4,9,13,14,16-19,25].

The tumor recurrence rate of 20.0% [n = 2/10 (R0 + Rx); Table 5] appears well comparable with data from the literature (Bohnacker *et al*[5], 15%)[10,13,14,16-18,25] as well as within the range of results in local surgical resection and kephal pancreatoduodenectomy.

The endoscopic approach in uT1 carcinoma (n_{uT1} = 12 out of n = 18 including all tumor stages, Table 5) correlates with recommendations of specific guidelines that endoscopic papillectomy can be reasonable in patients with uT1 and increased perioperative risk because of considerable comorbidity[2,6,10,12].

To our knowledge, this is one of the rare reports on the systematic investigation of endoscopic papillectomy in suspicious tumor lesions of the papilla in a representative number of patients[4,9,10,19,22,25] and their variety of tumor (-like) lesions as shown since there is a lack of extensive experiences because of their low incidence and the fact that optimal management of such tumor lesions has not yet been established[28].

The results justify endoscopic papillectomy as a reasonable, suitable and safe therapeutic measure in experienced hands[2,4,9,13-19,24] for the management of the broad spectrum of possible findings at the papilla or the minor papilla[27] in our institution and as occuring in daily clinical practice.

Finally, endoscopic papillectomy is considered a reasonable alternative to the "precut" or to PTCD. For comparison, endoscopic drainage provides a success rate of 95.4% (mortality, 0%) in acute cholangitis according to the literature whereas surgical intervention is associated with a lower success rate of 58% but a mortality of up to 12.4%.

However, there are limitations of the study with regard to the overall treatment results, which can be related to: the impact of the learning curve despite only two experienced interventional endoscopists performed papillectomy; the pitfalls of papillectomy such as potential of no achievable R0 resection despite curative intention, and the complication profile (including bleeding and perfora-

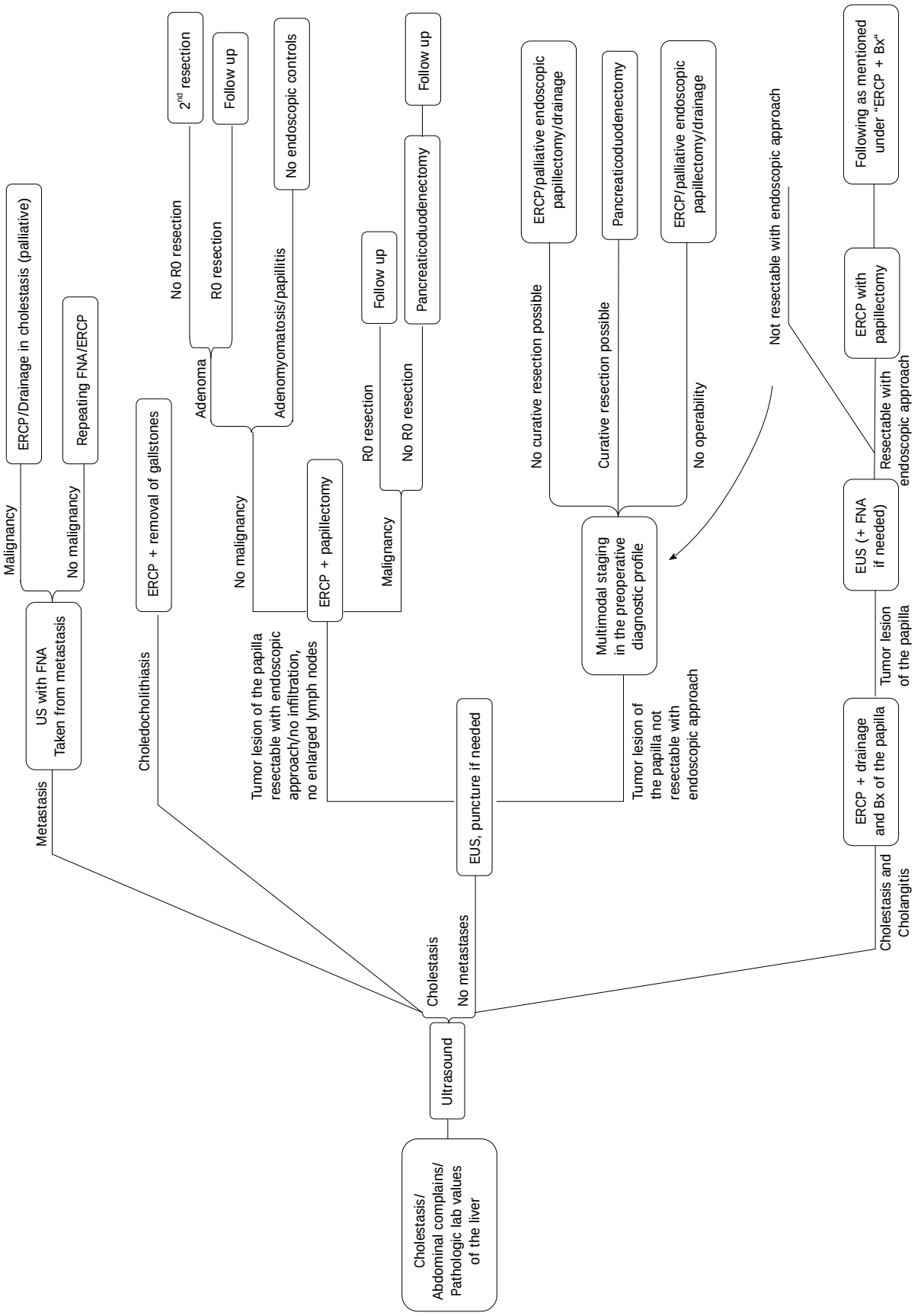

Figure 3 Suggested institutional algorithm on the diagnostic and therapeutic endoscopic approach in suspicious tumor-like lesions of the papilla of Vater. ERCP: Endoscopic retrograde cholangiopancreatography. EUS: Endoscopic ultrasonography.

tion with possible need of surgery); and no strict study inclusion criteria since study design represents a "systematic prospective bicenter observational study" reflecting daily clinical practice and consecutive but not selected patients.

In conclusion, endoscopic papillectomy is a challenging interventional approach but a suitable patient- and local finding-adapted diagnostic and therapeutic tool with adequate risk-benefit ratio in experienced hands.

COMMENTS

Background

The adequate management of diseases of the papilla of Vater (papilla) is challenging. There are several morphological changes and lesions of the papilla such as functional dysfunction, inflammation, stenosis or malignant tumor growth. For instance, a stenosis of the papilla is subdivided based on an autopsy registry as follows: Benign lesions, 42.7% (frequency); adenoma (premalignant lesion), 19.6%; carcinoma, 37.7%. For the treatment of tumor lesions of the papilla, it is required in addition to a sufficient histopathological investigation to achieve an adequate pretherapeutic tumor staging, which allows a decision-making toward the appropriate treatment (surgical intervention, papillectomy, papillotomy) according to the patient´s specific finding. These requirements can be fulfilled by endoscopic ultrasonography (EUS) for the majority of tumor (-like) lesions. For the specific clinical status of the single patient (*e.g.*, high risk because of accompanying diseases) and to cover the need of lower invasiveness and interventional trauma for a more favorite outcome as well as earlier reconvalescence, an additional approach to open surgery (providing transduodenal papillectomy/ampullectomy but with a substantial complication rate) is required-this might be provided by the very specific interventional endoscopic approach, named endoscopic papillectomy.

Research frontiers

To provide a substantial contribution to the important field of interventional endoscopy with a low number of valuable studies and case numbers on (endoscopic) papillectomy/tumor (-like) lesions of the papilla, the aim of the study was to investigate the clinical value of endoscopic papillectomy (to broaden the spectrum of therapeutic options in managing tumor lesions of the papilla of Vater) indicated by feasibility and safety of the procedure in various diseases of the papilla in a representative number of patients in a setting of daily clinical and endoscopic practice and care by means of a systematic prospective observational study, which can be considered one of the rare study approaches existing so far and emphasizing this measure of interventional endoscopy on one hand and, on the other hand, this type of study to sufficiently characterize daily clinical (endoscopic) practice in addition to rather specifically initated comparative (controlled randomized) studies.

Innovations and breakthroughs

Endoscopic papillectomy (based on sufficient preinterventional diagnostics by, among others (in particular), EUS as a sufficient diagnostic measure to preoperatively clarify diseases of the papilla including suspicious tumor stage in conjunction with postinterventional histopathological investigation of a specimen) is a challenging interventional approach. Thus, endoscopic papillectomy can be considered a valuable addition in the (diagnostic and) therapeutic management of (peri-) ampullary (tumor-like) lesions if treated patients are systematically and prospectively analyzed for quality assurance and adequately followed, *e.g.*, with appropriate follow-up investigations within reasonable time intervals.

Applications

Again, endoscopic papillectomy is a challenging interventional approach but a suitable patient- and local finding-adapted diagnostic and therapeutic tool with adequate risk-benefit ratio in experienced hands. Derived from this, an increasing number of interventional endoscopists may (based on and derived from the experiences analyzed in the systematic clinical prospective observational study presented here) begin with the endoscopic approach of papillectomy in their own endoscopic practice.

Terminology

Papilla of Vater: important anatomic structure at the mouth of the bile duct and/or pancreatic duct with possible inflammatory and neoplastic lesions leading to unspecific or varying symptomatology. Papillectomy: interventional (challenging endoscopic) or open surgical procedure intending to completely remove papilla of Vater in specific, in particular, neoplastic [benign or malignant (in early lesions)] findings to provide low invasiveness and complication rate but making sure a case-, finding- and risk-adapted approach. Endoscopic ultrasonography: very suitable diagnostic tool, in particular, for the periampullary region but also feasible for image-guided interventional endoscopic procedures such as papillectomy (or removal of small tumor lesions, biopsy, puncture, injection). Adenoma is considered a benign neoplastic lesion originating from adenoid structures such as the superficial layer of the gastrointestinal (GI) tract and, in particular, is an important lesion of the anatomic region such as papilla of Vater or the periampullary region.

Peer review

The manuscript provides additional information to the existing literature obtained by a systematic prospective observational study characterizing in particular daily clinical practice in interventional endoscopy of a GI endoscopy center on feasibility and safety of endoscopic papillectomy for various tumor (-like) lesions of the papilla in a representative number of patients, which can be considered one of the rare study approaches existing so far and emphasizing this measure of interventional endoscopy on one hand and, on the other hand, this type of study to sufficiently characterize daily clinical (endoscopic) practice in addition to rather specifically initated comparative (controlled randomized) studies. In detail, endoscopic papillectomy was found to be feasible and safe in experienced hands. The clinical researchers and experienced/advanced GI endoscopists should be encouraged to further pursue this type of study, lesions and patients experiencing this challenging type of tumor (-like) lesion of the papilla, for which only a few experts can provide similar results and expertise.

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BRIEF ARTICLE

Phytoestrogens/insoluble fibers and colonic estrogen receptor β: Randomized, double-blind, placebo-controlled study

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Abstract

AIM: To assess the safety and effect of the supplementation of a patented blend of dietary phytoestrogens and insoluble fibers on estrogen receptor (ER)-β and biological parameters in sporadic colonic adenomas.

METHODS: A randomized, double-blind placebo-controlled trial was performed. Patients scheduled to undergo surveillance colonoscopy for previous sporadic colonic adenomas were identified, and 60 eligible patients were randomized to placebo or active dietary intervention (ADI) twice a day, for 60 d before surveillance colonoscopy. ADI was a mixture of 175 mg milk thistle extract, 20 mg secoisolariciresinol and 750 mg oat fiber extract. ER-β and ER-α expression, apoptosis and proliferation (Ki-67 LI) were assessed in colon samples.

RESULTS: No adverse event related to ADI was recorded. ADI administration showed a significant increases in ER-β protein (0.822 ± 0.08 *vs* 0.768 ± 0.10, $P = 0.04$) and a general trend to an increase in ER-β LI (39.222 ± 2.69 *vs* 37.708 ± 5.31, $P = 0.06$), ER-β/ER-α LI ratio (6.564 ± 10.04 *vs* 2.437 ± 1.53, $P = 0.06$), terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (35.592 ± 14.97 *vs* 31.541 ± 11.54, $P = 0.07$) and Ki-67 (53.923 ± 20.91 *vs* 44.833 ± 10.38, $P = 0.07$) approximating statistical significance. A significant increase of ER-β protein (0.805 ± 0.13 *vs* 0.773 ± 0.13, $P = 0.04$), mRNA (2.278 ± 1.19 *vs* 1.105 ± 1.07, $P < 0.02$) and LI (47.533 ± 15.47 *vs* 34.875 ± 16.67, $P < 0.05$) and a decrease of ER-α protein (0.423 ± 0.06 *vs* 0.532 ± 0.11, $P < 0.02$) as well as a trend to increase of ER-β/ER-α protein in ADI *vs* placebo group were observed in patients without polyps (1.734 ± 0.20 *vs* 1.571 ± 0.42, $P = 0.07$).

CONCLUSION: The role of ER-β on the control of apoptosis, and its amenability to dietary intervention, are supported in our study.

Key words: Estrogen receptor-β; Estrogen receptor-α; Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; Sporadic adenomatous polyposis; Phytoestrogens; Insoluble fibers

Core tip: Active dietary intervention, a mixture of phytoestrogens and insoluble fibers, was administered in

patients with sporadic colonic adenomas in a randomized, double-blind placebo-controlled trial. Dietary supplementation induced an increase in estrogen receptor (ER)-β protein and the ER-β/ER-α ratio with a general trend towards an increase in epithelial proliferation and apoptosis. These results, even if limited by the small number of subjects and short period of dietary supplementation, suggest the possibility of an interaction with epithelial apoptosis by means of mediating ER-β levels.

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INTRODUCTION

There is considerable evidence, both observational and interventional, to suggest that estrogens may have a chemopreventive activity for colorectal cancer (CRC). Women have a lower rate of colonic adenomas and cancers than men before menopause but the differences progressively lessen after menopause[1]. The mortality from CRC has been decreasing progressively in women compared to men since 1950[2] and this decrease is correlated with the time frames of increasing use of hormone replacement therapy (HRT), as confirmed by controlled trials[3,4].

Any chemopreventive effect of estrogens for CRC would likely be mediated by the interaction of circulating estrogens with estrogen receptors (ERs) in the colonic epithelium[5]; the presence of these receptors has been investigated by different methods, in many papers since the 1980s[6-8]. There are two distinct types of estrogen receptors (ERs) in human tissues. ER-α is primarily expressed in the breast and endometrium, whereas ER-β is found in a wide variety of tissues that are traditionally considered non-hormonal, including the colon. ER-α and ER-β activation may lead to biologically opposite patterns, as demonstrated in cancer cell lines (HC 11, LoVo)[9,10]. Thus, it is reasonable to hypothesize that estrogens could have dissimilar biological consequences in different tissues according to the amount and type of ERs[11]; the ER-β/ER-α ratio has therefore been developed to measure this phenomenon[12]. There is a substantial body of evidence suggesting that the level of ER-β expression itself, and/or the ER-β/ER-α ratio, is related to colonic carcinogenesis in both humans and animal models of CRC. ER-β is abundantly expressed in the normal colon but its expression is progressively decreased in adenomas and CRC in relation to the disease aggressiveness[13-15]. Similarly, familial adenomatous polyposis shows progressively lowered ER-β levels and a reduced ER-β/ER-α ratio in pre-neoplastic and neoplastic tissue[14]. Finally, the results of different studies suggest that some herbal supplements may exert significant and potentially beneficial effects on decreasing the amount of precancerous lesions by inducing apoptosis in the large intestine[16-19]. In an adenomatous polyposis coli$^{Min/+}$ (Apc$^{Min/+}$) mouse model, dietary supplementation significantly counteracted the intestinal tumorigenesis and increased the ER-β expression in the colon[20]. Moreover, observational studies suggest that phytoestrogens intake in whole grain, as well as enterolignans and silibinin, may be associated with a decreased incidence of advanced colon adenomas and cancers in both men and women[21-24].

On these bases, we conducted a randomized, double-blind placebo-controlled study to determine whether short term administration of dietary phytoestrogens and insoluble fibers, (silymarin, secoisolariciresinol diglycoside from flaxseed and insoluble fibers from oat extract, (Eviendep® CMD Pharma Limited. A Nestle Health Science Company, London, United Kingdom) can selectively alter ERs expression in the normal appearing colonic mucosa of patients undergoing surveillance colonoscopy after a previous polypectomy. Epithelial proliferation and apoptosis were assessed at the same time.

The expression of these biomarkers was evaluated in two steps: firstly analysis after administration of the active dietary intervention (ADI, Eviendep) *vs* the placebo, and then a further subdivision into 4 subgroups: ADI *vs* placebo, with, or without polyp recurrence.

MATERIALS AND METHODS

Study design and patient population

The study was approved by the Ethics Committee of the University Hospital of Bari (No. 1410). All patients signed informed consent, in accordance with the Helsinki Declaration, revision 1983. The study was registered at Clinical Trial.gov (ID: NCT01402648). In accordance with the CONSORT statement flowchart, the study design and flow is described in Figure 1.

All subjects with endoscopic records, males or postmenopausal females (defined as the absence of a menstrual cycle for at least 2 years prior to enrollment) aged at least 50 years, who had undergone a previous endoscopic polypectomy and were enrolled in follow-up for surveillance colonoscopy to be performed 3 or 5 years later, according to standard guidelines, were screened for study eligibility. Six hundred patients, potentially eligible by chart-review, were contacted by the EC authorized Clinical Investigators by telephone-interview to explain the study and invite them to participate.

Inclusion criteria were based on biochemical evaluation of blood samples *i.e.*, hemoglobin $\geq$ 12.0 g/dL; platelets $\geq$ 120000/mm^3; international normalized ratio $\leq$ 1.5; alanine aminotranferease or aspartate aminotransferase $\leq$ 1.5 times the upper limit of normal values (ULN); alkaline phosphatase $\leq$ 1.5 times ULN; bilirubin $\leq$ 1.5 times ULN; blood urea nitrogen $\leq$ 40 mg/dL and normal blood pressure or controlled hypertension.

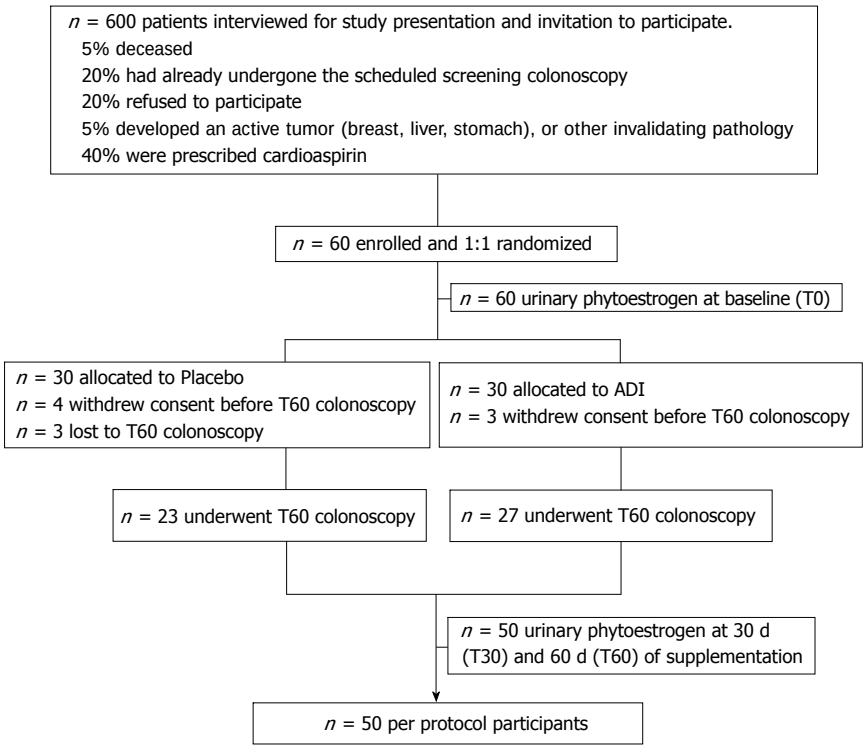

Figure 1 Diagram of study design.

The following were exclusion criteria: chronic inflammatory bowel disease, intestinal and/or extra-intestinal malignant neoplasms, acute or chronic renal disease, anemia, coagulation disorders or a body mass index (BMI) > 30 kg/m^2, anti-cancer treatment and/or systemic corticosteroids within 6 mo of enrollment; anticoagulants or platelet anti-aggregants and antibiotics within 30 d of enrollment, HRT, selective estrogen receptor modulator, *e.g.*, tamoxifene and related compounds or other supplemented phytoestrogens, aspirin and nonsteroidal antiinflammatory drugs within the previous 6 mo.

Sixty consenting, eligible patients, out of 600 interviewed, were randomly allocated to placebo (PL) or ADI (Eviendep) in a 1:1 ratio at baseline (T0), *i.e.*, 60 d in advance of their scheduled surveillance colonoscopy (T60). The period of 60 d of supplementation was chosen as adequate for complete turnover of the colonic epithelial cells (migration from the crypt and release into the lumen) approximately eight times and, therefore, for the reliability of the biological evaluation[25].

ADI was provided as a sachet composed of 750 mg insoluble and indigestible oat fiber (cellulose, hemicellulose and lignin), 50 mg flaxseed dry extract (containing 20% secoisolariciresinol diglycoside), and 175 mg milk thistle extract (70% silymarin by UV and 30% silibinin by high performance liquid chromatography). PL sachets contained maltodextrins (910 mg), and 100% of ADI excipients. The ADI and PL were provided by the Sponsor in identical boxes and sachets, and labeled with the protocol code and the allocated participant number. The ADI and PL were stored and distributed, according to the assigned treatment group, to the participants through the Hospital Pharmacy. The patients and investigators were blinded to assignment. There were no dietary restrictions.

Computer-generated randomization, study monitoring, database acquisition and statistical analysis were conducted through a contract with medical trial analysis (Ferrara, Italy), an independent Clinical Research Organization.

Safety was assessed by vital signs (heart rate, blood pressure); and blood homeworks at baseline (T0), and after 30 d (T30) and 60 d (T60).

Phytoestrogen intake, and compliance were assessed by urinary measurements *i.e.*, enterolignans enterodiol (ED) and enterolactone (EL), at T0, T30 and T60[19,20]. A few d before colonoscopy, all patients were asked to avoid fruit and vegetable intake and received bowel cleansing with PEG 4000 (1120 g/4 L water solution).

Endoscopy and histology

The number, location and size of all visualized polyps were recorded during endoscopy. A standard protocol for polyp removal was followed. Diminutive polyps (0.5 cm) were ablated with electrocoagulation, whereas all polyps > 0.5 cm in size were removed and submitted for histological assessment. Eight biopsies of the normal appearing sigmoidal mucosa were collected from all participants; seven of the biopsies were frozen in liquid nitrogen for biochemical and biologic endpoint analyses and one was well-oriented on blotting paper and then fixed in 4% formalin for immunostaining. The polyps were classified as hyperplastic or dysplastic by a gastrointestinal pathologist.

Biochemical and biologic endpoint analyses

Colonic biopsies were assessed for ER-β and ER-α

mRNA by reverse transcriptase (RT)-polymerase chain reaction (PCR), and protein by Enzyme-Linked ImmunoSorbant Assay. Total RNA was extracted from biopsies using the RNA easy mini kit (Qiagen), according to the manufacturer's instructions. RNA concentration and quality were assessed by spectrophotometric readings at 260 and 280 nm. ER-β and ER-α cDNA were generated by Reverse Transcription of 0.5 μg of total RNA in a 20 μL reaction volume (iScript Select cDNA Synthesis Kit, Biorad), according to the manufacturer's instructions. Two rounds of amplification (PCR-1 and PCR-2) were performed. The outer and inner primers used to detect ER-β and ER-α mRNAs at exon 3 have been previously described[26]. PCR-1 was performed in 50 μL final volume (iTaq DNA Polymerase Kit-Biorad) containing 2 μL cDNA and 40 pmol of outer primers through a denaturation step (95 ℃ for 3 min), 30 cycles (94 ℃ for 30 s, 55 ℃ for 30 s, 72 ℃ for 30 s) and a final primer extension step (72 ℃ for 10 min). For PCR-2, PCR-1-derived ER-β and ER-α were diluted tenfold, and a 3 μL aliquot was amplified with 40 pmol of inner primers as per PCR1 conditions. β-actin cDNA (0.5 μL) served as internal control. DNA fragments were separated on a 2% agarose gel stained with ethidium bromide and size assessed by comparison with 100 bp DNA marker using Molecular Imager ChemiDOC XRS+ (Biorad). After normalization to the fluorescent β-actin PCR band intensity, ER-β and ER-α mRNA were expressed as arbitrary units of fluorescence.

For the extraction of proteins, colonic biopsies were homogenized in lysis buffer [100 mmol/L Tris-HCl (pH 7.5), 300 mmol/L NaCl, 4 mmol/L EDTA, 2% NP40, 0.5% Na deoxycholate, 1 mmol/L sodium orthovanadate and a protease inhibitor cocktail-Roche]. Supernatants were collected after centrifugation (13000 *g* for 25 min) and the total protein concentration was measured by the Bradford method (Biorad). ER-β and ER-α were measured using QuantiSir specific gene knockdown quantification specific kits (Epigentek, Brooklyn, NY, United States), following the manufacturer's instructions. ERs content was normalized to GAPDH and expressed as optical densities (*A*) by computer-assisted densitometry.

Human urine spot samples (fasting) were extracted and measured by mass spectroscopy using a waters quattro premier mass spectrometer and waters acquity equipment. The lower and upper limit of quantitation (LLOQ and ULOQ) were 0.5 ng/mL and 2000 ng/mL for EL and ED, respectively. Two full calibration lines prepared in surrogate matrix (phosphate buffered saline; PBS), corresponding to five different EL and ED concentrations over the LLOQ and ULOQ, served as quality control (QC) samples. Duplicate QC samples prepared in the calibrated range were run together with the clinical samples. The concentration of ED and EL (ng/mL) and the coefficient of variation [CV (%) = SD of results/mean of results × 100] were measured.

Immunohistochemistry and immunofluorescence

Immunohistochemistry evaluated ERs expression, cell proliferation by Ki-67 and apoptosis by both terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and caspase-3. In all cases, IHC data was expressed as labeling index (LI), *i.e.*, the percent of immunostained colonocytes over the total counted cells along the length of 10 well oriented crypts. A further evaluation was based on the intensity of IHC staining divided into weak, moderate and strong. Both LI and intensity staining were calculated by two independent observers (MPS, ST) in a blinded fashion. Diagnostic agreement was revealed by a weighted *k* statistics coefficient > 0.8.

IHC was performed only on the sections of biopsies taken from non-adenomatous mucosa[4,14]. After permeabilization and antigen retrieval under shaking conditions in TBS buffer with TWEEN 0.025% for ER-β (15 min) and by microwave irradiation in citric buffer at pH 6.0 for ER-α (3 cycles of 5 min), the slides were covered with 5% goat serum for 30' at room temperature (RT) to block non-specific binding. The slides were incubated with primary antibodies diluted 1:50 in PBS, at 4 ℃ overnight (anti-ER-β: Novocastra Menarini, Milano, Italy and anti-ERα: Santa Cruz, CA, United States). Negative controls were obtained by dipping slides in PBS without primary antibodies. Reactions were detected by a polymer-based visualization kit (EnVision, Dako, Glostrup, Denmark). Slides were then incubated with the chromogen 3, 3-diaminobenzidine-tetrahydrochloride (DAB, Vector laboratories) for 40 min at RT, and Harris hematoxylin (Sigma) served for nuclear counterstaining.

Ki-67 expression was evaluated by monoclonal antibody (clone MIB-1, Dako, Glostrup, Denmark). The sections were treated in a microwave oven twice for 5 min in citrate buffer (pH 6.0) at high power (750 W) before primary antibody incubation for 1 h at RT. The secondary peroxidase-conjugated antibody (EnVision, Dako, Glostrup, Denmark) was applied to the sections. Peroxidase activity was visualized by diaminobenzidine chromogen (DAB, Vector laboratory) and counterstained with hematoxylin.

TUNEL was investigated by the *in situ* cell death detection kit, Roche. In brief, sections were treated with 0.1 mol/L citrate buffer (pH 6.0) cooled to an internal temperature of sub-boiling in the microwave oven at 350 W for 10 min, incubated with TUNEL probe at 37 ℃ for 1 h and counterstained with TOPRO 3 (Invitrogen Molecular Probes), diluted at 1:5000. All sections were observed at × 400 magnification by confocal microscopy (Leica TSC SP2 confocal laser scanning microscope).

Caspase-3 was detected with a methodological approach allowing evaluation of both its exclusive expression and co-expression with ER-β. In detail, a rabbit polyclonal antibody from cell signaling (clone D 175) was used. Antigen retrieval was performed by rocking the slides in TBS buffer with TWEEN 0.025% for 10 min followed by microwave irradiation in citric buffer at pH 6.0 for 10 min at 750 watts; slides were incubated for 1 h at room temperature in 1% BSA blocking solution and then dipped in a mixture of the two primary antibodies (ER-β 1:50, Caspase-3 1:30) at 4 ℃ overnight.

Table 1 Urinary enterolignans (ng/mL) in study groups at different timepoints (mean ± SD)

	Placebo (n = 23)	ADI (n = 27)	P value
ED + ELT0	697.8 ± 963.3	410.3 ± 336.9	0.270
ED + ELT30	472.4 ± 475.5	3300.1 ± 1535.5	< 0.001
ED + ELT60	116.0 ± 235.2	327.5 ± 313.1	< 0.001

ED: Enterodiol; EL: Enterolactone; ADI: Active dietary intervention.

Table 2 T60 colonoscopy: Estrogen receptor-β and estrogen receptor-α expression values in the normal sigmoid mucosa of study groups (mean ± SD)

	Placebo (n = 23)	ADI (n = 27)	P value
ER-β protein	0.768 ± 0.10	0.822 ± 0.08	0.04
ER-β mRNA	0.994 ± 0.99	1.266 ± 1.24	0.10
ER-β protein	0.510 ± 0.11	0.490 ± 0.12	0.50
ER-β mRNA	0.139 ± 0.28	0.230 ± 0.24	0.20
ER-β/ER-α protein	1.571 ± 0.42	1.734 ± 0.20	0.07

ER: Estrogen receptor; ADI: Active dietary intervention.

The sections were covered with the secondary antibody, Alexa 555 fluorescent-conjugated Goat anti-Rabbit (Invitrogen), diluted to 1:100 in PBS. TOPRO-3 (Invitrogen-Molecular Probes) diluted in PBS and incubated for 10' at room temperature, providing nuclear counterstaining. Cells were counted at × 400 magnification, by confocal microscopy (Leica TSC SP2 confocal laser scanning microscope).

Statistical analysis

Sample size estimation was based on the assumption of an equivalence margin between the study arms of 0.05 and an actual difference ($mean_{ADI}$ - $mean_{PL}$) of 1, SD_{ADI} = 2 and SD_{PL} = 2. Sixty patients were required to complete the study for an 80% powered study and a two-tailed 0.05 *a* error. The non parametric, two sided Wilcoxon Rank Sums test weighted ERs and biomarkers expression in study groups. ANOVA, χ^2 or *t* test were used to compare demographics and all the other functional parameters. Spearman's correlation was applied to assess relationships among ERs and the other biomarkers with the common diet and the allocated supplements. All analyses were performed using SAS version 8.2 (Statistical Analysis Software, Cary, NC). Diagnostic agreement was tested by calculating the weighted *k* statistics coefficient interpreted in accordance with the benchmarks of Landis and Koch. *A* value below 0.4 indicated poor agreement, a value between 0.4 and 0.8 moderate to good agreement, and a value of more than 0.8 excellent agreement[26].

RESULTS

A flow diagram of the study is shown in Figure 1. A total of 60 eligible consenting subjects were identified from 600 interviewed potential participants. They were enrolled and randomly assigned 1:1 to placebo or ADI. The groups were matched for age, gender, BMI, and season of enrollment; this last character displayed the absence of biases in phytoestrogen intake.

Ten out of 60 subjects dropped out, as reported in Figure 1. The final per protocol population consisted of 50 participants who completed the 60 d treatment with dietary supplements and underwent surveillance colonoscopy.

Endoscopy and histology findings

In 21 subjects (42%) polyps were found; the majority of these (58%) were electrocoagulated while the remainders were processed for histological examination. None showed high grade dysplasia.

Adverse events and compliance

No adverse events or biochemical modifications were reported in either the ADI or the placebo group.

Urinary enterolignan levels (ED, EL and ED + EL: ng/mL) were comparable between the study groups at baseline (Table 1). ADI treatment induced a significant increase in all urinary enterolignan levels at T30 and T60.

At T60 an expected fall in urinary lignans occurred in both groups due to the dietary restriction of fruits, vegetables and the colonoscopy bowel cleansing.

Molecular estrogen receptors assay

The effect of ADI on ER-β and ER-α protein and mRNA content at T60 is shown in Table 2. ADI induced a statistically significant increase of ER-β protein. ER-β mRNA levels were higher in the ADI than placebo group but the difference did not reach significance. ER-α protein and mRNA content were similar between the two groups; the ER-β/ER-α protein ratio showed a trend to an increase in the ADI group.

The median value of ER-β and ER-α proteins, referred to all 50 patients enrolled, were 0.82 and 0.47 *A*, respectively. The patients distribution around these numbers was different for the two proteins. The ADI group showed a spread above the value of ER-β in a higher percentage as compared to the placebo group (62.96% *vs* 34.78%; $P < 0.05$) while, on the contrary, in the ADI group the median value of ER-α protein decreased as compared to the placebo group (66.7% *vs* 34.8%; $P = 0.02$).

Treatment-related immunohistochemical biomarkers

Analysis of agreement between the observers provided κ = 0.87 (95%CI: 0.76-0.98). The assessment of intensity staining did not affect the results of LI, as the percentage of weakly stained cells was less than 10% and the results were referred only to moderate-strong intensity.

Table 3 reports the effect of ADI on the LI of ER-β, ER-α, Ki-67, TUNEL and caspase-3. The ADI treated patients had a trend towards an increase in ER-β LI (P = 0.06), the ER-β/ER-α LI ratio (2.5 fold compared to PL; P = 0.06), TUNEL (P = 0.07) and Ki-67 (P = 0.07) as compared to the placebo group, although none of these differences reached statistical significance.

Table 3 T60 colonoscopy: Labeling index (mean ± SD)

LI	Placebo (n = 23)	ADI (n = 27)	P value
ER-β	37.708 ± 5.31	39.222 ± 2.69	0.06
ER-β	20.416 ± 10.71	16.481 ± 10.67	0.20
ER-β/ER-α	2.437 ± 1.53	6.564 ± 10.04	0.06
TUNEL	31.541 ± 11.54	35.592 ± 14.97	0.07
Caspase-3	29.717 ± 7.98	32.937 ± 13.54	0.10
Ki-67	44.833 ± 10.38	53.923 ± 20.91	0.07

LI: Labeling index; ER: Estrogen receptor; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; ADI: Active dietary intervention.

Spearman correlation showed that, in the ADI group, the ER-β LI was directly correlated with the TUNEL LI (r = 0.406, P = 0.03) and caspase-3 LI (r = 0.529, P < 0.004). The relationship between ER-β and apoptosis was confirmed by the IHC evidence of ER-β caspase 3 co-localization, as clearly shown in Figure 2. In the PL group these correlations showed the same direction but did not reach statistical significance (r = 0.379, P = 0.07 and r = 0.134, P = 0.55, respectively).

Biomarker expression in patients with or without polyp recurrence

The expression of biomarkers in the normal mucosa of patients with (recurrent) and without (non-recurrent) polyps, was assessed. Table 4 shows that in patients without recurrence, there was a significant increase of the ER-β pattern (mRNA, protein and LI) and a decrease of ER-α protein in the ADI *vs* the placebo group. An increased ER-β/ER-α protein ratio (P = 0.01, not shown in the table) was also observed.

In patients with recurrence, a higher ER-β protein (P = 0.04) and a lower ER-α LI (P = 0.02), were also demonstrated in the ADI group.

DISCUSSION

There are substantial observational human data to suggest the possibility that ERs expression might be a marker of colon cancer risk. The $Apc^{Min/+}$ mouse model of intestinal neoplasia has been used to study this association. Barone *et al*[20] reported that the ER-β level and ER-β/ER-α ratio was substantially lower in the normal small intestinal mucosa of $Apc^{Min/+}$ mice than in syngenic wild type mice, and there was a consequent decrease of intestinal apoptotic activity. This fall was regulated by silymarin and an insoluble fibers (consisting of 6% lignin, known to be intestinally converted to the active enterolignans, enterodiol and EL) mixture. In this combination, silymarin is a potent ER-β agonist[27,28] and lignans have a substantial phytoestrogenic activity[29]. The silymarin/lignan combination also markedly decreased the number and size of intestinal tumors in $Apc^{Min/+}$ mice[20]. Moreover, it was demonstrated that oophorectomy in female $Apc^{Min/+}$ mice led to an increased number of polyps, which could be abolished by the administration of estrogens[3] or a ER-β selective agonist[4] like coumestrol. Similarly, in intact male $Apc^{Min/+}$ mice, ER-β up-regulation *via* administration of a dietary ER-β selective agonist can reduce the number of adenomas with high grade dysplasia[22]. Giroux *et al*[31] addressed this relationship directly, showing that ER-β deficiency induced by ER-β knockout in female $Apc^{Min/+}$ mice led to enhanced small intestine tumorigenesis.

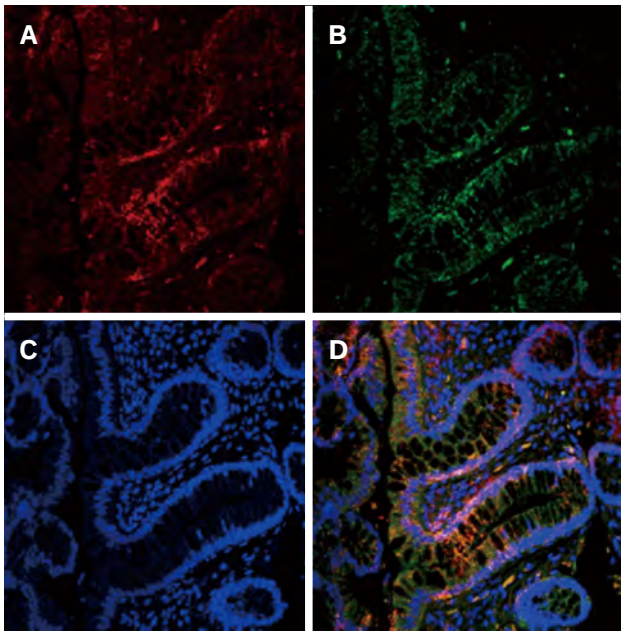

Figure 2 Colocalization estrogen receptor-β receptor and caspase-3. A: Nuclear estrogen receptor (ER)-β expression (red signal); B: Nuclear caspase-3 expression (green signal); C: Nuclear contrast with TOPRO-3 (blue signal); D: merge of the three signals. Both molecules are localized in the upper third of the crypts (Confocal microscope × 400).

The current study was designed to investigate whether a similar dietary supplement could modify ERs levels in humans. ADI (Eviendep® CMD Pharma Limited, United Kingdom), a dietary supplement containing a patented combination of insoluble fibers and dietary phytoestrogens (silymarin and lignans) can substantially increase phytoestrogen levels in humans. We determined whether ADI affects the balance of expression of the ERs, as well as between proliferation and apoptosis, in the colonic mucosa of men and post-menopausal women undergoing surveillance colonoscopy for previous adenomas. The expression of these parameters was evaluated in two study steps, firstly analysis of the situation after active treatment *vs* placebo, and then a further analysis of the same biomarkers in 4 subgroups: supplement *vs* placebo, with, or without recurrence.

In the first step, in comparison with the placebo group, the ADI group showed a mild but significant increase in the mean ER-β protein content (P = 0.04), as well as a trend towards increase of the ER-β/ER-α ratio (P = 0.07), while ER-α protein (P = 0.5) and the mRNA content (P = 0.2) were similar between the two

Table 4 Estrogen receptor-β and estrogen receptor-α, apoptosis and mitosis in non-recurrent patients

Non- recurrent	ER-β protein	ER-β mRNA	ER-β LI	ER-β protein	ER-β mRNA	ER-β LI	TUNEL LI	Caspase-3 LI	Ki-67 LI
ADI (median)	0.805	2.278	47.533	0.423	0.295	15.333	43.777	39.444	55.33
SD	0.13[1]	1.19[2]	15.47[2]	0.06[2]	0.26	9.76	17.24[3]	15.6[3]	20.41
PL (median)	0.773	1.105	34.875	0.532	0.159	16.750	31.375	29.53	45.31
SD	0.13	1.07	16.67	0.11	0.32	8.86	12.93	9.13	11.29

[1]P = 0.04, [2]P = 0.02, [3]P = 0.05 *vs* placebo group. LI: Labeling index; ER: Estrogen receptor; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; ADI: Active dietary intervention.

groups. In our study, we found that ADI induced a statistically significant increase of ER-β protein, whilst ER-β mRNA levels were higher than in placebo group but the difference did not reach significance. Moreover, both the protein and mRNA were significantly increased in the subjects without the finding of polyps at endoscopy. A possible explanation of our result may be due to an increased synthesis as well as a reduced degradation of ER-β protein. Indeed, the transcription, synthesis and degradation of ER protein are processes subjected to mechanisms of complex control through highly regulated adjustment systems. Degradation, in particular, takes place in the cell by a cytosolic complex, the proteasome. It is mediated by ubiquitin that binds to the ligand binding domain of the receptor (ubiquitination)[32,33]. It is possible that these mechanisms may be different in the colonic mucosa prepared or not the development of polyps. Furthermore, TUNEL (P = 0.07) and Ki-67 (P = 0.07) approximated statistical significance. Finally, correlation tests suggested a link between ER-β and apoptosis, thus confirming previous experiences[20]. Moreover, it has been shown that ER-β *in vitro* can up-regulate Lo Vo cells, which in turn activate the caspase 3 and 8 to induce apoptosis in Lo Vo cells, *i.e.*, transient transfected cellular elements used with the aim of evaluating a relationship between ERs and apoptosis[9]. Therefore, in the context of our randomized, double-blind placebo-controlled study, short-term exposure to ADI increased ER-β without substantially affecting ER-α in the normal colonic mucosa of patients who had had a previous polypectomy, regardless of whether they had adenomas at T60 surveillance colonoscopy. The overall increase in the ER-β/ER-α ratio in the ADI group was associated with an increased apoptotic rate in the normal appearing colonic mucosa. Indeed, the real goal was the assessment of biomarkers (estrogen receptors and indicators of proliferation/apoptosis) after a short period of diet. Merely for diet duration, highly significant results were not in our expectations, although a trend of our dietary supplementation to modify the biomarkers is clear from our study.

The second step led us to conclude that ADI administration was unable to affect polyp recurrence, but it should be remembered that the period of administration was not designed for this purpose. Of course, we failed to prove ADI clinical effects since the aim of our study could not be to demonstrate that our dietary supplementation had a chemopreventive effect on the growth of intestinal polyps. Indeed, a long time is needed for the development of these lesions (years), whilst the duration of the diet was very short (2 mo). However, we demonstrated that ADI significantly reduces ER-α and increases ER-β independently of polyp recurrence. Moreover, ADI induces apoptosis through TUNEL and caspase 3 expression. To our knowledge this is the first demonstration that ER-β expression can be modified in humans. If loss of ER-β is a marker of risk for CRC, high levels of ER-β, induced by ADI, might have chemoprotective effects and the mixture of sylimarin and lignans could increase ER-β signaling by increasing and activating the receptor signaling pathway.

As far as the safety of ADI treatment is concerned, there were no adverse events nor changes in blood chemistry. Moreover, the absence of an ER-α expression (mRNA, protein and immunochemically stained cells) increase after ADI supplementation empirically supported this evidence.

As expected, ADI substantially increased phytoestrogen levels, as indirectly measured by urinary enterolignan (ED and EL) levels. Although total urinary enterolignans were decreased in both the ADI and placebo groups at T60 due to dietary restriction of fruits and vegetables prior to colonoscopy, they were significantly increased (8 fold) at T30 in the ADI group. This assay was conducted to demonstrate the adherence to the diet of the subjects as well as to avoid that external dietary factors could affect our results. This evaluation could be an indirect index of phytoestrogen level increase in ADI group. Additionally, literature evidences showed that a high estrous cycle duration, increased corticosterone and 17 β-estradiol levels with an overexpression of receptors ER-α and ER-β in animal model[34] thus suggesting that increased estrogen levels may raise the expression of specific receptors.

Our results suggest that dietary supplements may be able to modulate ER-β, a potentially important biomarker of colon cancer risk. Additional studies of this possibility seem warranted. In conclusion, the role of ER-β in the control of apoptosis and its amenability to dietary intervention are supported by our study. Our data could encourage further, perhaps larger, studies to be undertaken with the aim of demonstrating the secondary chemopreventive potential of ADI against CRC.

COMMENTS

Background

Studies in the adenomatous polyposis coli ($Apc^{Min/+}$) mouse model suggest that estrogen receptor β (ER-β) expression is lower in the intestinal mucosa as compared to APC wild type mice, while dietary supplementation significantly counteracted the intestinal tumorigenesis by increasing ER-β expression. This plot is a start-up to apply in human studies as well.

Research frontiers

Estrogens may have chemopreventive activity for colorectal cancer (CRC), of which sporadic adenoma is a direct precursor. This study assessed the safety and effect of supplementation of a patented blend of dietary phytoestrogens and insoluble fibers, active dietary intervention (ADI), on ER-β expression and on cellular proliferation and apoptosis in patients with recurrent sporadic colonic adenomas. The study was not designed to obtain a chemoprevention for the short diet duration.

Innovations and breakthroughs

ADI is an innovative, brand new mixture which has proved safe in humans and effective in increasing ER-β expression, that may offset intestinal carcinogenesis in patients with a positive history of recurrence for sporadic adenomas

Applications

In the future authors would like to analyze the possibility of prolonging treatment with ADI for longer than in this study (*i.e.*, 2 mo) and to perform endoscopy before and after treatment, compatibly with the compliance of enrolled patients. This could help to increase the strength and to enhance the statistical significance of the results already obtained; the treatment may be shown to play a role in the prophylaxis of adenoma recurrence.

Terminology

ERs, estrogen receptors, α and β, are widespread in human tissue. ER-α is primarily expressed in the breast and endometrium, whereas ER-β is present in a wide variety of tissues that are traditionally considered non-hormonal, including the colon. ER-α and ER-β activation may lead to biologically opposite patterns, as demonstrated in cancer cell lines

Peer review

The protective role of estrogen is a well-known topic in the literature, the interaction of a modified diet (ADI) with the expression of the receptors is an issue that may pose in the future of the implications on chemoprevention of CRC. The work is well structured methodologically, certainly a larger sample could provide greater and more extensive statistical significance

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BRIEF ARTICLE

Optical diagnosis of colorectal polyps using high-definition i-scan: An educational experience

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Abstract

AIM: To examine performances regarding prediction of polyp histology using high-definition (HD) i-scan in a group of endoscopists with varying levels of experience.

METHODS: We used a digital library of HD i-scan still images, comprising twin pictures (surface enhancement and tone enhancement), collected at our university hospital. We defined endoscopic features of adenomatous and non-adenomatous polyps, according to the following parameters: color, surface pattern and vascular pattern. We familiarized the participating endoscopists on optical diagnosis of colorectal polyps using a 20-min didactic training session. All endoscopists were asked to evaluate an image set of 50 colorectal polyps with regard to polyp histology. We classified the diagnoses into high confidence (*i.e.*, cases in which the endoscopist could assign a diagnosis with certainty) and low confidence diagnoses (*i.e.*, cases in which the endoscopist preferred to send the polyp for formal histology). Mean sensitivity, specificity and accuracy per endoscopist/image were computed and differences between groups tested using independent-samples *t* tests. High *vs* low confidence diagnoses were compared using the paired-samples *t* test.

RESULTS: Eleven endoscopists without previous experience on optical diagnosis evaluated a total of 550 images (396 adenomatous, 154 non-adenomatous). Mean sensitivity, specificity and accuracy for diagnosing adenomas were 79.3%, 85.7% and 81.1%, respectively. No significant differences were found between gastroenterologists and trainees regarding performances of optical diagnosis (mean accuracy 78.0% *vs* 82.9%, P = 0.098). Diminutive lesions were predicted with a lower mean accuracy as compared to non-diminutive lesions (74.2% *vs* 93.1%, P = 0.008). A total of 446 (81.1%) diagnoses were made with high confidence. High confidence diagnoses corresponded to a significantly higher mean accuracy than low confidence diagnoses (84.0% *vs* 64.3%, P = 0.008). A total of 319 (58.0%) images were evaluated as having excellent quality. Considering excellent quality images in conjunction with high confidence diagnosis, overall accuracy increased to 92.8%.

CONCLUSION: After a single training session, endoscopists with varying levels of experience can already

provide optical diagnosis with an accuracy of 84.0%.

Key words: Colonoscopy; High-definition i-scan; Optical diagnosis; Colorectal polyps; Training

Core tip: Several studies examined the feasibility of optical diagnosis of colorectal polyps using chromoendoscopy, either dye-based or digital techniques, while only a few studies examined the performances using the high-definition (HD) i-scan technology. In addition, experience on prediction of polyp histology using HD i-scan has been reported in an expert setting only, was based on various classification criteria, and paid only a limited attention to the impact of training. In the current study, at our university hospital, we investigated the effect of training on the diagnostic performances of endoscopists with varying levels of experience. We found that, after a short didactic training session, all endoscopists, trainees as well as gastroenterologists, can predict polyp histology with a mean accuracy of 84%.

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INTRODUCTION

Image enhanced endoscopy techniques, such as dye-based or digital based chromoendoscopy [*i.e.*, narrow band imaging (NBI), high-definition (HD) i-scan or Fujinon Intelligent Color Enhancement (FICE)] have become largely available in daily practice, yet their additional diagnostic value remains unclear[1,2]. Several studies have demonstrated that expert endoscopists can accurately differentiate adenomatous from non-adenomatous polyps (*i.e.*, optical diagnosis) using such technologies[3-5], thereby enabling an individualized treatment, in which small adenomas are resected without pathologic examination, while small non-adenomatous polyps of the rectosigmoid are left *in situ*[6,7]. In addition, optical diagnosis might offer an alternative to histologic diagnosis in case polyps cannot be retrieved for histopathology[8]. This practical approach may downsize the pathology costs and reduce the risk of complications, which in turn would increase the efficiency and cost-effectiveness of colonoscopic procedures[6,9].

A large number of studies have focused on the contribution of image enhanced endoscopy techniques in the detection of colorectal polyps[10-14], while information on their role in histologic characterization is still expanding. Feasibility studies on optical diagnosis have been performed using chromoendoscopy[15,16] or NBI[3,14-18], especially in expert setting, while only a few studies examined the feasibility of HD i-scan technology[5,17,19-22], and none of them has been conducted outside an expert setting. Of note, in a study examining diagnostic performances for optical diagnosis of small colorectal polyps using HD i-scan, Chan *et al*[22] found a 30% difference in accuracy (63% *vs* 93%) between 2 experienced endoscopists, highlighting the need for training and use of standardized criteria. It is important to understand whether incorporating basic principles of optical diagnosis in the education of practicing endoscopists is sufficient to attain and maintain skills in optical diagnosis[2,7]. At our institution, training on recognition of colorectal lesions, with focus on non-polypoid adenomas, is currently incorporated in the educational curriculum[23]. In the current study, we further extended this training by developing a short training module on optical diagnosis using HD i-scan images. We sought to examine the performances in predicting polyp histology in a group of endoscopists with varying levels of experience. We hypothesized that endoscopists can accurately predict polyp histology after a short training session, irrespective of their prior endoscopy practice experience.

MATERIALS AND METHODS

We conducted a prospective educational study at the Maastricht University Medical Center, the Netherlands. No ethical review was required by the local Institutional Review Board.

Colonoscopy and image collection

We created a library of digital photographs using HD i-scan colonoscopes (Pentax, 90i series, 1.3×10^6 pixels). Twin-pictures [surface enhancement (SE); tone enhancement (TE)] were obtained from consecutive colorectal polyps. Location, size and morphology of colorectal polyps were registered. Colorectal polyps were subdivided according to location into proximal lesions (*i.e.*, located proximal from the rectosigmoid) and distal lesions (*i.e.*, detected in the rectosigmoid), as described previously[18,24]. The size of the polyp was categorized as diminutive (*i.e.*, < 6 mm) or non-diminutive (*i.e.*, $\geqslant 6$ mm). Moreover, colorectal lesions were classified according to morphology into polypoid and non-polypoid (*i.e.*, lesions with a height of less than half of their diameter)[25]. All colonoscopies were performed by one colonoscopist (Sanduleanu S), with previous experience on image enhanced endoscopy techniques[26-28], including the HD i-scan technology. After digital documentation, all colorectal polyps were removed and sent for histopathological assessment. Colorectal polyps were assessed by two experienced gastrointestinal pathologists and classified according to the World Health Organization classification[29]. Tubular adenomas, tubulovillous adenomas, villous adenomas and carcinomas were categorized as adenomatous whereas hyperplastic polyps, other (*i.e.*, inflammatory of lesions) and normal tissue were categorized as polyps with a non-

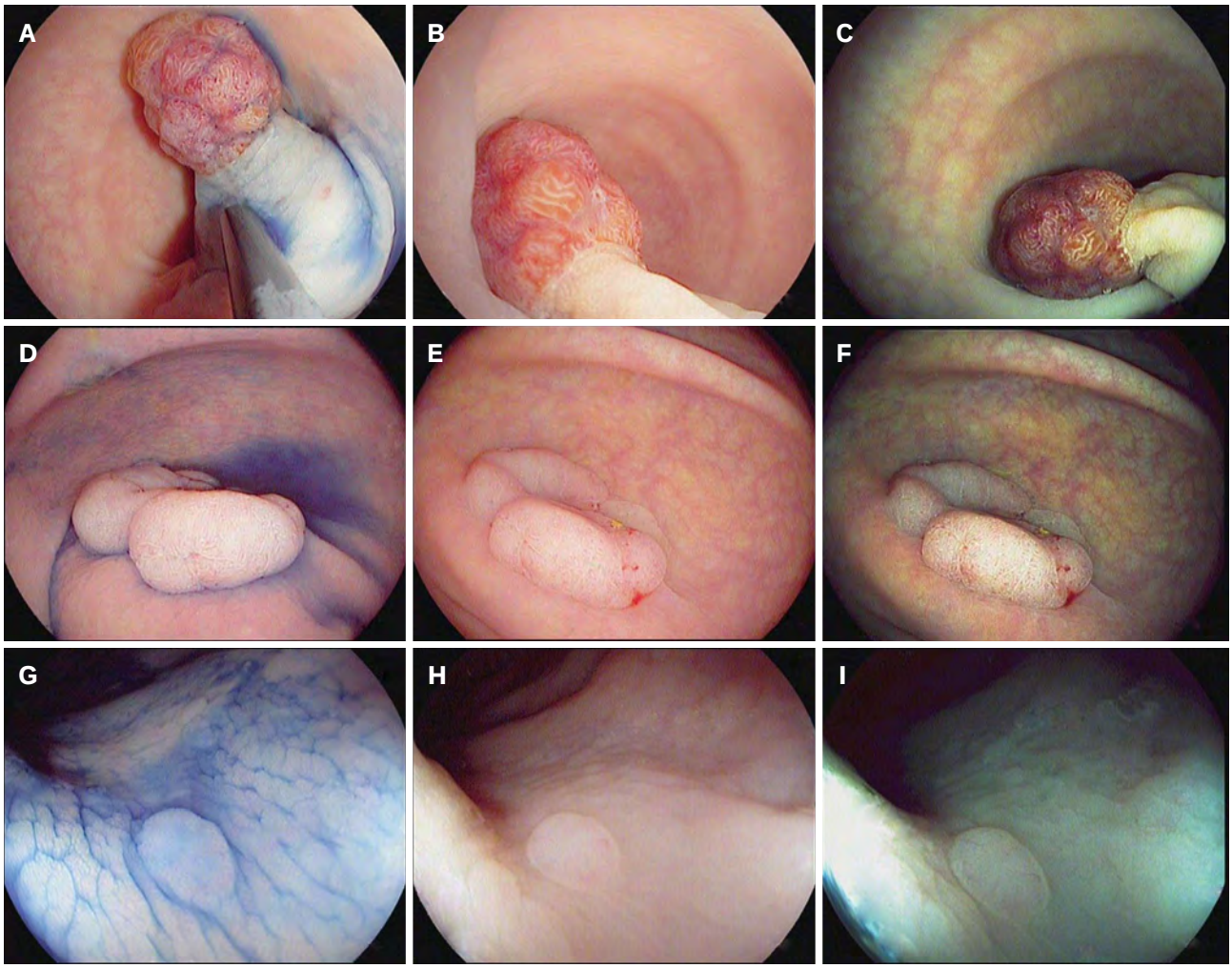

Figure 1 Characterization of colorectal polyps using chromoendoscopy with indigo carmine 0.4% (A, D and G) and high-definition i-scan, surface enhancement (B, E and H)/tone enhancement (C, F and I), the i-scan classification for endoscopic diagnosis. A-C: 20 mm sized pedunculated polyp (Paris Ip). Image enhanced endoscopy shows reddish color, prominent vessels and a type Ⅳ pit pattern of the epithelial surface. Histopathology showed a tubulovillous adenoma with low-grade dysplasia; D-F: 40 mm sized non-polypoid (Paris IIa) lesion. Image enhanced endoscopy shows reddish color, dilated, irregular vessels and a type Ⅳ pit pattern of the epithelial surface. Histopathology showed a tubulovillous adenoma with high-grade dysplasia; G-I: 3 mm sized non-polypoid (Paris IIa) lesion. Image enhanced endoscopy shows pale color, isolated, lacy vessels and a type Ⅱ pit pattern. Histopathology showed a hyperplastic polyp.

adenomatous histology. Sessile serrated adenomas/polyps were categorized as non-adenomatous polyps, in line with recent insights on the pit pattern of these lesions[30-32].

Training and development of classification table

Pilot phase: We established features associated with adenomatous and non-adenomatous histology, using a different set of 20 colorectal polyps which were examined using both chromoendoscopy and HD i-scan (Figure 1). We built upon previously described, international pit pattern classifications using (digital) chromoendoscopy, namely the Kudo classification and the NBI international colorectal endoscopic classification (NICE classification)[33,34]. We developed a simple classification (i-scan classification for endoscopic diagnosis, ICE-classification), in which the following parameters were separately rated: color, epithelial surface pattern and vascular pattern (Table 1). The quality of images was evaluated by 2 independent examiners and categorized as excellent or good.

Study: For the purpose of this educational study, we assembled an image set of twin pictures (SE; TE) from 50 colorectal polyps to create a test which can be accomplished within 1 h. We developed a training module (Microsoft PowerPoint) including the following information: (1) potential clinical relevance of optical diagnosis; (2) basic principles of the HD i-scan technology; (3) differential criteria of adenomatous *vs* non-adenomatous polyps using HD i-scan; and (4) clinical examples. Eleven endoscopists, working at our university center, with varying levels of experience, but who did not routinely use HD i-scan prior to this study were invited to participate. All endoscopists received a 20-min didactic training session aiming to familiarize them with the ICE classification of colorectal polyps using HD i-scan. Hereafter, the endoscopists evaluated an image set of 50 colorectal polyps, placed in random order by a computer-generated random number sequence, assessing polyp histology with high- or low confidence levels. High confidence was defined as

Table 1 Classification system for diagnosis of non-adenomatous and adenomatous colorectal polyps using high-definition i-scan (ICE classification)

	Non-adenomatous	Adenomatous
Color	Pale	Reddish
	Similar to adjacent mucosa	Different from adjacent mucosa
	Indiscrete borders	Clearly demarcated
Surface pattern	Round pits of uniform size, no definite pits	Oval, tubular or branched pits
Vessel pattern	Isolated, lacy vessels	Dilated, irregular vessels

cases in which the endoscopist could assign a diagnosis with certainty and low confidence in cases in which the endoscopist preferred to send the polyp for formal histopathology.

Statistical analysis

Numbers (percentages) were used to describe categorical variables. Sensitivity, specificity and accuracy in predicting polyp histology were calculated using formal histopathology as gold standard. The positive and negative predictive values (PPVs and NPVs) are highly dependent on the prevalences of adenomatous and non-adenomatous polyps. As our image set may not truly reflect the prevalence of these lesions in the general population, we further calculated the PPVs and NPVs based on our sensitivity and specificity data combined with literature data about the prevalence of adenomatous and non-adenomatous polyps in the general population (*i.e.*, approximately 50% of all colorectal polyps detected during colonoscopy are adenomatous, while 50% non-adenomatous[8,35,36]). A two-step procedure was used to account for the dependency between repeated measurements. First, mean sensitivity, specificity and accuracy per endoscopist were computed and the differences between groups (*i.e.*, gastroenterologists *vs* trainees) were tested using independent-samples *t* tests. High *vs* low confidence diagnoses were compared using the paired-samples *t* test. For the effect of location, size, morphology and image quality, the data were first summarized per image and differences between groups (*i.e.*, distal *vs* proximal lesions, diminutive *vs* non-diminutive lesions, polypoid *vs* non-polypoid lesions and excellent *vs* good quality images) were compared using the independent-samples *t* test. We finally used *k* statistics to assess the agreement of predicted histology with formal histopathology. We considered a *kappa* value < 0.20 to indicate poor agreement, 0.21-0.40 slight agreement, 0.41-0.60 moderate agreement, 0.61-0.80 substantial agreement and 0.81-1.00 almost perfect agreement[37]. Two-sided *P* values ≤ 0.05 were considered statistically significant. Statistical analyses were conducted using the SPSS version 20.0.

RESULTS

A total of eleven endoscopists (4 gastroenterologists and 7 trainees) participated. Median (range) duration of colonoscopic experience was 12 years (range 7-15 years) for the participating gastroenterologists and 3 years (range 0-4 years) for the trainees. A total of 50 twin pictures (Figure 2) were incorporated into the test set, consisting of 14 (28.0%) non-adenomatous and 36 (72.0%) adenomatous polyps. Table 2 depicts the endoscopic and pathologic characteristics of the polyps incorporated into our test set. The majority (65.3%) of colorectal polyps were diminutive in size and 49.0% were located in the distal colon. Moreover, 29 (59.2%) cases had a polypoid and 20 (40.8%) a non-polypoid endoscopic appearance[38]. Image quality was rated as excellent in 29 (58.0%) cases and good in the remaining 21 (42.0%) cases.

Table 2 Endoscopic and pathologic characteristics of the colorectal polyps incorporated in our test set *n* (%)

Characteristic	Polyps
Total number of colorectal polyps	50
Location[1]	
Proximal colon	25 (51.0)
Distal colon	24 (49.0)
Size[1]	
Diminutive	32 (65.3)
Non-diminutive	17 (34.7)
Morphology[1]	
Polypoid	29 (59.2)
Non-polypoid	20 (40.8)
Histopathology	
Tubular adenoma	29 (58.0)
Tubulovillous adenoma	6 (12.0)
Carcinoma	1 (2.0)
Hyperplastic polyp	8 (16.0)
Sessile serrated adenoma/polyp	1 (2.0)
Other (*i.e.*, inflammatory)	2 (4.0)
Normal tissue	3 (6.0)
Final histopathology	
Non-adenomatous	14 (28.0)
Adenomatous	36 (72.0)
Image quality	
Excellent	29 (58.0)
Less than excellent	21 (42.0)

[1]Information was missing in 1 case.

Endoscopic features of non-adenomatous and adenomatous polyps

The endoscopic characteristics of non-adenomatous and adenomatous polyps are depicted in Figure 2. The frequencies of endoscopic features predictive of adenomatous and non-adenomatous polyps observed by the eleven endoscopists are shown in Table 3. Reddish color, surface epithelium different from the surrounding mucosa, clear demarcation and oval, tubular or branched pits were frequently seen in adenomatous polyps, whereas thick vessels were observed in only 40.9% of all adenomatous polyps. In contrast, pale color, surface epithelium similar to the adjacent mucosa, indiscrete borders, round pits of uniform size or no definite pits and isolated, lacy vessels were all frequently noticed in non-adenomatous polyps. The surface pattern remained unclear in 9.3% of

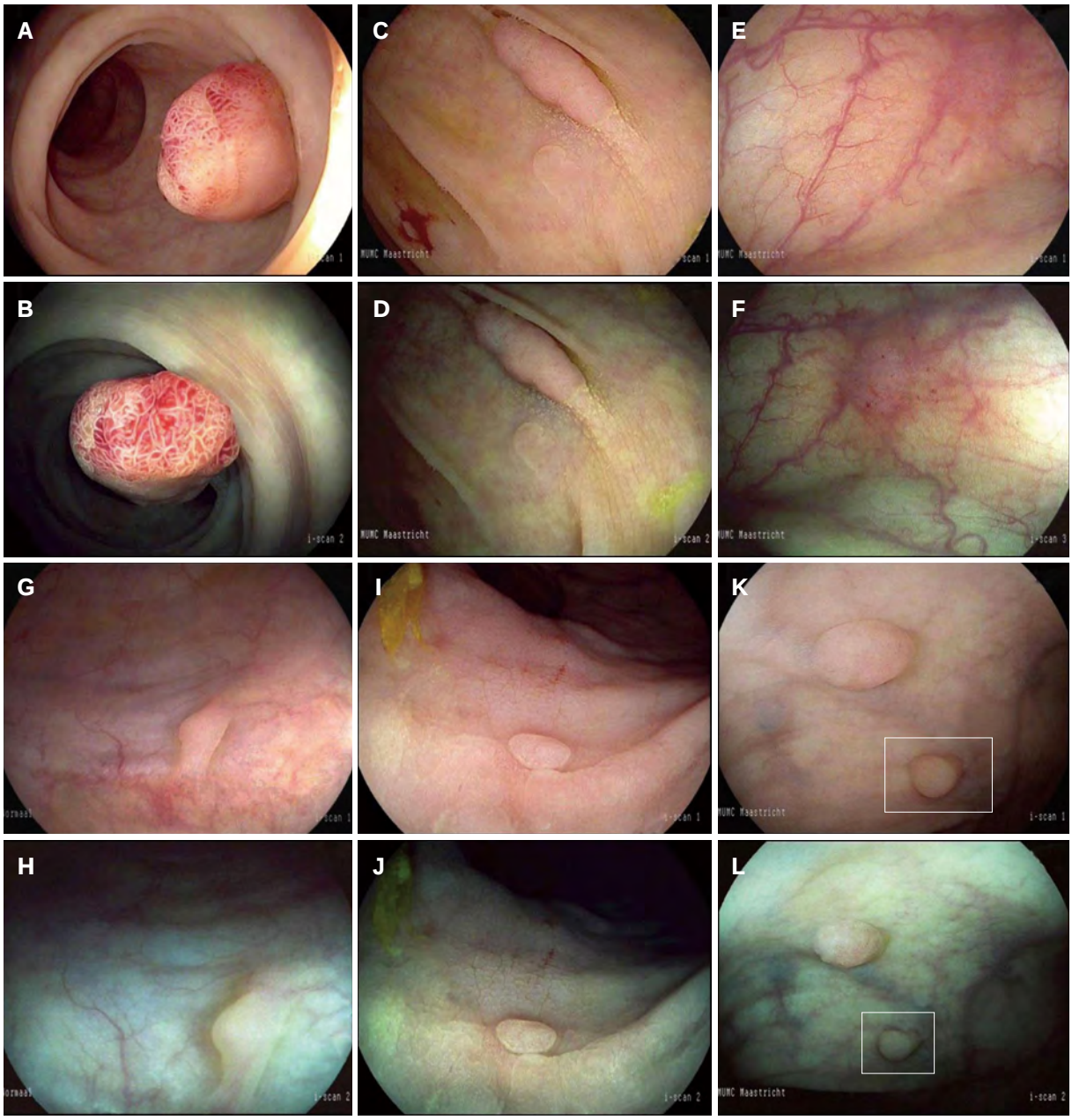

Figure 2 Endoscopic features of non-adenomatous and adenomatous polyps. High-definition (HD) i-scan surface enhancement (SE) (A) and tone enhancement (TE) (B) of a 12 mm pedunculated polyp located in the rectum characterized by a reddish color, prominent vessels and a branch-like (type Ⅳ) pit pattern. All endoscopists diagnosed this lesion as adenomatous with high confidence. Histopathology showed a tubulovillous adenoma with low-grade dysplasia. HD i-scan SE (C) and TE (D) of a 20 mm non-polypoid lesion in the ascending colon characterized by a reddish color, irregular vessels and a tubular (type Ⅲs) pit pattern. Adenomatous histology was predicted by all endoscopists with high confidence. Histopathology showed a tubular adenoma with low-grade dysplasia. HD i-scan SE (E) and TE (F) of a 4 mm non-polypoid lesion located in the proximal colon characterized by a reddish color, surface epithelium different from the surrounding mucosa and indiscrete borders while the vascular pattern could not be clearly observed. Polyp histology was predicted with low confidence by 6 of the 11 endoscopists. Histopathology showed a tubular adenoma with low grade dysplasia. SE (G) and TE (H) of a 3 mm sessile lesion in the sigmoid characterized by a pale color, no vessels and no definite pit pattern. Non-adenomatous histology was predicted with high confidence by 10 of the 11 endoscopists. Histopathology showed normal mucosa. SE (I) and TE (J) of a 3 mm non-polypoid lesion in the rectum characterized by a pale color, no vessels and round pits of uniform size. Non-adenomatous histology was predicted with high confidence by all endoscopists. Histopathology showed a hyperplastic polyp. SE (K) and TE (L) of a 3 mm sessile lesion in the rectum characterized by a pale color and epithelium similar to the adjacent mucosa while the surface pattern and vessel pattern are unclear. Histopathology was predicted with low confidence by 5 out of 11 endoscopists. Histopathology showed a hyperplastic polyp.

all adenomatous polyps and 15.6% of all non-adenomatous polyps, whereas a clear vascular pattern could not be observed in 16.2% of the adenomas.

Diagnostic performances in optical diagnosis using HD i-scan

A total of 550 images were evaluated by the eleven en-

Table 3 Frequencies of endoscopic features predictive of adenomatous and non-adenomatous polyps

	Adenomatous polyps	Non-adenomatous polyps
Features predictive of adenomas		
Reddish	62.6%	2.6%
Different adjacent mucosa	74.7%	11.7%
Clearly demarcated	72.5%	33.1%
Oval, tubular or branched pits	71.7%	11.0%
Thick vessels	40.9%	8.4%
Features predictive of non-adenomas		
Pale	32.3%	92.9%
Similar adjacent mucosa	22.5%	85.7%
Indiscrete borders	26.0%	65.6%
Round pits of uniform size, no definite pits	18.9%	73.4%
Isolated, lacy vessels	42.9%	87.0%
Features unclear		
Color	5.1%	4.5%
Adjacent mucosa	2.8%	2.6%
Demarcation	1.5%	1.3%
Surface pattern	9.3%	15.6%
Vessel pattern	16.2%	4.5%

Table 4 Diagnostic performances of the eleven endoscopists in predicting polyp histology subdivided according to level of experience, location, size, morphology and image quality

	Sensitivity	Specificity	Accuracy	PPV[1]	NPV[1]
Overall	79.3 ± 7.0	85.7 ± 9.6	81.1 ± 4.7	84.7	80.5
Experience					
GE	76.4 ± 6.6	82.1 ± 9.2	78.0 ± 3.7	81.0	77.7
Trainees	81.0 ± 7.1	87.8 ± 9.9	82.9 ± 4.5	86.9	82.2
P value	0.320	0.378	0.098		
Location					
Proximal	74.6 ± 30.9	80.0 ± 29.7	75.6 ± 30.1	78.9	75.9
Distal	84.2 ± 29.0	88.9 ± 8.8	86.0 ± 23.4	88.4	84.9
P value	0.353	0.547	0.187		
Size					
Diminutive	63.2 ± 33.3	90.2 ± 7.8	74.2 ± 29.1	86.6	71.0
Non-diminutive	97.2 ± 7.2	27.3[2]	93.1 ± 18.3	57.2	90.7
P value	< 0.001		0.008		
Morphology					
Polypoid	81.8 ± 25.1	85.1 ± 20.0	83.1 ± 23.0	84.6	82.4
Non-polypoid	75.4 ± 35.0	87.9 ± 13.9	77.3 ± 32.8	86.2	78.1
P value	0.536	0.829	0.470		
Image quality					
Excellent	89.3 ± 23.1	93.5 ± 6.9	90.3 ± 20.3	93.2	89.7
Good	63.6 ± 33.3	77.9 ± 23.4	68.4 ± 30.5	74.2	68.2
P value	0.010	0.117	0.007		

Data are expressed as mean ± SD. [1]Based on our sensitivity and specificity data and literature data regarding prevalences of non-adenomatous and adenomatous polyps; [2]Based on 1 case only, no statistics performed. GE: Gastroenterologist; PPV: Positive predictive value; NPV: Negative predictive value.

doscopists. Mean sensitivity, specificity and accuracy for diagnosing adenomas were 79.3%, 85.7% and 81.1%, respectively (Table 4). The corresponding PPV and NPV were 84.7% and 80.5%, respectively.

Factors influencing the diagnostic performances of the endoscopists

The effects of the level of experience, polyp characteristics and image quality for the prediction of polyp histology on the diagnostic performances of the endoscopists are shown in Table 4. We found no significant difference in mean sensitivity (76.4% *vs* 81.0%, $P = 0.320$), mean specificity (82.1% *vs* 87.8%, $P = 0.378$) and mean accuracy (78.0% *vs* 82.9%, $P = 0.098$) for predicting polyp histology between gastroenterologists and trainees. Of note, mean accuracy for prediction of histology was significantly higher in non-diminutive *vs* diminutive polyps (93.1% *vs* 74.2%, $P = 0.008$). With regard to location, colorectal polyps located in the distal colon were predicted with a higher mean accuracy as compared to colorectal polyps located in the proximal colon, although not statistically significant (86.0% *vs* 75.6%, $P = 0.187$). With regard to the morphology, no significant differences were observed between polypoid and non-polypoid lesions (83.1% *vs* 77.3%, $P = 0.470$). Of all images, 319 (58.0%) were considered to be of excellent quality. As expected, excellent quality images were predicted with a significantly higher mean accuracy as compared to good quality images (90.3% *vs* 68.4%, $P = 0.007$).

High confidence diagnoses

A total of 446 (81.1%) diagnosis could be made with high confidence. High confidence diagnoses corresponded to a significantly higher mean accuracy (84.0% *vs* 64.3%, $P = 0.008$) than low confidence diagnoses. A total of 281 (88.1%) excellent quality images were diagnosed with high confidence. High confidence diagnosis in combination with excellent quality images resulted in a significantly higher mean accuracy (92.8% *vs* 62.3%, $P = 0.014$) as compared to low confidence diagnosis in combination with high quality images. PPVs and NPVs of high confidence diagnoses were 90.2% and 82.8%, respectively.

Agreement with formal histology

The *kappa* value, reflecting agreement of the endoscopist prediction with formal histology, ranged from 0.453 to 0.737 among the eleven endoscopists (Table 5). Analysis of high confidence diagnoses only improved the kappa value ranging from 0.519 to 0.821 indicating a moderate to substantial agreement.

A total of 319 (58.0%) images were rated as excellent quality images. Taking into consideration these images only, the kappa values ranged from 0.621 to 0.828. When considering high confidence diagnoses in combination with excellent quality images, the kappa values ranged from 0.709 to 0.900 indicating substantial to almost perfect agreement.

DISCUSSION

The present study shows that after a short didactic training session on optical diagnosis using HD i-scan, endoscopists can already predict colorectal polyp histology,

Table 5 Kappa values reflecting agreement of the endoscopist prediction with formal histopathology

	Kappa range	Interpretation[1]
All images (n = 50)		
All predictions	0.453-0.737	Moderate–substantial agreement
High confidence predictions	0.519-0.821	Moderate-substantial agreement
Excellent quality images (n = 29)		
All predictions	0.621-0.828	Substantial-almost perfect agreement
High confidence predictions	0.709-0.900	Substantial-almost perfect agreement

[1]Interpretation according to Landis and Koch (reference 37).

with a mean accuracy of 84.0% for high confidence diagnosis. In addition, we found that specific polyp characteristics might negatively affect the diagnostic performances of endoscopists, in particular a diminutive size.

It is important to systematically evaluate the benefit of new image enhanced endoscopy techniques, as these have become widely available and might enable refinement of diagnosis and a more efficient treatment of colorectal polyps in routine practice[1,2]. Accurate optical diagnosis may allow the endoscopist to leave diminutive non-adenomatous polyps of the rectosigmoid in place and remove and discard diminutive adenomas, thereby reducing the pathology costs and potential risks of complications[6,7]. In addition, these technologies may offer a substitute for histopathology in case colorectal polyps are not retrieved for formal histopathology[8] (*i.e.*, about 8% of all colorectal polyps are histologically uninterpretable in routine practice due to lack of retrieval after polypectomy or damaged material[39]).

Several studies demonstrated optical diagnosis is feasible using chromoendoscopy[15,16] or NBI[3,14-17], while only few studies examined the feasibility using HD i-scan technology[5,17,19-22], and none of them was conducted outside an expert setting. We therefore developed a simple classification system of colorectal polyps using HD i-scan (ICE classification) and evaluated the diagnostic performances for prediction of histology in a group of non-expert endoscopists.

In line with previously reported data[5,17,19-22], the current study shows that endoscopists can predict polyp histology with a mean accuracy of 84.0% (high confidence diagnosis) using HD i-scan still images. Hoffman *et al*[5,19] reported accuracy rates ranging from 96% to 99% in two single centre studies. All procedures in these studies were conducted by endoscopists with high-level of expertise on image enhanced endoscopy techniques[40], including HD i-scan, and using electronic magnification, which might explain the high accuracy rates observed in these studies[5,19]. The results of our study are consistent with the findings reported in a study by Pigò *et al*[21] in which an experienced endoscopist predicted the histology of 150 colorectal polyps during colonoscopy with a sensitivity, specificity and accuracy of 95%, 82% and 92%, respectively. Subsequently, still images of these 150 colorectal polyps were evaluated by four other endoscopists after a short teaching session with an overall sensitivity of 88%, specificity of 62% and accuracy of 82%. When evaluating good/excellent quality images only, their overall accuracy improved to 94%, which is in line with our data. A possible explanation for the higher accuracy rates reported by real-time visual assessment compared to still images is perhaps the dynamic observation of lesions (*e.g.*, air insufflation, closer inspection) which may provide additional details. It is therefore plausible that our data underestimate the true diagnostic performances of the endoscopists in routine practice, when polyp histology is predicted by real-time visual assessment.

The present study indicates that a 20 min didactic training session is an effective and efficient way to familiarize endoscopists with the basic principles of optical diagnosis. This is in agreement with existing literature data showing a short and rapid learning curve for accurate evaluation of still images using new image enhanced endoscopy techniques[41,42]. Although several studies showed experienced endoscopists can accurately predict polyp histology[5,14,17], recent data reported lower performances of endoscopists in a community setting[18]. It is possible such lower performances and operator-dependency may reflect different levels of training[6,22], thus emphasizing the need for learning programs comprising both an *ex vivo* and *in vivo* phase. By analogy with our previous experience regarding training in detection and management of non-polypoid lesions[23], we suggest the following steps might be considered. First, a short didactic training session might be offered to the endoscopists to familiarize them with the basic principles of optical diagnosis. Second, video training can help to further shape their skills. Finally, individual feedback during colonoscopy by an experienced endoscopists and self-learning (*i.e.*, comparison of optical diagnosis with formal histopathology) might be important to achieve and maintain proficiency in optical diagnosis.

In this study we paid special attention to identify specific factors which may negatively impact prediction of histology, as this may help to establish targets for improvements. We found that colorectal polyps of diminutive size, proximally located or with non-polypoid morphology are more likely predicted with a lower accuracy. In a recent study by our group, proximal neoplasms appeared to be in general more frequently diminutive and have a non-polypoid shape[27]. Taken together these findings emphasize the need for careful inspection of proximal lesions, which might be both more challenging to detect and also to characterize histologically. Optimal bowel preparation, which may be more difficult to achieve in the proximal colon[43] has definitely a key role in reaching these targets.

In this study, we developed a simple classification system (*i.e.*, the ICE classification) of colorectal polyps by means of HD i-scan, applying similar criteria to those described by the NICE classification using NBI[34]. Although several studies now investigated the performance

characteristics of optical diagnosis using different image enhanced technologies[4,17,26], the outcomes are difficult to compare due to heterogeneity in the diagnostic criteria used. Implementation of a simple, universal classification system, irrespective of the endoscopic technology used, is essential for comparing the outcomes across studies.

According to the American Society for Gastrointestinal Endoscopy recommendations, a NPV of at least 90% should be attained for a safe implementation of a leave in place approach for diminutive non-adenomatous polyps in routine practice[44]. These benchmarks can be already achieved by expert endoscopists[45], yet data in a non-expert setting are scarce and controversial. In our experience, excellent quality images in conjunction with high confidence diagnosis resulted in a mean accuracy of 92.8%. Studies are currently underway at our institution to assess the performance characteristics of real-time optical diagnosis in a non-expert setting.

Some methodological aspects of this study need further consideration. As a strength, endoscopists with varying levels of experience, and who were previously familiarized on the recognition and management of non-polypoid colorectal neoplasms participated[27,28]. Second, we used a simple classification system (ICE classification), based on previously defined and validated criteria[34]. Third, we examined the influence of polyp characteristics, image quality and use of confidence levels on the diagnostic performances to highlight potential targets for training. As a limitation, we used a selection of images consisting of small and larger colorectal polyps whereas optical diagnosis seems to be applicable for small and diminutive polyps only, given the low rate of advanced histology in these lesions[8]. We assume, however, that simple observation and recognition of the pit-patterns may be the first step when developing educational skills in optical diagnosis. Second, the diagnostic performances of the endoscopists involved in this study did not reach the threshold recommended for a safe implementation of a leave in place approach of non-adenomatous polyps[44]. This was not unexpected, as HD i-scan was only recently introduced at our institution, and hence, the endoscopists might have been still early in their learning curves. Third, the diagnostic performances might have been underestimated as endoscopists predicted polyp histology based on still images instead of real-time visual assessment. Finally, in this study we assigned the sessile serrated adenomas/polyps to the group of non-adenomatous polyps. However, as only 1 out of the 50 colorectal polyps examined turned to be a sessile serrated adenoma/polyp, its re-classification into adenomatous lesion is unlikely to change the results and conclusions of this study.

In conclusion, the present study indicates that optical diagnosis using HD i-scan is feasible for endoscopists with varying levels of experience, with a mean accuracy of 84.0% for high confidence diagnosis. A short training module is an effective tool to familiarize the practicing endoscopists with the basic principles of optical diagnosis, although continuous training and practice may be needed to optimize the skills in optical diagnosis.

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COMMENTS

Background

Image enhanced endoscopy techniques, such as chromoendoscopy either dye-based or digital [narrow band imaging, high-definition (HD) i-scan or Fujinon Intelligent Color Enhancement] techniques became largely available. Such technologies offer the opportunity to accurately differentiate adenomas from non-adenomatous polyps, allowing their individualized treatment, which in turn may increase the efficiency of colonoscopic procedures.

Research frontiers

Currently, data about the diagnostic performances using HD i-scan are scarce. None of these studies was conducted outside an expert setting, nor applied standardized diagnostic criteria, and none specifically addressed the impact of training.

Innovations and breakthroughs

In this study, authors found that, after a short didactic session, all endoscopists, trainees as well as gastroenterologists, could already predict polyp histology with a mean accuracy of 84%. Authors additionally propose a simple classification of diagnostic criteria of colorectal polyps using HD i-scan (i-scan classification for endoscopic diagnosis), which might represent a practical tool for optical diagnosis in the community setting.

Applications

This study highlights the importance of training in attaining practical skills in optical diagnosis using new image enhanced endoscopy techniques.

Terminology

The HD i-scan technology is a new image enhanced endoscopy technique that allows digital chromoendoscopy *via* the Pentax EPKi processor.

Peer review

They present the result of examining performances regarding prediction of polyp histology using HD i-scan in a group of endoscopists with varying levels of experience. The authors conclude that endoscopists with varying levels of experience can already provide optical diagnosis with an accuracy of 84.0%. This is a relevant study as optical diagnosis of colorectal polyps might offer an alternative to histologic diagnosis and may downsize the pathology costs and reduce the risk of complications. This manuscript is a well-written paper, however, it may be refined with a minor revision by authors.

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BRIEF ARTICLE

Low rates of adherence for tumor necrosis factor-α inhibitors in Crohn's disease and rheumatoid arthritis: Results of a systematic review

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Abstract

AIM: To investigate adherence rates in tumor necrosis factor-α (TNF-α)-inhibitors in Crohn's disease (CD) and rheumatoid arthritis (RA) by systematic review of medical literature.

METHODS: A structured search of PubMed between 2001 and 2011 was conducted to identify publications that assessed treatment with TNF-α inhibitors providing data about adherence in CD and RA. Therapeutic agents of interest where adalimumab, infliximab and etanercept, since these are most commonly used for both diseases. Studies assessing only drug survival or continuation rates were excluded. Data describing adherence with TNF-α inhibitors were extracted for each selected study. Given the large variation between definitions of measurement of adherence, the definitions as used by the authors where used in our calculations. Data were tabulated and also presented descriptively. Sample size-weighted pooled proportions of patients adherent to therapy and their 95%CI were calculated. To compare adherence between infliximab, adalimumab and etanercept, the adherence rates where graphed alongside two axes. Possible determinants of adherence were extracted from the selected studies and tabulated using the presented OR.

RESULTS: Three studies on CD and three on RA were identified, involving a total of 8147 patients (953 CD and 7194 RA). We identified considerable variation in the definitions and methodologies of measuring adherence between studies. The calculated overall sample size-weighted pooled proportion for adherence to TNF-α inhibitors in CD was 70% (95%CI: 67%-73%) and 59% in RA (95%CI: 58%-60%). In CD the adherence rate for infliximab (72%) was highercompared to adalimumab (55%), with a relative risk of 1.61 (95%CI: 1.27-2.03), whereas in RA adherence for adalimumab (67%) was higher compared to both infliximab (48%) and etanercept (59%), with a relative risk of 1.41 (95%CI: 1.3-1.52) and 1.13 (95%CI: 1.10-1.18) respectively. In comparative studies in RA adherence to infliximab was better than etanercept and etanercept did better than adalimumab. In three studies, the most consistent factor associated with lower adherence was female gender. Results for age, immunomodulator use and prior TNF-α inhibitors use were conflicting.

CONCLUSION: One-third of both CD and RA patients treated with TNF-α inhibitors are non-adherent. Female gender was consistently identified as a negative determinant of adherence.

Key words: Adherence; Tumor necrosis factor-α inhibitors; Systematic review; Crohn's disease; Rheumatoid arthritis

Core tip: This study assessed adherence with tumor ne-

crosis factor-α (TNF-α) inhibitors in Crohn's disease (CD) and rheumatoid arthritis (RA) by systematic review. We found only two-third of the patients with CD and RA receiving TNF-α inhibitors adherent to therapy. Definitions of measurement of adherence varied widely between studies and there is no clarity on what levels of adherence are required for optimal results of therapy. Future research on adherence should focus on therapy outcome, by using uniform definitions of adherence.

Fidder HH, Singendonk MMJ, van der Have M, Oldenburg B, van Oijen MGH. Low rates of adherence for tumor necrosis factor-α inhibitors in Crohn's disease and rheumatoid arthritis: Results of a systematic review. *World J Gastroenterol* 2013; 19(27): 4344-4350 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i27/4344.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i27.4344

INTRODUCTION

Crohn's disease (CD) and rheumatoid arthritis (RA) are chronic inflammatory conditions characterized by episodes of remission and flare-ups that have a major impact on the patient's physical, emotional and social well-being. The management of these diseases has been profoundly modified by the introduction of tumor necrosis factor-α (TNF-α) inhibitors, *i.e.*, infliximab, adalimumab and etanercept (only in RA), and these agents have become an integral part of the therapeutic arsenal. In RA TNF-α inhibitors have shown to rapidly improve symptoms, retard radiographic disease progression and improve functional status and health-related quality of life[1]. Also in CD TNF-α inhibitors are highly efficacious for induction and maintenance therapy and reduce rates of hospitalization and surgery[2].

Clinical effectiveness of TNF-α inhibitors is dependent on adequate adherence, and failure to stick to the prescribed drug regimen contributes to failure of treatment and disease recurrence. For both RA and CD, good adherence is associated with more effective treatment, including limited loss of response[3,4]. Fernández-Nebro *et al*[4] reported that in CD the probability of premature failure of TNF-α inhibitors was 61% less in patients with good adherence.

For patients with inflammatory bowel disease, reported non-adherence rates for oral medication range in most studies between 30%-45%[5]. Low adherence has an impact on healthcare budgets by increasing number of treatment failures, subsequent diagnostic procedures and unnecessary change of therapy. A Cochrane review on adherence concluded that improving medicine intake may have a far greater impact on clinical outcomes than an improvement in treatments[6]. In line with this statement Kane *et al*[7] pointed out that all-cause and CD-related medical costs were 81% and 94% higher, respectively, for non-adherent patients in comparison to adherent patients.

Although it has been 15 years since TNF-α inhibitors were introduced for the treatment of CD and RA, our understanding of patient's compliance to TNF-α inhibitors is minimal and reported rates of adherence vary widely, depending on the definition of adherence. In order to assess the adherence rates for TNF-α inhibitors in CD and RA, we systematically reviewed literature and performed meta-analysis.

MATERIALS AND METHODS

We conducted a structured search of PubMed to identify potentially relevant English-language publications that assessed adherence to TNF-α inhibitors in CD. In our search strategy, the following keywords and search strings were used: (infliximab OR Remicade OR adalimumab OR Humira OR etanercept OR Enbrel OR anti-TNF OR biological) AND Crohn AND (adherence OR compliance). In the same way, we conducted a search of PubMed to identify potentially relevant publications that assessed adherence with TNF-α inhibitors in RA, thereby substituting the search term "Crohn" for "RA". During the whole process the exact reporting guidelines as described in the PRISMA statement (www.prisma-statement.org) were followed.

Two investigators (Fidder HH and Singendonk MMJ) independently reviewed identified titles and abstracts of all citations in the literature search results. Potentially relevant studies were retrieved and the following predefined inclusion criteria were applied: (1) original research article; (2) adult patients; (3) definition of adherence provided; (4) methodology of measurement of adherence described; and (5) data based on number of patients. From selected abstracts, full articles were retrieved, reviewed and included if they contained data regarding adherence to TNF-α inhibitors in CD or RA. Disagreements between reviewers were adjudicated by discussion and consensus with a third-party arbiter (MvO).

The following information was extracted for each selected study: TNF-α inhibitors used, study design, sample size, definition of adherence measurement and the levels of adherence reported. We also sought for determinants of adherence in included studies, and tabulated the following factors of interest by using the presented OR: gender, age, duration of disease and therapy, prior and concomitant therapy. The data obtained from the selected articles describing adherence with TNF-α inhibitors were tabulated and also presented descriptively.

Given the large variations in definitions and methodologies of measurement of adherence and patient samples of the studies included, we used the definitions of adherence used by the authors in order to calculate the sample size-weighted pooled proportions of patients that were adherent to therapy and to compare adherence rates between adalimumab, infliximab and etanercept. To portray these data for each therapeutic agent, we graphed them alongside two axes; adherence rate re-

Table 1 Characteristics of included studies on Crohn's disease and rheumatoid arthriti

	Number of patients	Anti-TNF treatment	Definition of adherence	Adherence
Crohn's disease				
Kane *et al*[7]	571	Infliximab	≥ 7 infusions in first year of treatment	66%
Billioud *et al*[17]	108	Adalimumab	Neither delaying nor missing > 1 injection in 3 mo	55%
Kane *et al*[18]	274	Infliximab	No "No show" designation during study period	85%
Rheumatoid arthritis				
Borah *et al*[19]	2537	Etanercept	Medication possession ratio ≥ 0.80	71%
	1292	Adalimumab		67%
Harley *et al*[20]	853	Etanercept	Medication possession ratio ≥ 0.80	68%
	141	Infliximab		81%
Li *et al*[21]	1359	Etanercept	Proportion of days covered ≥ 0.80	32%
	1012	Infliximab		43%

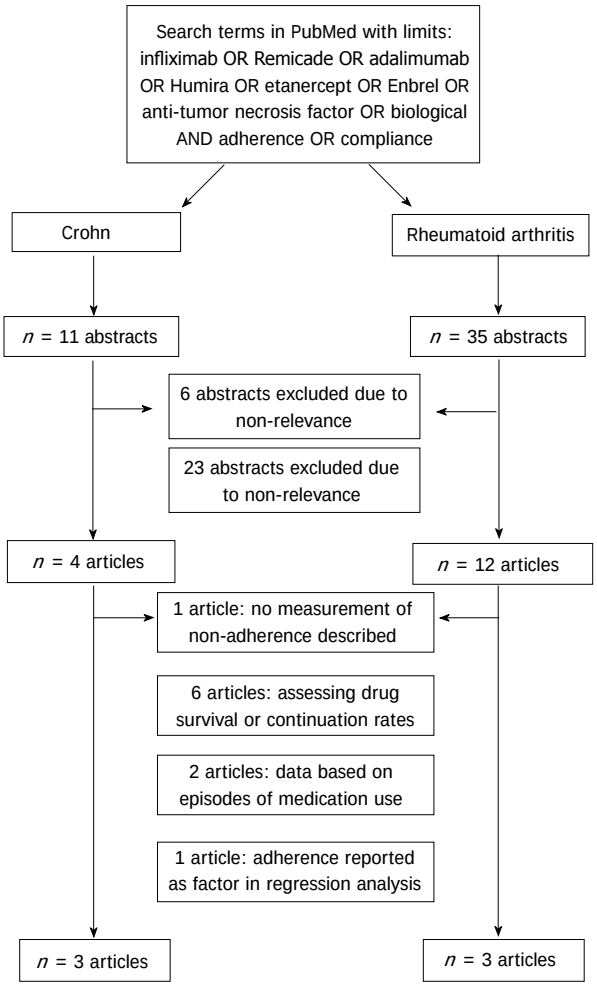

Figure 1 Results of literature search.

ported by the selected study for each agent and number of patients included.

RESULTS

Literature search

The search identified 11 studies regarding CD and 35 regarding RA, of which respectively 7 and 32 articles were excluded in two selection procedures. The main reason for exclusion of studies on CD was the absence of data on adherence or compliance (Figure 1)[8]. Exclusion of studies on RA was mainly based on the fact that these studies assessed drug survival or continuation rates only, but not compliance and/or adherence[4,9-13]. Three other studies were excluded: two studies based their data on episodes of medication use instead of number of patients[14,15] and one study only reported adherence as a factor in regression analysis[16]. Six articles that met our inclusion criteria remained, three on CD[7,17,18] and three on RA[19-21]. Characteristics of included studies are shown in Table 1.

CD

Two out of three studies on CD reported on adherence to infliximab[7,18] and one on adalimumab[17]. There were no comparative studies. We identified considerable variation in the definitions and methodologies of measuring adherence between studies. The adherence rate for all TNF-α inhibitors as calculated by the sample size-weighted pooled proportion was 70% (95%CI: 67%-73%). For adalimumab, the reported adherence rate was 55%[17] and for infliximab the reported rates were 66%[7] and 85%[18] (Table 1). Adherence to adalimumab (55%) was statistically significantly lower than infliximab (72%) therapy, with a relative risk of 0.76 (95%CI: 0.64-0.91) (Figure 2)[7,17,18].

RA

For RA we only found comparative studies: two of the included studies assessed adherence to both etanercept and infliximab[20,21] and one study compared etanercept with adalimumab[21]. In RA, measurement of adherence was based on medication possession ratios in two studies and one study measured adherence as the proportion of days covered (PDC). Medication possession rate (MPR) is defined as the sum of days supply for all fills in period divided by the number of days in a period. PDC is the number of days in a period covered by medication divided by the days in a period. In all studies patients were considered adherent if MPR or PDC was ≥ 0.8.

Reported adherence rates ranged from 32% to 81% (Table 1)[19-21]. The overall adherence rate was 59% (95%CI: 58%-60%), as calculated by the sample size-weighted pooled proportion. In the two studies compar-

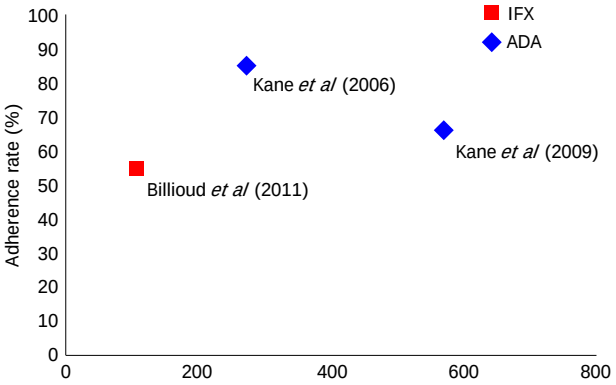

Figure 2 Adherence rates reported by the included studies on Crohn's disease for infliximab and adalimumab. The vertical axis rank-orders the therapeutic agents by the adherence rates and the horizontal axis by number of patients included. IFX: Infliximab; ADA: Adalimumab.

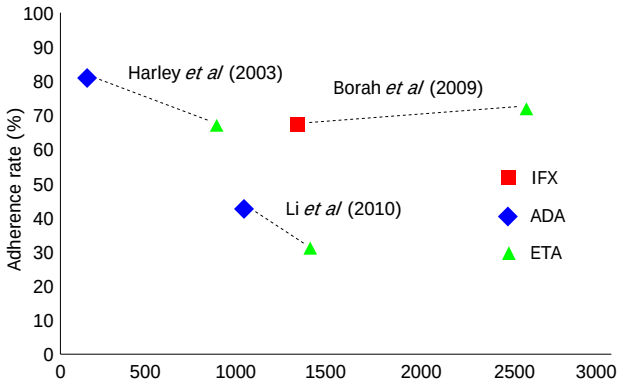

Figure 3 Adherence rates reported by the included studies on rheumatoid arthritis for infliximab, adalimumab and etanercept. The vertical axis rank-orders the therapeutic agents by the adherence rates and the horizontal axis by number of patients included. IFX: Infliximab; ADA: Adalimumab; ETA: Etanercept.

ing infliximab to etanercept adherence to infliximab was consistently higher (Figure 3)[20,21]. In the study comparing etanercept to adalimumab, patients using etanercept were slightly more adherent than adalimumab users[19]. After pooling the published adherence rates, we found the highest adherence rate for adalimumab (67%) compared to both infliximab (48%) and etanercept (59%), with a relative risk of 1.41 (95%CI: 1.3-1.52) and 1.13 (95%CI: 1.10-1.18) respectively.

Predictors

Five studies have formally explored possible predictors of (non)-adherence to TNF-α inhibitors (Table 2)[7,19-21]. The most consistent factor associated with lower adherence (reported by three studies) was female gender[7,20,21]. Increasing duration of therapy was reported as a factor negatively associated with adherence and duration of disease as factor associated with better adherence[7,18]. Results for age, immunomodulator use and prior TNF-α inhibitors use were conflicting[7,19].

DISCUSSION

We systematically reviewed adherence rates to TNF-α inhibitors in CD and RA. Although literature on adherence rates to TNF-α inhibitors in other rheumatological diseases exists, we did not assess adherence for these diseases given the relatively small patient numbers. Given the central position of TNF-α inhibitors in the management of CD and RA and the importance of adherence for effective treatment, the total number of six studies that adequately assessed adherence to anti-TNF therapy was surprisingly low. Our analysis of the included studies on CD and RA has three key findings. First, we found that adherence to TNF-α inhibitors in CD and RA is low, with only two-thirds of the patients being adherent to therapy. Second, adherence rates for adalimumab were lower compared to infliximab in CD. Last, we found that female gender was consistently associated with non-adherence to TNF-α inhibitors.

Our findings of rather low adherence to TNF-α inhibitors are in line with figures reported for adherence to oral medication in inflammatory bowel disease, that range between 28% and 93% of patients adherent to prescribed therapy[5,22,23]. In a comparative cohort study mesalazine and azathioprine were associated with the lowest compliance[24]. In RA the adherence rates for TNF-α inhibitors has been reported between 30% and 80%, depending on definitions used[25]. The low adherence to TNF-α inhibitors are especially worrisome since long treatment intervals are associated with infusion reactions and loss of response as result of increased antibody formation against TNF-α inhibitors[26-28]. Moreover, non-adherence in adalimumab treated patients predicts higher hospitalization rates and increased medical service costs[7]. Adherence to continuous maintenance treatment with TNF-α inhibitors is important for the efficacy of treatment.

Although the different routes and schedules of administration of TNF-α inhibitors and the different measures of adherence across studies may impede a direct comparison, we found lower adherence rates with adalimumab and etanercept. In RA, pooling the adherence rates gave higher adherence for adalimumab over infliximab but all comparative studies reported higher adherence rates for infliximab as well. Differences in patient numbers between studies and a difference between the number of studies used for calculating the pooled adherence rates for the single treatment modalities are underlying this conflicting finding. In addition, Li *et al*[21] assesses adherence rates with etanercept and infliximab by using the PDC, which is a more conservative estimate for adherence compared to the MPR. Discrepant adherence between treatment options may be explained by a number of reasons including dosing frequency and route of administration. Etanercept and adalimumab are self-administered subcutaneously, whereas infliximab is administered intravenously, by a healthcare professional in a clinical setting. As patients need to visit infusion sites, adherence is more controllable in favor of infliximab. Indeed, in the two comparative studies between infliximab and etanercept[20,21], higher adherence was found for the intravenously administered infliximab. In the study of

Table 2 Determinants of adherence in Crohn's disease and rheumatoid arthritis

	Studies on Crohn's disease			Studies on rheumatoid arthritis	
	Kane *et al*[7]	Billioud *et al*[17]	Kane *et al*[18]	Borah *et al*[19]	Li *et al*[21]
Female gender	OR < 1		OR < 1; P < 0.05		OR < 1
Increasing age		OR < 1			OR > 1
Immunomodulator use	OR > 1		OR < 1		OR > 1; P < 0.05[1]
Prior biologic use	OR < 1; P < 0.05			OR > 1; P < 0.05	
Increasing duration of therapy			OR < 1; P < 0.05		
Increasing disease duration		OR > 1; P < 0.05			

[1]Significant at P < 0.05 for age 55-64 years (OR = 1.49).

Borah *et al*[19] adherence of etanercept - which is injected once or twice a week - was slightly higher than adalimumab, which is mostly self-administered using a bi-weekly schedule. But still, even for the more controllable intravenously administered modalities, adherence rates are well below 100%.

In order to improve adherence, it is essential to identify and understand risk factors for non-adherence. Although several factors were reported as determinants of adherence by the reviewed studies, we did not find any of these factors consistently associated with non-adherence, with the exception of female gender. This is in contrast with a previous study on oral therapies in inflammatory bowel disease that identified female gender as a positive determinant of adherence[29]. Also in other fields of medicine attempts to identify clinical, demographic and treatment factors that consistently predict adherence have proven quite disappointing[6]. Jackson *et al*[5] who systematically reviewed factors associated with non-adherence to oral medication specifically for inflammatory bowel disease pointed out that that simple factors such as demographics or treatment regimens could not reliably predict adherence. Far more important determinants were psychological distress, patients' beliefs about therapy, and doctor-patient interactions[5]. For the clinician, it is essential to be aware of the importance of psychological factors in non-adherence and that significant improvement in terms of adherence may be achieved by fine-tuning doctor-patient communication and addressing patients' individual beliefs about disease and medications.

Our study has several strengths and limitations. We provided a detailed and systematic review of published literature and studied adherence rates on both CD and RA with TNF-α inhibitors. Koncz *et al*[30] reviewed compliance and persistence for TNF-α inhibitors only in RA patients. Contrary to this review we included only studies that assessed adherence rates based on number of patients included and reported individual adherence results. Furthermore, we identified potential predictors of adherence. In the evaluation of potential predictors of adherence, we had no access to original research data and therefore we were dependent on the analyses performed by others and could not perform an individual patient data meta-analysis. The major drawback of this approach is the lack of agreement in terminology and methodologies for measurement of therapy behaviour, making the results of studies assessing this issue difficult to interpret and compare. Compliance, persistence and adherence are definitions used for assessing this. The medication possession ratios of infliximab, etanercept and adalimumab ranged between 63% and 90% and PDC around 40%. The PDC provides a more conservative estimate of adherence compared to MPR when patients are switching drugs or using dual-therapy in a class. The differences between these terms have been defined in a report for the National Institute for Health Research by Mikkelsen *et al*[31] Compliance can be defined as "the extent to which the patients" behaviour matches the prescriber's recommendations' quantified as "a percentage of number of doses taken or therapy days available in relation to a fixed period of time". Persistence refers to how long the patient takes the medicine for and is therefore measured by units of time. Adherence covers both these aspects of medication taking behaviour. Although these definitions seem clear, methodologies of measurement vary and therefore hinder comparability of findings. These differences in measurement of adherence cannot be shrugged aside, but at this stage, based on currently available literature, we provided more insight in the TNF-α therapy behaviour of patients with CD and RA. Despite the mentioned limitations, it is still clear that adherence with TNF-α therapy is low for all different modalities. However, there is no clarity on what levels of adherence are required for optimal results of therapy yet and these levels might vary depending on disease activity and localization. Therefore, in the future adherence should be assessed in combination with therapy outcome in order to determine optimal treatment schedules by using uniform definitions of compliance, adherence and persistence.

In conclusion, through systematic review we found that only two-third of the patients with CD and RA receiving TNF-α inhibitors were adherent to therapy. Developing methods that properly assess medication adherence could provide tools for improvement of therapy outcome. Although female gender was identified as a negative determinant of adherence, one should be aware that mechanisms underlying adherence are complicated and probably not determined by simple patient's and treatment characteristics.

COMMENTS

Background

Crohn's disease (CD) and rheumatoid arthritis (RA) are chronic inflammatory conditions characterized by episodes of remission and flare-ups that have a major impact on the patient's physical, emotional and social well-being. The management of these diseases has been profoundly modified by the introduction of tumor necrosis factor-α (TNF-α) inhibitors.

Research frontiers

Clinical effectiveness of TNF-α inhibitors is dependent on adequate adherence, and failure to stick to the prescribed drug regimen contributes to failure of treatment and disease recurrence. For both RA and CD, good adherence is associated with more effective treatment, including limited loss of response.

Innovations and breakthroughs

The analysis of the included studies on CD and RA has three key findings. First, the authors found that adherence to TNF-α inhibitors in CD and RA is low, with only two-thirds of the patients being adherent to therapy. Second, adherence rates for adalimumab were lower compared to infliximab in CD. Last, they found that female gender was consistently associated with non-adherence to TNF-α inhibitors.

Applications

The authors found that only two-third of the patients with CD and RA receiving TNF-α inhibitors were adherent to therapy. Developing methods that properly assess medication adherence could provide tools for improvement of therapy outcome.

Peer review

This article investigated adherence rates inTNF-α-inhibitors in CD and RA by systematic review of medical literature, and is informative and well-presented.

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BRIEF ARTICLE

A new pancreaticojejunostomy technique: A battle against postoperative pancreatic fistula

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Abstract

AIM: To present a new technique of end-to-side, duct-to-mucosa pancreaticojejunostomy with seromuscular jejunal flap formation, and insertion of a silicone stent.

METHODS: We present an end-to-side, duct-to-mucosa pancreaticojejunostomy with seromuscular jejunal flap formation, and the insertion of a silicone stent. This technique was performed in thirty-two consecutive patients who underwent a pancreaticoduodenectomy procedure by the same surgical team, from January 2005 to March 2011. The surgical procedure performed in all cases was classic pancreaticoduodenectomy, without preservation of the pylorus. The diagnosis of pancreatic leakage was defined as a drain output of any measurable volume of fluid on or after postoperative day 3 with an amylase concentration greater than three times the serum amylase activity.

RESULTS: There were 32 patients who underwent end-to-side, duct-to-mucosa pancreaticojejunostomy with seromuscular jejunal flap formation. Thirteen of them were women and 19 were men. These data correspond to 40.6% and 59.4%, respectively. The mean age was 64.2 years, ranging from 55 to 82 years. The mean operative time was 310.2 ± 40.0 min, and was defined as the time period from the intubation up to the extubation of the patient. Also, the mean time needed to perform the pancreaticojejunostomy was 22.7 min, ranging from 18 to 25 min. Postoperatively, one patient developed a low output pancreatic fistula, three patients developed surgical site infection, and one patient developed pneumonia. The rate of overall morbidity was 15.6%. There was no 30-d postoperative mortality.

CONCLUSION: This modification appears to be a significantly safe approach to the pancreaticojejunostomy without adversely affecting operative time.

Key words: Whipple; Pancreaticojejunostomy; Technique; Seromuscular jejunal flap; Pancreatic fistula

Core tip: Pancreaticojejunostomy represents one of the most challenging technical aspects of the Whipple procedure, mainly due to its failure, and to the resulting morbidity and mortality rates. Several technical variations have been proposed, in an effort to minimize postoperative pancreatic fistula rates. The technique we describe is an end-to-side, duct-to-mucosa two-layer pancreaticojejunostomy intended to promote enhanced healing process, through the creation of a seromuscular

jejunal flap. This technique appears to be safe and reliable; however, these are preliminary results.

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INTRODUCTION

The first pancreaticoduodenectomy was performed by a German surgeon, Kausch, in 1909[1-3]. It has been considered the surgical procedure of choice for ampullary cancer, after Whipple *et al*[4] had described three cases in 1935. Nowadays, it has become the standard procedure in the management of pancreatic head and periampullary carcinoma[2]. In recent years, the mortality rate of pancreatico-duodenectomy has been decreased to below 5%[5-7]. However, the postoperative morbidity rate remains high, ranging from 30% to 50%[7-10]. Pancreatic fistula[11,12] is the most common complication and its reported incidence varies from 2% to 40%[8,11,13]. Several different anastomotic surgical techniques have been used, in order to minimize pancreatic fistula occurrence after pancreaticoduodenectomy, although it is still debated which of them has any clear advantage[2,9,10,14,15]. We present a modification for duct to mucosa end-to-side pancreaticojejunostomy, with a seromuscular jejunal flap, in order to increase the safety of the anastomosis.

MATERIALS AND METHODS

During the period January 2005 to March 2011, 32 consecutive patients underwent pancreaticoduodenectomy by the same surgical team. There were 13 women and 19 men, with a mean age of 64.2 years (range 55-82 years). The underlying diseases of these patients are shown in Table 1. The surgical procedure performed in all cases was classic pancreaticoduodenectomy, without preservation of the pylorus. The diagnosis of pancreatic leakage was defined as a drain output of any measurable volume of fluid on or after postoperative day 3 with an amylase concentration greater than three times the serum amylase activity[11].

Technique

A scalpel is used to sharply transect the pancreas at the level of the portal vein. Hemostasis of the bleeding points of the pancreatic stump is achieved either with 4-0 non-absorbable suture and/or with electrocautery. After the pancreaticoduodenectomy specimen has been removed, the pancreatic remnant is dissected free of the underlying structures for a distance of approximately 2 cm. The transected jejunum is brought through the bed of the resected duodenum (*i.e.*, posterior to the mesenteric vessels).

Table 1 Underlying diseases of the patients who underwent end-to-side, duct to mucosa pancreaticojejunostomy with seromuscular jejunal flap formation after pancreaticoduodenectomy

Disease	Patients (*n*)
Pancreatic head carcinoma	15
Ampullary carcinoma	12
Distal common bile duct carcinoma	5
Total	32

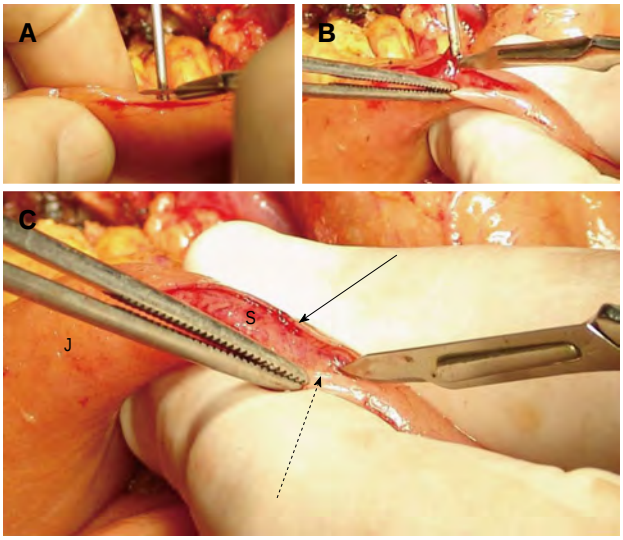

Figure 1 Preparation of the jejunal stump. A: Incision on the jejunum; B: Seromuscular flap formation; C: The seromuscular layers are dissected free from the submucosa. J: Jejunum; S: Submucosa; Arrow: Posterior seromuscular flap; Dotted arrow: Anterior seromuscular flap.

The jejunal seromuscular layer is incised starting about 2 cm distal to the jejunal stump, along the antimesenteric border. The length of this incision is just smaller than the cephalo-caudal diameter of the pancreatic stump. Using a scalpel the seromuscular layer of the jejunum is dissected free from the underlying submucosa, towards both sides of the aforementioned incision, in order to create two seromuscular flaps (*i.e.*, one dorsal and one ventral flap), and to expose the underlying submucosa, which must remain intact (Figure 1). The extent of the dissection is determined by the antero-posterior diameter of the pancreatic stump, in order to fit the surface of the pancreatic cut edge on the surface area of the exposed mucosa.

Following this, a segment of nelaton catheter is inserted into the main pancreatic duct, and is fixed with a 4-0 absorbable monofilament suture (polydioxanone, PDS Ⅱ, Ethicon, Inc.). The tube girth is selected to exactly fit the diameter of the main pancreatic duct. On the intraductal part of the stent, several holes are created on different positions, at a distance of 1 cm from each other, in order to ensure uninhibited outflow of the pancreatic fluid. The extraductal part of the stent left is about 5 cm in length.

The next step is to create the first of all four suturing

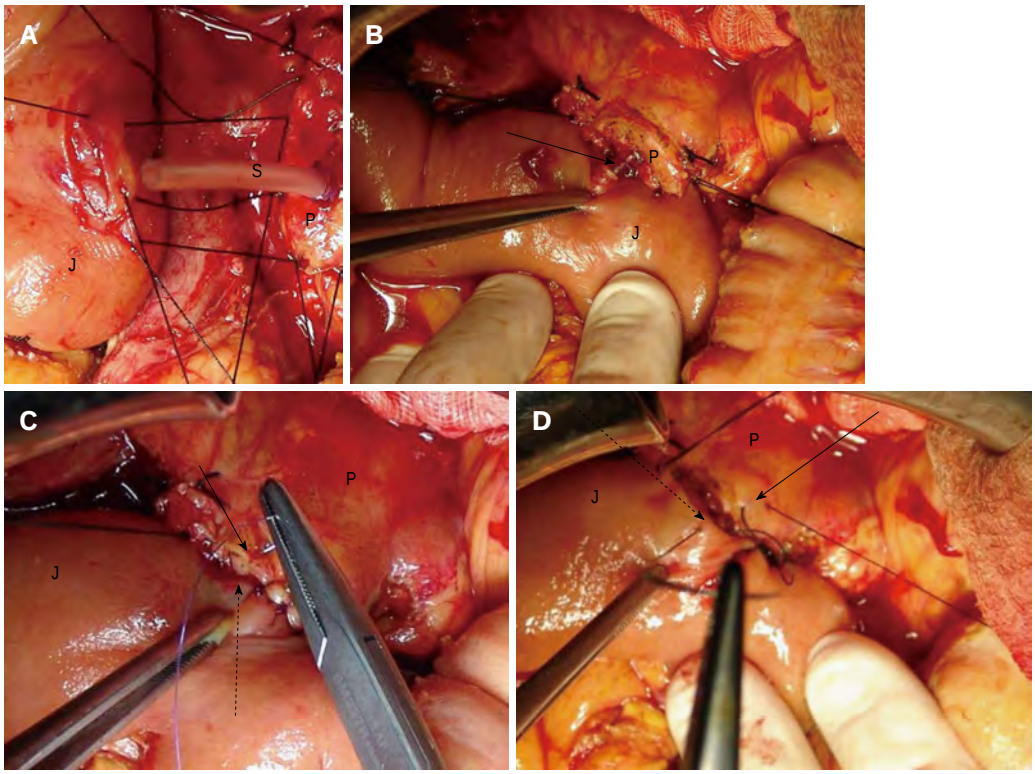

Figure 2 The posterior, anterior external layer of sutures and stent in place. A: The posterior (dorsal) external layer of sutures; B: Stent in place. Arrow: The stent enters the jejunal small opening, which has been created in the middle of the dissected submucosal surface; C: The anterior (ventral) internal layer of sutures. Arrow: Anterior pancreatic cut edge border; Dotted arrow: Anterior seromuscular flap; D: The anterior (ventral) external layer of sutures. Arrow: Anterior pancreatic part of the capsular parenchyma; Dotted arrow: Anterior part of the jejunal seromuscular layer. J: Jejunum; S: Stent; P: Pancreatic stump cut surface.

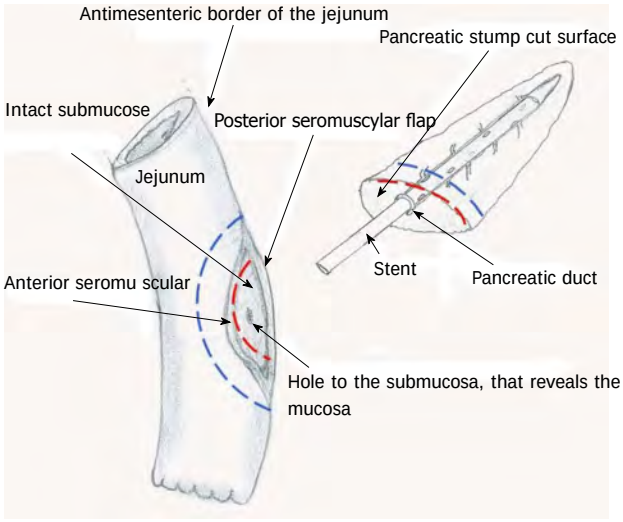

Figure 3 This is a schema that shows the dissected surface of the jejunum and the cut surface of the pancreas that are approximated. The red dotted line represents the ventral internal suturing layer (*i.e.*, the 3rd one). The blue dotted line represents the ventral external suturing layer (*i.e.*, the 4th one).

layers. The dorsal part of the jejunal seromuscular layer and the dorsal part of the capsular parenchyma of the pancreatic stump are sutured with 3-0 silk interrupted stitches of 0.5-1 cm distance from each other. This is the dorsal external suturing layer (Figure 2A).

Then, the dorsal cut edge border of the pancreatic stump is sutured to the edge of the dorsal jejunal seromuscular flap with 4-0 polydioxanone sutures (PDS) Ⅱ interrupted stitches 0.5 to 1 cm apart. This is the dorsal internal layer.

A small hole in the jejunal mucosa is made, in accordance with the diameter of the main pancreatic duct. The free end of the stent tube is advanced through this hole into the jejunal lumen (Figure 2B). The mucosa at the site of the hole and the edge of the main pancreatic duct are sutured with two interrupted stitches of 4-0 PDS Ⅱ.

The third layer is created by the approximation of the ventral cut edge border of the pancreatic stump and the edge of the ventral jejunal seromuscular flap with 4-0 PDS Ⅱ interrupted stitches, 0.5-1 cm apart (Figure 2C). This is the ventral internal layer.

The final layer of sutures, the ventral external layer, is created by suturing the ventral part of the jejunal seromuscular layer and the ventral part of the capsular parenchyma of pancreatic stump, with 3-0 silk interrupted stitches (Figure 2D). Figure 3 shows a drawing of the jejunal and pancreatic sites of anastomosis.

Statistical analysis

There were only descriptive measures used, since there was no control group in this study and its main purpose was to describe a surgical technique.

RESULTS

There were 32 consecutive patients that underwent pan-

creaticoduodenectomy with the above described pancreaticojejunostomy technique. There were 13 women and 19 men, with a mean age of 64.2 years (range 55-82 years). The underlying diseases of these patients are shown in Table 1. The mean operative time was 310.2 ± 40.0 min, and the mean time needed to perform the pancreaticojejunostomy was 22.7 min (range 18-25 min). One patient developed low output pancreatic fistula. Three patients developed surgical site infection and one patient developed pneumonia, postoperatively. The overall morbidity rate was 15.6%. There was no postoperative mortality.

DISCUSSION

Pancreaticojejunostomy represents one of the most challenging technical aspects of the Whipple procedure, because of its failure rates, as well as the resulting morbidity and mortality. Several technical variations have been proposed, in an effort to minimize postoperative pancreatic fistula rates[1,2,9,10,14-20]. The most important risk factors identified are technique, soft pancreatic texture and main pancreatic duct diameter of 3 mm or less[13,21-26]. The technique we describe is an end-to-side, duct-to-mucosa two-layer pancreaticojejunostomy. Each step of the procedure already described adheres to a rationale focused on the elimination of pancreatic leakage. First, the exposure of intact jejunal mucosa was thought to promote vascularization and enhance the healing process between the mucosa and the cut surface of the pancreatic stump[27]. Such an approach has been employed in the past with favorable results, but still carried a significant fistula occurrence.

With this in mind, we incorporated the dissection of the seromuscular flaps and their fixation to the border of the pancreatic stump aiming to offer a more reliable sealing of the anastomosis. In the same context, we proposed the internal layer of sutures, which was not previously employed, in order to keep the two traumatic surfaces firmly in contact to further favor the healing process between them. Finally, stenting the main pancreatic duct ensures duct patency, while eliminating undesired distention. In one of our cases, the stent was present in situ even on the six-year follow-up.

In conclusion, this technique appears to be safe and reliable. Because this is a preliminary report of a small series, it is of essential importance that it is evaluated via a prospective study in a larger series, before firm conclusions can be drawn.

Furthermore, while of sound reasoning, the assumption that healing is significantly augmented by exposing the intestinal submucosa is yet to be experimentally proved.

COMMENTS

Background

Pancreatic fistula following pancreaticoduodenectomy is a serious complication associated with high morbidity rates. Various factors have been implicated as contributors to pancreatic anastomotic leak, the incidence of which has been as high as 28% in some series.

Research frontiers

Pancreatic texture, main pancreatic duct diameter and anastomotic technique are considered the most significant factors contributing to higher risk for pancreatic fistula. Among them, anastomotic technique is the only factor that can be evolved.

Innovations and breakthroughs

The authors present a modification for duct to mucosa end-to-side pancreaticojejunostomy, with a seromuscular jejunal flap, in order to increase the safety of the anastomosis.

Applications

This modification appears to be a significantly safe approach to the pancreaticojejunostomy without adversely affecting operative time.

Peer review

In this manuscript, authors have presented the new procedure of pancreaticojejunostomy in pancreaticoduodenectomy to prevent pancreatic fistula. They concluded the new procedure could decrease the occurrence rate of pancreatic fistula. The results are basically interesting for readers.

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BRIEF ARTICLE

Gastrointestinal side effects in children with Wilson's disease treated with zinc sulphate

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Abstract

AIM: To investigate the side effects of a zinc sulphate therapy in a cohort of Polish pediatric patients with Wilson's disease.

METHODS: We retrospectively analyzed a cohort of 53 pediatric patients with Wilson's disease treated at the Children's Memorial Health Institute in Warsaw, Poland between the years 1996 and 2011 with zinc sulphate. Patients were diagnosed with Wilson's disease according to the scoring system of Ferenci, with 49 cases confirmed by mutation analysis. Data about the dosage scheme of zinc sulphate, side effects and efficacy and toxicity of the treatment were collected and recorded in the patient's medical chart at each visit to the hospital.

RESULTS: Mean age of diagnosis for the entire cohort was 10 years (range, 2.5-17 years). Duration of treatment with zinc sulfate was 83.3 wk (range, 8-344 wk). Side effects, all of gastrointestinal origin, were observed in 21 patients (40% - 9 males and 12 females), irrespective of the duration of therapy. Thirteen out of 21 patients were over the age of 10 years. The most common ATP7B mutation was p.H1069Q. Esophagogastroduodenoscopy, performed in 7 patients (33.3%) suffering from persistent and severe abdominal pain, revealed gastrointestinal ulcerations or erosions with negative *Helicobacter pylori* tests in all subjects investigated. The above mentioned 7 patients were treated with proton pump inhibitors. Three of those experienced resolution of symptoms, whereas proton-pump inhibitors failed to alleviate symptoms of the remaining four children and conversion of therapy to *D*-penicillamine was needed.

CONCLUSION: Zinc sulphate appears to cause significant gastrointestinal side effects, which children on therapy for Wilson's disease should be closely monitored for.

Key words: Wilson's disease; Zinc; Abdominal pain; Gastrointestinal ulcer; Therapy

Core tip: The present study demonstrates a considerably higher rate and severity of gastrointestinal adverse effects secondary to zinc sulphate therapy in pediatric patients as previously reported. A total of 40% of our treated patients experienced gastrointestinal symptoms, of which the more severe cases were associated with endoscopically evident gastric ulcerations and erosions. Furthermore it was shown, that proton pump inhibitors were not effective in treating patients with severe zinc associated gastrointestinal side effects, requiring a switch to an alternative treatment regimen.

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INTRODUCTION

Wilson's disease is an autosomal recessive disorder of copper metabolism, which results in impaired copper excretion by the liver. Toxic copper accumulates both in the liver and in the central nervous system. Various therapies are used, which include copper chelators like *D*-penicillamine and trientine or tetrathiomolybdate, as well as zinc preparations, such as zinc sulphate and zinc acetate. Liver transplantation is used mainly in acute liver failure. In Poland trientine is not registered and the most popular drugs are *D*-penicillamine (Cuprenil®, Teva Pharmaceuticals Polska) and zinc sulphate (Zincteral®, Teva Pharmaceuticals Polska). Zinc acetate (Wilzin®, Orphan Europe) is registered but not commonly used due to its high costs, which are not covered by the state and often unaffordable for patients.

The most common side effects of zinc therapy described are nausea and abdominal pain. Other complications, secondary to zinc-induced copper deficiency such as hematologic abnormalities, specifically leukopenia (fever, chills, sore throat), neutropenia (oropharyngeal ulcers) and sideroblastic anemia (fatigue, general weakness) are very uncommon but may occur[1-3]. A mild, harmless increase in serum amylase and lipase concentrations without clinical or radiologic evidence of pancreatitis[4] and a 20% reduction of high-density lipoprotein cholesterol in male patients (associated with reduction in total cholesterol) have been described[5]. Zinc may have immunosuppressant effects and reduce leukocyte chemotaxis, however one study found no adverse effects on lymphocyte function with chronic use[6].

Although many potential adverse effects of zinc therapy have been described, zinc sulphate is regarded to be an overall safe and well-tolerated drug. According to Wiggelinkhuizen's systematic review[7], 12.5% of adult patients with Wilson's disease suffered from side effects secondary to zinc therapy, all of gastrointestinal origin. There are only few studies focusing on pediatric patients, most of them reporting gastrointestinal adverse effects of zinc sulphate or acetate in children to be uncommon and mild, generally handled by changing dosage scheme or resolving spontaneously[8-12]. However, the analysis of our cohort with 53 pediatric patients with Wilson's disease led to differing results. Gastrointestinal side effects were detected in a considerably greater proportion of patients and range of severity was clearly wider, with some cases associated with severe and poorly tolerated symptoms and endoscopically evident ulcerations, a finding not been described earlier. Based on this detailed retrospective analysis of Polish children, we raised the concern about the safety of zinc sulphate therapy in pediatric patients with Wilson's disease.

Table 1 *ATP7B* mutations in patients with Wilson's disease treated with zinc sulphate

Mutation analysis	*n*
p.H1069Q/p.H1069Q	22
Diagnosis not confirmed by mutation analysis	1
p.H1069Q/-	7
p.H1069Q/p.Q1351X	2
p.H1069Q/p.A1135fs	2
p.V845fs/-	2
p.H1069Q/p.E507fs	1
p.H1069Q/p.C985Y	1
p.H1069Q/p.L1325fs	1
p.H1969Q/p.W779X	1
p.H1069Q/p.R969Q	1
p.H1069Q/p.T737I	1
p.H1069Q/p.P1273L	1
p.H1069Q/p.V772_I774del	1
p.H1069Q/p.Arg969Gln	1
p.H1069Q/p.G1341R	1
p.A1135fs/p.A1135fs	1
p.A1135fs/p.R1319X	1
p.G1158fs/p.G1158fs	1
p.W779X/p.W779R	1
p.Q1351X/-	1
p.A1135fs/-	1
p.N1270S/-	1

MATERIALS AND METHODS

The study included 53 patients with hepatic presentation of Wilson's disease treated at the Children's Memorial Health Institute in Warsaw, Poland between the years 1996 and 2011. Wilson's disease was diagnosed according to the scoring system of Ferenci[13,14], with confirmation by mutational analysis in 49 cases. All patients treated with zinc sulphate were selected to the main analysis. Zinc sulphate was used as first choice therapy for 50/53 cases and 3/53 patients were initiated on *D*-penicillamine and later switched to zinc sulphate as a maintenance therapy (Table 1). Data about dosage scheme of zinc sulphate and adverse effects during therapy were collected and recorded in the patient's medical chart at each hospital visit. The Local Ethics Committee approved the study. Zinc sulphate was administered at a dose of 135 mg of elemental zinc daily in three divided doses for children with a body weight more than 50 kg. Three patients (males, 14, 15 and 16 years old) received a different zinc sulphate regiment with 180 mg of elemental zinc daily. The dose for younger and smaller children dosage was twice daily 45 mg of elemental zinc. One 9-year-old patient was given a daily dose of 45 mg of elemental zinc. At each visit (for the first year of therapy once every 3 mo, subsequently twice a year) patients were followed for efficacy and toxicity of treatment by measuring liver function tests (alanine transaminase, bilirubin), complete blood count and coagulation parameters, and by physical examination. Adequacy of zinc therapy was monitored with zinc serum levels and with 24-h urinary excretion of copper. Zinc serum

Table 2 Characteristic of patients with side effects during zinc therapy

No.	Sex	Age of onset of symptoms (yr)	Age of diagnosis (yr)	Mutation analysis		Zinc sulphate therapy-dosage scheme (mg)	Treatment history	Cause of conversion to *D*-penicillamine	Cause of conversion to zinc acetate	Cause of additional intervention	Endoscopic examination
1	M	7	8	p.V845fs	-	2 × 45	ZS - P - ZA	Abdominal pain	Rash		No
2	F	Asymptomatic, positive family history	15	p.V845fs	-	3 × 45	ZS - P - ZA	Abdominal pain	Abdominal pain		No
3	M	Asymptomatic, positive family history	6	p.Q1351X	-	3 × 45	ZS - P	Abdominal pain			No
4	M	7	16	p.W779X	p.W779R	3 × 45	ZS - P	Abdominal pain, loss of appetite			No
5	F	7	9	p.H1069Q	p.H1069Q	3 × 45	ZS - P	Vomiting			No
6	M	Lack of data	12	p.H1069Q	p.P1273L	3 × 45	ZS + PPI			Abdominal pain, symptoms of GERD	No
7	M	11	12	-	-	3 × 45	ZS - ADS			Abdominal pain	No
8	F	11	11	p.N1270S	-	3 × 45	ZS - P	Nausea			No
9	M	12	13.5	p.G1158fs	p.G1158fs	5 × 45	ZS - P	Nausea, vomiting			No
10	F	13	14	p.H1069Q	p.Q1351X	3 × 45	ZS - ZA		Nausea		No
11	F	Lack of data	12	p.H1069Q	p.T737I	3 × 45	ZS - P	Nausea			No
12	M	8	13	p.H1069Q	p.V772_I774del	3 × 45	P - ZS - ADS			Nausea	No
13	F	7	7	p.H1069Q	p.G1341R	2 × 45	ZS - P	Abdominal pain			No
14	M	7	7	p.A1135fs	-	2 × 45	ZS - ZA - P	Elevated transaminases	Abdominal pain		No
15	F	Asymptomatic, positive family history	5	p.H1069Q	p.H1069Q	2 × 45	ZS + PPI - P	Abdominal pain			Yes
16	F	8	8	p.H1069Q	p.H1069Q	2 × 45	ZS +PPI - P	Abdominal pain			Yes
17	F	14	14	-	-	3 × 45	ZS + PPI			Abdominal pain	Yes
18	F	12	12	-	-	3 × 45	ZS +PPI - P	Abdominal pain			Yes
19	M	Asymptomatic, positive family history	Lack of data	-	-	2 × 45	ZS +PPI - P	Abdominal pain			Yes
20	F	10	10	p.H1069Q	-	3 × 45	ZS + PPI			Abdominal pain	Yes
21	F	9	9	-	-	1 × 45	ZS + PPI			Abdominal pain	Yes

ZS: Zinc sulphate therapy; P: *D*-penicillamine therapy; ZA: Zinc acetate therapy; PPI: Proton pump inhibitor therapy; ADS: Alternative dosage scheme; GERD: Gastroesophageal reflux disease; M: male; F: Female.

levels less than 125 μg/dL and urine copper above 75 μg/d were defined as treatment failure, suggesting non-response or non-compliance. Patients with poor tolerance for zinc sulphate were started on zinc acetate or *D*-penicillamine.

Statistical analysis

The data were collected from patient's medical charts and analyzed retrospectively. Patients who underwent upper tract endoscopy due to persistent abdominal pain were carefully described. The frequency of findings was presented in numbers and in percentages. Conclusions were based on careful description of findings, as low numbers of patients with presented features did not allow performing statistical analysis.

RESULTS

Characteristics of patients presenting with side effects during treatment with zinc sulphate are illustrated in Table 2. Mean age of diagnosis for our cohort of 53 patients was 10 years (range, 2.5-17 years). Median duration of treatment with zinc sulfate was 83.3 wk (range, 8-344 wk). Side effects secondary to zinc sulphate were observed in 21 children (21/53, 40% of investigated children, 9 males, 12 females; 13/21, 62% aged over 10 years), all symptoms were of gastrointestinal origin: abdominal pain, nausea or vomiting. Adverse ef-

Table 3 Characteristic of patients with persistent abdominal pain

No.	Abdominal pain before zinc sulphate therapy	Duration of zinc sulphate therapy before abdominal pain occurred (wk)	EGD before intervention	Intervention	Abdominal pain after intervention	EDG after intervention	Additional intervention	Abdominal pain after additional intervention	EGD after additional intervention
1	Yes	Lack of data	Gastritis with mucosal ulceration	PPI	Yes	Ulcer scar	No	-	-
2	No	96	Gastritis with mucosal ulceration	PPI	No	No	No	-	-
3	No	28	Gastritis with mucosal ulceration	PPI	Yes	Gastritis with mucosal ulceration	Conversion to *D*-penicillamine	No	No
4	No	144	Gastritis with mucosal ulceration	PPI	Yes	Gastritis	Conversion to *D*-penicillamine	No	No
5	No	12	Gastritis with mucosal erosion	PPI	No	No	No	-	-
6	No	96	Gastritis with mucosal ulceration	PPI	Yes	No	Conversion to *D*-penicillamine	No	Normal findings
7	No	96	Gastritis with mucosal erosion	PPI	Yes	No	Conversion to *D*-penicillamine	No	Normal findings

EGD: Esophagogastroduodenoscopy; PPI: Proton pump inhibitor.

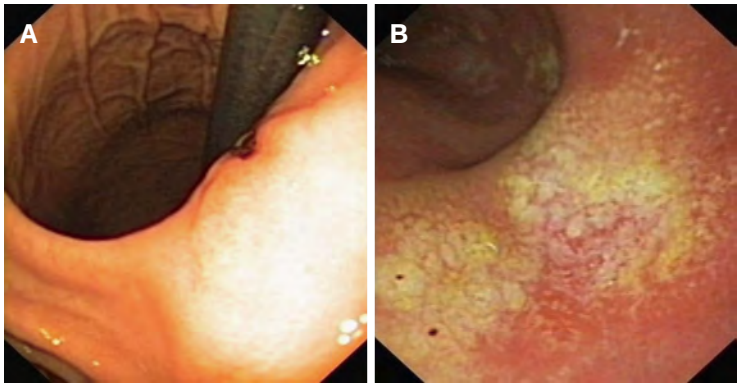

Figure 1 Endoscopic image. A: Endoscopic image of patient 6. Deep ulcer on the minor curvature of the stomach; B: Endoscopic image of patient 1. Flat mucosal ulcerations of the greater curvature of the stomach, about 1 cm in diameter covered with a flat fibrous coating.

fects associated with alternative therapies were noted as well within this group of 21 patients: rash (1 patient) and abdominal pain (1 patient) on *D*-penicillamine and elevated transaminases with zinc acetate treatment (1 patient).

Esophagogastroduodenoscopy (EGD) was performed in 7 children (7/21, 33%, 6 females, 1 male) experiencing persistent and severe abdominal pain. Gastritis with ulcerations or erosion was evident in all cases. There was no history of nonsteroidal antiinflammatory drugs use in any of the cases. Histopathology of biopsies showed mild to moderate lymphocytic infiltrations. All subjects investigated tested negative for *Helicobacter pylori* (*H. pylori*). Tests were performed during endoscopy by urea test (Table 3 and Figure 1). Three of these seven patients experienced resolution of symptoms on proton pump inhibitors (PPIs) (3 mo therapy: 1 patient; 6-wk therapy: 1 patient; unknown therapy duration due to lack of data: 1 patient). Adverse effects while on PPIs or recurrence of symptoms with discontinuation of PPIs were not observed. Effectiveness of therapy was confirmed by follow up EGD in one case.

Treatment with PPIs failed to alleviate symptoms of the remaining four patients, requiring a switch to *D*-penicillamine. Follow up EGDs were performed on two of these four patients.

One of the remaining 14 patients presented with GERD symptoms and improved clinically on PPIs, an EGD was not performed.

Three other patients experienced amelioration of symptoms following a conversion to zinc acetate. Two of the latter mentioned three cases had initially been converted to *D*-penicillamine. However side effects, such as rash in one case and abdominal pain in the other, required a second switch to zinc acetate. With eight patients, amelioration of clinical symptoms was achieved after converting therapy to *D*-penicillamine. One of those eight cases, although remission of gastrointestinal symptoms had been attained after switching from zinc sulphate to acetate, elevated transaminases necessitated another switch to *D*-penicillamine. Two other patients improved on the alternative zinc sulphate dosage scheme.

Table 4 illustrates the distrubution of mutations between the two groups: patients with and without side effects during zinc sulphate therapy. However conclusions regarding susceptibility towards adverse effects cannot be drawn from this data.

Table 4 Characteristic of mutation analysis in two groups of patients with Wilson's disease: with and without side effects during zinc sulphate therapy

Mutation analysis	No. of patients during zinc sulphate therapy	
	Without side effects	With side effects
Homozygous for p.H1069Q mutation	16	3
Compound heterozygous for p.H1069Q mutation	8	5
Carrier for p.H1069Q mutation	4	1
Carrier for a mutation other than p.H1069Q	0	5
Mutation other than p.H1069Q on two alleles	2	2
Lack of data	2	5

DISCUSSION

The first line therapy for asymptomatic patients and for those with mild hepatic disease is zinc sulphate. It is also being used as a maintenance therapy for patients, whose hepatic symptoms improved under *D*-penicillamine[15,16]. Another indication for zinc sulphate is the presentation of neurological symptoms[16,17]. The guidelines of the American Association for the Study of Liver Diseases recommend a daily dose for older children and adults of 150 mg in three divided doses[18,19]. However, compliance with the three times per day dosage may be problematic, and it has to be taken at least twice daily to be effective[15,18,19]. For smaller children, with a body weight under 50 kg, the dose is 75 mg/d in three divided doses[18-20]. The dose for children under 5 years of age is not well defined. It is worth noting that taking zinc with food interferes with its absorption[21] and therefore with the effectiveness of treatment. Adequacy of treatment with zinc sulphate can be estimated by measuring serum zinc levels or 24-h urinary zinc excretion. Other goals of treatment monitoring are to ensure patients compliance with therapy, and identify adverse side effects. Patients should be monitored at least twice a year. More frequent observation is needed during the initial phase of treatment, for patients experiencing worsening of symptoms or side effects and for those suspected of noncompliance with therapy[18,19]. Brewer *et al*[20] advise to test zinc serum and urine copper levels during therapy. A zinc serum level less than 125 μg/dL generally indicates poor compliance. According to the authors the best monitoring tool may be following urine copper levels, with maximum levels around 50 μg/d. Roberts *et al*[18,19] recommend that urinary copper excretion should not exceed 75 μg/d on a stable treatment. Many studies report sustained elevation of alanine aminotransferase during zinc therapy, which may indicate poor compliance and which may results in fewer and milder side effects. Our patients mostly adhered to zinc therapy but many of them presented sporadically with increased copper excretion in the urine indicating treatment failure.

There are only few studies focusing on side effects of zinc therapy in pediatric patients with Wilson's disease, and those available studying only small numbers of patients. Abdel Ghaffar *et al*[11] described nine Egyptian children with Wilson's disease on a zinc sulphate monotherapy and reported vomiting and epigastric pain, which improved when zinc was taken with a small amount of protein. Most studies however analyzed effectiveness and safety of zinc acetate, which is known to be better tolerated than zinc sulphate. Brewer *et al*[20] treated 34 children with zinc acetate, with the only side effect being mild gastric irritation in four patients. These were handled by taking the first dose mid-morning, rather than on arising. In cases more difficult to treat, zinc was taken with a small protein meal. Only mild nausea was described by Mizuochi *et al*[10] in three of four children treated with zinc acetate, while Brewer *et al*[20] did not observe any adverse effects secondary to zinc acetate in eleven pediatric patients.

More data is available about zinc associated adverse effects in adults. Clinical data of 117 Wilson's disease patients were analyzed retrospectively by Bruha *et al*[22]. Side effects associated with zinc sulphate were mild with only 5 patients requiring a switch to zinc acetate (four cases due to gastrointestinal intolerance, one patient due to eosinophilia). No adverse events were noted for zinc acetate therapy.

In a systematic study of Japanese patients with Wilson's disease zinc acetate was shown to be safe and efficacious. Although more than 50% (20 patients) of their patients experienced adverse effects, including gastrointestinal symptoms and decreased blood iron levels, reported events were mild and patients were able to be continued on the zinc treatment[23].

The present study demonstrates side effects secondary to zinc sulphate therapy in 40% of treated patients, considerably higher compared to previous reports[6,7,11,24]. Complaints were of gastrointestinal origin: epigastric pain, vomiting, nausea or loss of appetite. Seven of these 21 symptomatic children were diagnosed with gastric ulcerations or erosions with negative *H. pylori* tests and were started on proton pump inhibitors. While three of those patients were relieved of their symptoms, PPIs failed to be effective for the other four, requiring a conversion of their therapy from zinc sulphate to *D*-penicillamine (Table 2). Most studies report gastrointestinal adverse effects of zinc sulphate or acetate in children to be mild and generally handled by changing the dosage scheme or even resolving spontaneously[10,11]. This maneuver proved to be effective in only two of our patients. One child with abdominal pain experienced relief of symptoms when zinc was taken with a small protein meal given early in the morning and in one case first dose of zinc was taken 30 min after the meal. Still, there are very few reports on side effects of zinc therapy in children. Adult patients with Wilson's disease experienced mild side effects secondary to zinc sulphate and treatment was converted from zinc sulphate to acetate or gluconate[9,22], in selected cases a switch to *D*-penicillamine was necessary[24]. Treatment with zinc acetate resulted in only mild complaints and patients were able to continue therapy without any modifications. In his systematic review of zinc sulphate therapy, Wiggelinkhuizen describes that

12.5% (28/224) of patients suffered from side effects, all of gastrointestinal origin and in only two cases discontinuation of therapy was required[7]. However, in the present study, therapy had to be converted from zinc sulphate to *D*-penicillamine in twelve patients and to zinc acetate in three other probands. One child (Table 2) was converted to *D*-penicillamine after a failed attempt to treat with zinc acetate and two patients were switched to zinc acetate after unsuccessful *D*-penicillamine therapy.

We described the distribution of genetic mutations between the two groups of patients, those who experienced side effects and the ones that remained asymptomatic throughout the therapy (Table 4). However, due to the small number of patients, conclusions regarding susceptibility towards adverse effects cannot be drawn from this data.

EGD in children is indicated, only in the presence of severe symptoms and in younger children endotracheal intubation is required to perform an endoscopy. Therefore, there are very little data available on endoscopic findings especially in younger children. We decided to perform endoscopy only in those cases where side effects were expected to cause a withdrawal of treatment. To our knowledge this is the first study to document gastric ulcerations as a significant complication of zinc treatment in Wilson's disease patients. In the present study we recorded severe side effects secondary to zinc sulphate in seven out of 21 patients and EDG performed in all cases revealed gastritis with mucosal ulceration or erosion. *H. pylori* test was negative in all subjects investigated. Histopathology of biopsies was unspecific, showing mild to moderate lymphocytic infiltrations. A case report from 1978 by Moore[25], about a 15 year old girl who had been taking zinc sulphate for acne, was the first to describe hemorrhagic gastric erosions associated with zinc sulphate therapy. The ulcerative effect of zinc sulphate was thought to be secondary to the corrosive zinc chloride, which is most likely formed by the action of gastric hydrochloric acid on zinc sulphate[26]. This could explain its isolated effect on the gastric mucosa, leaving the duodenal mucosa intact.

Taking into account the fact that peptic ulcer disease is uncommon in children with an estimated prevalence of 1 in 3000 hospital admissions[27], the high frequency of peptic ulcer disease observed in our study was disquieting. Epidemiological studies show a constant increase in incidence and prevalence of peptic ulcer disease in children[28]. Symptoms in children with suspected peptic ulcer disease commonly include pain associated with food intake, vomiting, bleeding, and a positive family history, and are crucial factors for the diagnosis of peptic ulcer disease in childhood. Poorly localized abdominal pain of a dull character is the most common symptom, but may be localized to the epigastric or periumbilical area in some cases. Unequivocal epigastric pain is relatively uncommon in children and should always prompt further investigation[27]. Esophagogastroduodenoscopy is the diagnostic procedure of choice for children with suspected peptic ulcer. Non-*H. pylori* ulcer disease can be treated effectively with acid-suppression (proton pump inhibitors, histamine 2 receptor inhibitors)[27]. Our data however demonstrate a failure of PPIs in severe cases of zinc sulphate related abdominal symptoms. Four of our seven patients with gastrointestinal ulcerations or erosions needed to be switched to *D*-penicillamine due to uncontrollable symptoms despite a treatment with PPIs.

Zinc sulphate is still a commonly used drug to treat Wilson's disease, especially in patients who otherwise cannot afford therapy. Therefore, it is very important to assess its efficacy and tolerability. Gastrointestinal side effects especially in children are not well studied. They may cause incompliance, which seems to be a major reason for treatment failure[29,30]. To address this problem we performed endoscopy in selected symptomatic patients and identified gastric ulcer disease as the underlying cause of symptoms in 7/21 patients.

The limitation of our study is the retrospective character of data analysis and the relatively small size of the patient group. However, this rare disease limits the options to perform randomized prospective trials.

Adverse reactions such as abdominal pain, nausea and even gastritis are common in children with Wilson's disease treated with zinc sulphate. They may occur at different stages of therapy. The high frequency of gastritis in patients with chronic abdominal pain indicates the need to perform gastroscopy in selected patients. Discontinuation of zinc sulphate is often inevitable and conversion to *D*-penicillamine or zinc acetate may be a safer option for these patients. Zinc sulphate appears to cause significant side effects, which should be seriously considered during monitoring the therapy of Wilson's disease.

COMMENTS

Background

Wilson's disease represents a metabolic disorder caused by excessive accumulation of copper in tissues. Penicillamine and zinc compounds are used in therapy. Due to costs and availability zinc sulphate is commonly used for treating Wilson's disease in Poland. It is regarded safe and well tolerated, but there are little data available on side effects, especially in children.

Research frontiers

The most common side effect of zinc therapy described is nausea and abdominal pain. Other uncommon complications are: hematologic abnormalities, specifically leucopenia, neutropenia and sideroblastic anemia, a mild increase in serum amylase and lipase concentrations without clinical or radiologic evidence of pancreatitis, 20% reduction of high-density lipoprotein cholesterol in male patients (but with reduction in total cholesterol) or reduction leukocyte chemotaxis. According to the results of Wiggelinkhuizen's systematic review in adult patients with Wilson's disease 12.5% of them suffered from side effects of zinc therapy, all of gastrointestinal origin. There are only few studies focusing on pediatric patients but most of them report gastrointestinal adverse effects of zinc sulphate or acetate in children also to be uncommon and mild, generally handled by changing dosage scheme or resolved spontaneously.

Innovations and breakthroughs

The present study demonstrates side effects secondary to zinc sulphate therapy in 40% of treated patients, which is higher compared to previous reports. The common complaints were of gastrointestinal origin: epigastric pain, vomiting, nausea or loss of appetite. The clinical observation led to conclusions that some side effects seemed to be poorly tolerated by children and endoscopic investigations revealed ulcerations which had not been described earlier. Apart from that authors have some evidence derived from this analysis that proton pump inhibitors may not be effective in zinc sulphate treated children experiencing persistent abdominal symptoms and modification of treatment is necessary.

Applications

The study results suggest that zinc sulphate can cause significant side effects as gastritis or gastric ulcer which should be seriously considered during monitoring the therapy of Wilson's disease in children. The high frequency of gastritis in patients with chronic abdominal pain indicates the need to perform gastroscopy in selected patients. Discontinuation of zinc sulphate is often inevitable and conversion to penicillamine or zinc acetate may be a safer option for these patients.

Peer review

The manuscript is tackling an important issue in the treatment and management of Wilson's disease patients. It has excellent introduction and very good discussion, and is well written.

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BRIEF ARTICLE

Underexpression of *LATS1* TSG in colorectal cancer is associated with promoter hypermethylation

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Abstract

AIM: To investigate large tumor suppressor 1 (*LATS1*) expression, promoter hypermethylation, and microsatellite instability in colorectal cancer (CRC).

METHODS: RNA was isolated from tumor tissue of 142 CRC patients and 40 colon mucosal biopsies of healthy controls. After reverse transcription, quantitative polymerase chain reaction (PCR) was performed, and *LATS1* expression was normalized to expression of the *ACTB* and *RPL32* housekeeping genes. To analyze hypermethylation, genomic DNA was isolated from 44 tumor CRC biopsies, and methylation-specific PCR was performed. Microsatellite instability (MSI) status was checked with PCR using BAT26, BAT25, and BAT40 markers in the genomic DNA of 84 CRC patients, followed by denaturing gel electrophoresis.

RESULTS: Decreased *LATS1* expression was found in 127/142 (89.4%) CRC cases with the average ratio of the LATS1 level 10.33 ± 32.64 in CRC patients *vs* 32.85 ± 33.56 in healthy controls. The lowest expression was found in Dukes' B stage tumors and G1 (well-differentiated) cells. Hypermethylation of the *LATS1* promoter was present in 25/44 (57%) CRC cases analyzed. *LATS1* promoter hypermethylation was strongly associated with decreased gene expression; methylated cases showed 162× lower expression of *LATS1* than unmethylated cases. Although high-grade MSI (mutation in all three markers) was found in 14/84 (17%) cases and low-grade MSI (mutation in 1-2 markers) was found in 30/84 (36%) cases, we found no association with *LATS1* expression.

CONCLUSION: Decreased expression of *LATS1* in CRC was associated with promoter hypermethylation, but not MSI status. Such reduced expression may promote progression of CRC.

Key words: Large tumor suppressor 1; Colorectal cancer; Quantitative polymerase chain reaction; Reduced expression; Promoter hypermethylation; Microsatellite

instability; Salvador-Warts-Hippo pathway

Core tip: Searching for new colorectal cancer (CRC) molecular markers is a very important objective, because CRC is one of the most common malignancies in the world and one of the most fatal of human neoplasms. Decreased expression of large tumor suppressor 1 in CRC was associated with promoter hypermethylation, but not microsatellite instability status. Such reduced expression may promote progression of CRC.

Wierzbicki PM, Adrych K, Kartanowicz D, Stanislawowski M, Kowalczyk A, Godlewski J, Skwierz-Bogdanska I, Celinski K, Gach T, Kulig J, Korybalski B, Kmiec Z. Underexpression of *LATS1* TSG in colorectal cancer is associated with promoter hypermethylation. *World J Gastroenterol* 2013; 19(27): 4363-4373 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i27/4363.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i27.4363

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies in the world and one of the most fatal human neoplasms. Almost 1.2 million new cases occur annually, accounting for 608700 related deaths in 2008[1]. Although nearly 90% of patients may be successfully cured with surgery in early stages, CRC is frequently diagnosed in late stages, *i.e.*, Dukes' C and D, when the prognosis is poor[2,3]. Therefore, the search for CRC molecular markers, as well as elucidation of epigenetic factors that are responsible for variability in the expression of putative markers, is very important.

Human large tumor suppressor 1 (*LATS1*, also known as *WARTS*) was discovered in 1999[4] as a highly conserved homolog of the *Drosophila melanogaster* (*D. melanogaster*) lats gene[5]. *LATS1* encodes a serine/threonine kinase, which is involved in the regulation of various cellular processes. Before mitotic division, the presence of LATS1 is crucial for control of the R1 tetraploidy checkpoint[6]. During the early phase of mitosis, LATS1 associates with cell division control protein 2 homolog[7], and the progress of cytokinesis occurs only after association of the MOB kinase activator 1A cytoplasmic protein with LATS1[8,9].

More recently, genetic studies in *Drosophila* have identified LATS as a central mediator in a tumor suppressing pathway called the Salvador-Warts-Hippo (SWH) pathway[10,11]. The SWH pathway is also a critical factor in the regulation of organ size in *D. melanogaster* and mammals[12,13]. Moreover, deregulation of SWH pathway activity has been implicated in the genesis of multiple human cancers[11,14-16]. Several mammalian factors are involved in signal transduction in the SWH pathway, including the tumor suppressor proteins neurofibromin 2, Ras association family member 1-6, serine/threonine kinase 3, LATS1, and an oncogene called Yes-associated protein (YAP). YAP, a transcription coactivator that associates with various transcription factors, is overexpressed in human carcinomas including ovarian, liver, and prostate cancers[13]. LATS1 kinase is a main negative regulator of YAP. LATS1 inhibits the transcriptional activity/function of YAP *via* phosphorylation of Ser 127 in YAP[17]. Moreover, LATS1-phosphorylated YAP is involved in a p53-independent apoptosis pathway in which phosphorylated YAP plays a role in transcriptional activation of the pro-apoptotic gene, p53 up-regulated modulator of apoptosis[18]. Overexpression of *LATS1* in *LATS1*$^{-/-}$ mouse cells (by introducing human *LATS1* with adenovirus-mediated gene transfer) and HeLa cells suppresses tumorigenicity *in vivo* and *in vitro* by inducing apoptosis[18,19].

LATS1 is considered to play a suppressor role in some tumors. Decreased *LATS1* expression is found mainly in soft tissue-derived tumors, including sarcomas[20] and astrocytomas[21]. However, *LATS1* quiescence was also observed in breast[22], cervical[23] cancers and head and neck squamous cell carcinoma[24]. In the gastrointestinal tract, decreased *LATS1* expression has been recently observed in gastric cancer[25], but in a small sample of CRCs, *LATS1* overexpression was found[26].

Hypermethylation of CpG islands (GC-rich sequences) in regulatory portions of a gene is an important epigenetic mechanism responsible for decreased gene expression or gene silencing[27-30]. Aberrant methylation of CpG islands in the promoter region of *LATS1* has been found in breast and ovarian cancers[4,22,31] and soft-tissue sarcomas[20]. Our preliminary results suggested that *LATS1* expression is decreased in CRC and is associated with promoter hypermethylation[32]. In the present study, we used quantitative polymerase chain reaction (QPCR) to determine the expression profile of *LATS1* in a relatively large group of CRC patients. We also examined the hypermethylation status of the *LATS1* promoter as a putative epigenetic mechanism affecting gene expression.

MATERIALS AND METHODS

Patients

The study was approved by the local ethics committees, and informed, written consent regarding the use of tissue was obtained before surgery or colonoscopy from all CRC and control patients, respectively. The specimens were obtained from four gastrointestinal endoscopic units and surgical clinics located in geographically different parts of Poland from 2008 to 2011. Clinical and demographic data were collected at the time of enrollment (Table 1). The study included 142 patients with CRC (87 males and 55 females; mean age 68 ± 10.8 years; range, 37-90 years). No CRC patients had a second neoplastic disease. None of the patients had undergone previous chemo- or radiotherapy. Tumors located in the anal canal and anus were not included in this study. The control group comprised 40 healthy individuals (17 males and 23 females; mean age 53 ± 14.2 years; range, 21-76 years) who underwent colonoscopy as part of routine surveillance for CRC. None of the CRC patients or controls

suffered from inflammatory bowel disease or had a family history of CRC. Patients were not on medication at the time of investigation. Before medical examination, blood samples were collected for routine testing from all CRC patients.

Collection of colon samples

All steps of material collection, including patients' clinical data, tissue collection, storage, shipment, and laboratory processing, followed The Cancer Genome Atlas (TCGA) instructions and were standardized in all collaborative clinics[33,34]. Briefly, CRC samples were obtained during surgical hemicolectomy, and control group specimens were collected during colonoscopy. For histopathologic examination and molecular studies, samples (5 mm × 5 mm × 5 mm) from macroscopically altered tumor tissue were taken within 20 min after tumor resection. For control patients, one biopsy (2 mm × 2 mm × 2 mm) was fixed in 10% neutral buffered formalin, and two specimens from the adjacent location to the biopsy site were collected for nucleic acid analyses. The formalin-fixed samples were obtained for the routine histological survey; if the result of histological examination showed pathological condition of the patient's tissue, the adjacent biopsies were excluded from the control group analyzed in this study. Both tumor samples and mucosal biopsies were immediately placed in sterile vials containing RNAlater (Ambion-Life Technologies, Grand Island, NY, United States), incubated for 6 h at 4 ℃, and then stored at -25 ℃ until further analysis.

Nucleic acid extraction and reverse transcription

Total RNA was extracted from a portion of the tumor samples (ca. 3 mm × 5 mm × 5 mm) and the entire mucosal biopsies of control patients using a Total RNA kit (A&A Biotechnology, Gdynia, Poland). Isolated RNA was quantified with spectrophotometry (Nanodrop ND 1000, Thermo Fisher Scientific, Fitchburg, WI, United States). DNA was digested with RNase-free DNase I (Fermentas-Thermo Fischer Scientific, Fitchburg, WI, United States) for 30 min at 37 ℃. Then, the DNase was inactivated by adding EDTA and incubating at 65 ℃ for 10 min. Before storing at -85 ℃, RNA integrity was analyzed with agarose gel electrophoresis. Total RNA (2 μg) was reverse transcribed using 0.5 μg oligo(dT)$_{18}$ primers (Sigma-Aldrich, Munich, Germany) and 200 U RevertAid M-MuLV Reverse Transcriptase (Fermentas-Thermo Fischer Scientific, Fitchburg, WI, United States) in a total volume of 20 μL, and the resulting cDNA was stored at -25 ℃. In 84 of the CRC cases, 1 mL venous blood that was collected in sterile K2-EDTA vials was used for DNA isolation using a Blood Mini DNA kit (A&A Biotechnology, Gdynia, Poland). From these same patients, DNA was also extracted from a portion of the tumor samples (ca. 3 mm × 5 mm × 5 mm) adjacent to the tumor fragments used for the RNA study using the Genomic Mini AX Tissue kit (A and A Biotechnology, Gdynia, Poland) and stored at -25 ℃.

QPCR assay to determine the LATS1 mRNA level

Quantification of *LATS1* gene expression was carried out using iQ Cycler (Bio-Rad, Hercules, CA, United States) with Sybr® Green I as a fluorophore. *LATS1* expression was determined with Livak's comparative method $2^{-\Delta\Delta Ct}$[35] relative to the geometric mean of the expression levels of two housekeeping genes: β-actin (*ACTB*; GenBank acc. No. NM_001101.3) and ribosomal protein L32 (*RPL32*; NM_000994.3). These genes showed very stable expression in CRC in our previous studies[36,37] and studies of other investigators[38]. Except for the *ACTB* assay[39], all primers were designed by us using GenBank data. QPCR conditions were validated and showed 90%-100% efficiency for all assays. The amplification primer pairs were 5'-TGCACTGGCTTCAGATGGACAC-3' and 5'-ATGTGCTAGACATCGCTGGTGC-3'; for *LATS1* (functional transcript, ENSEMBL No. ENST00000543571, GenBank No. NM_004690.2), 5'-TGTGCCCATCTACGAGGGGTATGC-3' and 5'-GGTACATGGTGGTGCCGCCAGACA-3' for *ACTB*[39], and 5'-TGACAACAGGGTTCGTAGAAGAT-3' and 5'-GTTCTTGGAGGAAACATTGTGAG-3' for *RPL32*. The reaction mixture (17 μL) included 0.4 μL cDNA, 0.2 μmol/L each forward and reverse primers, and real-time PCR iQ SYBR Green SuperMix (Bio-Rad). All reactions were performed in duplicate. The amplification parameters were denaturation for 5 min at 95 ℃, followed by 38 cycles of denaturation for 15 s at 95 ℃, annealing for 20 s at 55 ℃ for *RPL32*, 57 ℃ for *LATS1*, and 60 ℃ for *ACTB*, extension for 15-25 s at 72 ℃, and fluorescence reading for 5 s at 77 ℃-80 ℃. Dynamic melting curve analysis was performed for all reactions. Data were automatically collected and analyzed with iCycler iQ Optical Software ver. 3.0a (Bio-Rad).

Microsatellite instability status analysis

Microsatellite instability (MSI) status was determined according to the National Cancer Institute Workshop on Microsatellite Instability for Cancer Detection and Familial Predisposition[40] and was based on polymorphism analysis of three markers: BAT26 for *MSH2*, BAT25 for the *c-kit* oncogene, and BAT40 for the *HSD3B2* suppressor gene. BAT sequences were obtained from the UniSTS database (http://www.ncbi.nlm.nih.gov/unists), and the methodology was based on our previous results[41]. Briefly, the PCR reaction contained 0.5 U Taq polymerase, PCR buffer (Fermentas-Thermo Fischer Scientific, Fitchburg, WI, United States), 200 nmol/L each primer pair, 0.1 mmol/L each dNTP, and 30 ng DNA in a final volume of 15 μL and was performed using the following parameters: denaturation for 5 min at 95 ℃, 35 cycles of denaturation for 15 s at 95 ℃, annealing for 20 s at 49 ℃ (BAT25, BAT26) or 54 ℃ (BAT40), and extension for 30 s at 72 ℃. Denaturing acrylamide gel electrophoresis was performed in a Sequi-Gen Ⅱ Sequencing Cell (Bio-Rad) followed by silver staining ($AgNO_3$; POCH, Gliwice, Poland) for identification of extra DNA bands, which were considered mutations in the selected BAT markers. Low-

and high-grade MSI (MSI-L and MSI-H, respectively) were confirmed by 1-2 and all 3 mutated markers, respectively. If no mutation was observed in the paired tumor and blood DNA samples, the sample was confirmed as microsatellite stable (MSS).

Bisulfite modification and methylation-specific PCR

Because the method of bisulfite conversion of DNA requires at least 1 μg DNA, we performed this analysis with only 44 tumor samples with sufficient material using the EZ DNA Methylation™ kit (Zymo Research, Orange, CA, United States). Briefly, 1 μg tissue DNA was denatured using 0.2 mol/L NaOH and subsequently incubated with a sodium salt of bisulfite ion (HSO_3^-) at 50 ℃ for 16 h. Next, the mixture was desulfonated, and DNA was purified on silica-membrane columns to a final volume of 10 μL. Bisulfite-modified DNA was stored at -25 ℃. The methylation status of the *LATS1* promoter region was determined with methylation-specific PCR (MSPCR). Bisulfite-modified DNA was amplified with primers specific for methylated or unmethylated sequences. The methylated DNA was amplified using M primers: sense 5'-TCGTTTTGTCGTTTAGGTTGG-3' and antisense 5'-CGACGTAATAACGAACGC-3', and unmethylated DNA was amplified using UM primers: sense 5'-TAGGTTGGAGTGTGGTGGT-3' and antisense 5'-CCCAACATAATAACAAACACCT-3'. All primer sequences were previously published[20-22] except for the M sense primer, which was redesigned *de novo* using the GenBank database and methPrimerDB online software. For the methylation assay, Human HCT116 DKO Non-methylated DNA and Human HCT116 DKO Methylated DNA (Zymo Research) after bisulfite modification were used as positive controls in MSPCR. Briefly, 0.6 μL bisulfite-modified DNA was amplified in a total volume of 15 μL containing reagents from the Zymo*Taq*™ DNA Polymerase kit (Zymo Research) and 400 nmol/L each primer. MSPCR reactions were as follows: denaturation for 5 min at 95 ℃, five cycles of denaturation for 30 s at 95 ℃, annealing for 30 s at 52 ℃, extension for 20 s at 72 ℃; 30 cycles of denaturation for 30 s at 95 ℃, annealing for 30 s at 50 ℃, extension for 20 s at 72 ℃; final extension for 5 min at 72 ℃. PCR products (10 μL) were run on a 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet illumination. Images were stored using a Gel Doc apparatus and software (Bio-Rad).

Statistical analysis

Normality of the QPCR data was assessed with the Shapiro-Wilk test. Parametric data such as red blood cells, hematocrit, hemoglobin, and *LATS1* mRNA levels between various groups were evaluated using the Mann-Whitney *U* test. Comparison of *LATS1* mRNA ratios between CRC subgroups with various histological and MSI grades and methylation status was calculated using the Kruskal-Wallis analysis of variance (ANOVA) test. Fisher's exact test was used to assess correlations between the methylation status and clinical-pathological variables. The statistical analyses were performed using Statistica ver. 10 program (Stat Soft Inc., Tulsa, OK, United States), and the level of significance was set at $P < 0.05$.

RESULTS

Relationship between LATS1 expression and clinical parameters

Clinico-pathological data including tumor stages according to tumor location, Dukes' classification, and G grade[42,43] are presented in Table 1. We found no statistical differences in geographic location of patients, sex, age, tumor location, and disease progression. We found a relationship between tumor location and erythrocyte counts, hematocrit level, and hemoglobin concentration; patients with a tumor on the right side were characterized by decreased values compared with patients whose tumor was on the left side ($P < 0.05$, Table 1). No associations between blood parameters and Dukes classification, TNM, and G grading of CRC were found.

Quantification of *LATS1* mRNA was performed in colorectal tumor samples from 142 CRC patients and compared with tissue samples from 40 healthy persons. Decreased *LATS1* gene expression was found in 127 of 142 (89.4%) tumors in the CRC cases ($P < 0.05$). Because QPCR data were not normally distributed (mean values: 10.33 ± 32.64 *vs* 32.85 ± 33.56, $P < 0.05$), the median expression ratio was 0.075 (range, 0.003-210.672) in CRC patients *vs* 40.097 (range, 0.004-98.228) in controls ($P < 0.05$). Thus, the average expression of *LATS1* was many times lower in tumor tissue than in normal colon mucosa of controls. No correlations between *LATS1* mRNA level and gender, age, or tumor location were found. Also, no statistical differences in the mRNA ratio were observed in patients who lived in different regions of Poland.

Comparison of *LATS1* expression levels with patients' clinico-pathological data revealed 8 times lower *LATS1* levels in Dukes' A stage compared to controls (Figure 1). The lowest *LATS1* expression was observed in Dukes' B stage, which was 42 times lower than in controls, whereas in more advanced CRC cases described as Dukes' C and D stage, *LATS1* expression was 24 and 14 times lower than in controls, respectively. We found a weak negative correlation between tumor progression and the *LATS1* mRNA level ($R^2 = -0.25$, $P < 0.05$, Spearman's test, plot not shown). When the histological G grade of cancer cells was considered, *LATS1* mRNA levels were significantly decreased in both G2 and G3 grades (Figure 1). However, due to the low number of G1 cases (well-differentiated cells) and G4 (undifferentiated cells) cases (three each), no comparison with grades G2 and G3 was possible.

MSI status and clinicopathological data

We analyzed 84 of the 142 CRC cases for MSI status. The highest rate of mutation was found in the BAT26 marker ($n = 28/84$; 33%), followed by 26 cases for BAT40 (31%) and 25 for BAT25 (30%) (Table 1). Our analysis revealed

Table 1 Clinical and histopathological characteristics of colorectal cancer patients and results of large tumor suppressor 1 mRNA quantification using quantitative polymerase chain reaction *n* (%)

Clinical parameter			Blood parameters (mean ± SD)				MSI results (*n* = 84)					QPCR results
			RBC (10^6/μL)	Ht	Hb (g/dL)	WBC (10^3/μL)	BAT 26	BAT 25	BAT 40	MSI-L	MSI-H	Downregulated cases *vs* control
	CRC 142 cases		4.26 ± 4.58	36% ± 5.2%	12 ± 2.1	7.41 ± 2.71	28/84 (33)	25/84 (30)	26/84 (31)	30/84 (36)	14/84 (17)	127/142 (88)[1]
Sex and age	M (*n* = 87)	67 ± 10.4 (37-89)	4.36 ± 0.50[2]	36% ± 6.0%	12 ± 2.4	7.26 ± 2.91	21/54 (39)	18/54 (33)	16/54 (30)	22/54 (41)	9/54 (17)	78/87 (90)
(mean ± SD, range, yr)	F (*n* = 55)	69 ± 11.4 (44-90)	4.18 ± 0.41	35% ± 4.6%	11 ± 1.8	7.53 ± 2.58	7/30 (23)	7/30 (23)	10/30 (33)	8/30 (37)	5/30 (17)	49/55 (88)
Tumor location	Right side	55 (39)	4.09 ± 0.46[2]	33% ± 4.7%[2]	10 ± 1.9[2]	7.18 ± 3.45	9/32 (28)	8/32 (25)	7/32 (22)	8/32 (25)	4/32 (12.5)	51/55 (93)
	Left side	87 (61)	4.36 ± 0.43[2]	38% ± 4.8%[2]	12 ± 1.8[2]	7.56 ± 2.17	19/52 (36.5)	17/52 (33)	19/52 (36.5)	22/52 (42)	10/52 (19)	76/87 (81)
	Ascending colon	46 (32)	4.08 ± 0.46	33% ± 5.0%[2]	11 ± 2.0[2]	7.26 ± 3.42	9/26 (35)	8/26 (31)	6/26 (23)	7/26 (27)	4/26 (15)	43/46 (93)[1]
	Transverse colon	11 (8)	4.12 ± 0.43	33% ± 4.2%[2]	10 ± 1.5[2]	7.43 ± 3.47	0/6 (0)	0/6 (0)	1/6 (17)	1/6 (17)	0/6 (0)	10/11 (91)[1]
	Descending/sigmoid colon	41 (29)	4.29 ± 0.53	36% ± 5.2%[2]	12 ± 2.1[2]	7.49 ± 2.35	6/23 (26)	7/23 (30)	9/23 (39)	9/23 (39)	4/23 (17)	32/41 (78)[1]
	Rectum	44 (31)	4.43 ± 0.33	39% ± 4.5%[2]	13 ± 1.6[2]	7.47 ± 2.08	13/29 (45)	10/29 (34)	10/29 (34)	13/29 (45)	6/29 (21)	42/44 (95)[1]
Dukes' stage	A	27 (19)	4.23 ± 0.49	38% ± 5.3%	12 ± 2.0	7.58 ± 2.08	2/19 (11)	2/19 (11)[2]	1/19 (6)[2]	4/19 (22)	0/19 (0)	22/27 (81)[1,2]
	B	41 (29)	4.30 ± 0.47	36% ± 5.5%	11 ± 2.1	7.38 ± 1.99	9/23 (39)	5/23 (22)[2]	7/23 (30)[2]	8/23 (35)	4/23 (17)	40/41 (98)[1]
	C	54 (38)	4.22 ± 0.47	36% ± 5.0%	12 ± 2.0	7.42 ± 3.41	12/34 (35)	14/34 (41)[2]	15/34 (44)[2]	14/34 (41)	8/34 (24)	49/54 (91)[1]
	D	20 (14)	4.34 ± 0.26	32% ± 4.4%	10 ± 1.9	7.13 ± 2.26	5/8 (62.5)	4/8 (50)[2]	3/8 (37.5)[2]	4/8 (50)	2/8 (25)	18/22 (82)[1]
Lymph node metastasis	Negative	68 (48)	4.28 ± 0.46	36% ± 5.5%	12 ± 2.1	7.44 ± 1.97	11/42 (26)	7/42 (17)[2]	8/42 (19)[2]	12/42 (29)[2]	4/42 (10)[2]	62/68 (91)[1]
	Positive	74 (52)	4.23 ± 0.45	35% ± 5.0%	11 ± 2.1	7.39 ± 3.28	17/42 (40)	18/42 (43)[2]	18/42 (43)[2]	18/42 (43)[2]	10/42 (24)[2]	65/74 (88)[1]
Histological	Well (G1)	3 (2)	4.17 ± 0.35	36% ± 4.7%	12 ± 1.4	7.44 ± 2.19	1/3 (33)	1/3 (33)	1/3 (33)	0/3 (0)	1/3 (33)	3/3 (100)[1]
differentiation (G stage)	Moderate (G2)	48 (34)	4.26 ± 0.47	36% ± 5.0%	11 ± 2.0	7.17 ± 2.29	8/31 (26)	6/31 (19)	8/31 (26)	9/31 (29)	4/31 (13)	46/48 (96)[1]
	Poorly (G3)	88 (62)	4.16 ± 0.45	35% ± 5.5%	11 ± 2.2	7.62 ± 3.54	18/47 (38)	18/47 (38)	17/47 (36)	20/47 (43)	9/47 (19)	84/88 (95)[1]
	Undifferentiated (G4)	3 (2)	4.36 ± 0.79	37% ± 6.6%	12 ± 2.3	9.16 ± 4.74	1/3 (33)	0/3 (0)	0/3 (0)	1/3 (33)	0/3 (0)	2/3 (66)[1]

[1]Statistically significant difference between analyzed subgroup and control; [2]Statistically significant difference between subgroups, Kruskal-Wallis analysis of variance test. RBC: Red blood cells count; Ht: Hematocrit; Hb: Hemoglobin; WBC: White blood cells; MSI: Microsatellite instability; BAT26: Marker for MSH2; BAT25: Marker for the c-kit oncogene; BAT40: Marker for the HSD3B2; MSI-L: Low grade MSI, mutation in 1-2 BAT markers; MSI-H: High grade MSI, mutation in 3 BAT markers; CRC: Colorectal cancer; M: Male; F: Female.

MSI-L in 30 cases and MSI-H in 14 cases, which equates to 44 (52%) cases with the MSI genotype and 40 (48%) with MSS. The BAT25 and BAT40 markers were different according to the Dukes' stage (Kruskal-Wallis ANOVA test, $P < 0.05$), followed by higher occurrence of mutations in CRC cases with positive lymph node metastasis (any T, N1-2, any M) *vs* negative metastasis (Kruskal-Wallis ANOVA test, $P < 0.05$). We found no relationship between MSI status and *LATS1* expression.

MSI status and LATS1 expression

To check if the MSI phenotype influenced the expression pattern of *LATS1*, we compared QPCR results of *LATS1* expression ratios in MSS, MSI-L, and MSH-H cases (Figure 2). Although we found that MSI-L and MSI-H samples were characterized by higher *LATS1* levels than MSS cases, we found no statistical differences among those ratios (Figure 2).

Methylation status of the LATS1 gene promoter and its relationship with mRNA levels, clinical data and MSI data

To assess the methylation profile of *LATS1* during CRC progression, we analyzed 44 CRC cases with different clinico-pathological outcomes. Hypermethylation of the *LATS1* promoter was present in 25/44 cases (57%), whereas in 19 CRC tumor samples, no hypermethylation was found. No correlations were observed between *LATS1* hypermethylation and gender, age, tumor location, Dukes' stage, or G grading. Hypermethylation of the *LATS1* promoter was found in all patients with Dukes' A and D stages, and in 31% and 58% cases with Dukes' B and C stages, respectively (Table 2).

Comparison of *LATS1* promoter methylation status with *LATS1* mRNA levels in the analyzed tumor specimens (Table 2 and Figure 3) revealed a very significant reduction in *LATS1* expression in hypermethylated cases *vs* non-hypermethylated cases ($P < 0.05$). The biggest difference was observed in Dukes' C stage in which the *LATS1*

Table 2 Relationship between large tumor suppressor 1 promoter methylation status, large tumor suppressor 1 mRNA level, and histopathological and microsatellite instability data in colorectal cancer *n* (%)

Clinical parameter		Total	M	Av. *LATS1* mRNA fold change, control *vs* M	UM	Av. *LATS1* mRNA fold change, control *vs* UM	Av. *LATS1* mRNA fold change, UM *vs* M	*P* value between UM and M groups
Tumor total		44	25 (57)	597	19	3.55	162	0.00005
Dukes' stage	A	4	4 (100)	556	0	No data	No data	-
	B	16	5 (31)	699	11	228	3	0.041
	C	19	11 (58)	469	8	1.53	305	0.009
	D	5	5 (100)	1263	0	No data	No data	-
Lymph node metastasis	Negative	20	9 (45)	632	11	75	8	0.015
	Positive	24	16 (67)	586	8	1.53	381	0.0002
Histological differentiation G stage	G1	2	1 (50)	801	1	538	1.5	NS
	G2	11	3 (27)	1216	8	1.5	802	0.018
	G3	28	20 (71)	538	8	166	3.65	0.015
	G4	3	1 (33)	699	2	92	8	NS
MSI status	MSS	27	19 (70)	7	8	1.7	4	NS
	MSI-L	11	7 (64)	8	4	2.4	3.3	NS
	MSI-H	6	4 (67)	12	2	3.5	3.4	NS

M: Hypermethylation of large tumor suppressor 1 (*LATS1*) promoter; UM: Unmethylation of *LATS1* promoter; MSI: Microsatellite instability; MSS: Microsatellite stable; MSI-L: Low grade MSI, mutation in 1-2 BAT markers; MSI-H: High grade MSI, mutation in 3 BAT markers; NS: Noy significant.

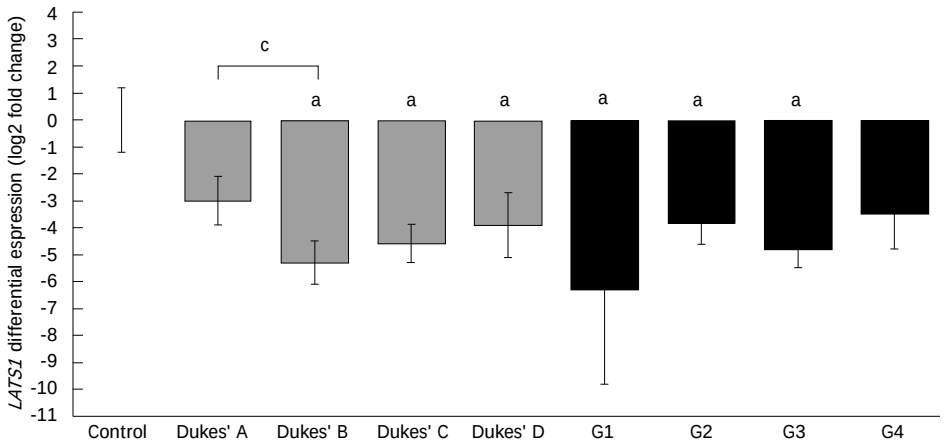

Figure 1 Large tumor suppressor 1 mRNA levels in colorectal cancer and control colon biopsies. Quantitative polymerase chain reaction results of large tumor suppressor 1 (*LATS1*) expression in 142 colorectal cancer (CRC) samples compared with 40 colon biopsies of healthy patients. CRC cases were divided according to clinicopathological data: tumor stage - Dukes' A (*n* = 27), B (*n* = 41), C (*n* = 54), D (*n* = 20); histological differentiation of tumor cells (G staging): G1 (*n* = 3), G2 (*n* = 48), G3 (*n* = 88), G4 (*n* = 3). Vertical bars represent *LATS1* fold ratio calibrated to the average C_t of control ($\Delta\Delta C_t^{LATS1} = \Delta C_t^{LATS1, sample} - \Delta C_t^{LATS1, control}$), error bars: SE. $^aP < 0.05$ *vs* control group; $^cP < 0.05$ between subgroups (Mann-Whitney *U* test).

expression level was 305 times lower in methylated CRC samples than in unmethylated samples ($P < 0.05$, plot not shown). The lowest *LATS1* ratio was found in Dukes' D stage (1263 times lower than in control). However, we found that all analyzed samples in this subgroup showed a hypermethylation pattern, and thus, statistical comparison between methylated and unmethylated cases in D stage could not be evaluated. Interestingly, *LATS1* expression in unmethylated CRC cases was not statistically different from that in controls.

When the methylation status of the *LATS1* promoter was analyzed in patients with and without the presence of metastatic cells in regional lymph nodes and/or distant organs, we found a strong relationship between the metastatic potency of cancer and reduced expression of *LATS1* and hypermethylation of its promoter. The *LATS1* ratio was 381 times lower in hypermethylated *vs* unmethylated in metastatic CRC cases ($P < 0.05$, dark grey boxes in Figure 3, Table 2), and only 8 times lower in hypermethylated *vs* unmethylated in non-metastatic CRC cases ($P < 0.05$, light grey boxes in Figure 3, Table 2). Moreover the expression of *LATS1* in unmethylated metastatic CRC samples was 49 times reduced as compared to unmethylated non-metastatic CRC cases ($P < 0.05$, Figure 3, Table 2).

Comparison of the histological grading of CRC (G), methylation status, and *LATS1* mRNA levels revealed the highest proportion of hypermethylation (71% of analyzed cases) in poorly differentiated (G3) CRC cases (Table 2). However, the difference in the *LATS1* expression level in methylated cases was only ca. 4 times lower than in unmethylated cases in the G3 subgroup ($P < 0.05$, Table 2, Figure 4). On the contrary, the difference in the *LATS1* mRNA level between methylated and unmethyl-

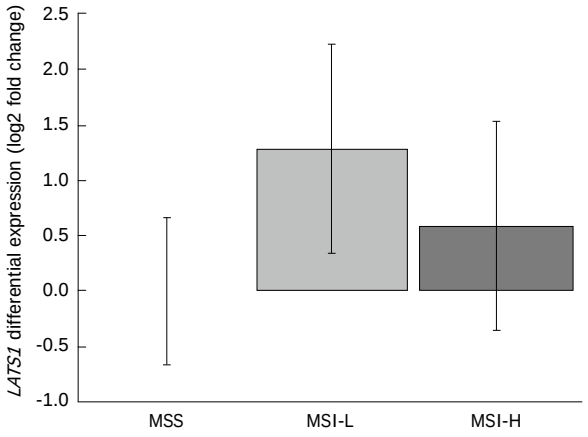

Figure 2 Microsatellite instability status and large tumor suppressor 1 expression. Comparison of large tumor suppressor 1 (*LATS1*) mRNA levels in cases divided by the mutations observed in the BAT26, BAT25, and BAT40 microsatellite markers. Samples were considered to have microsatellite stability (MSS; no mutation), microsatellite instability-low grade (MSI-L; 1-2 mutations, light grey box), and microsatellite instability-high grade (MSI-H; mutations in all three markers, dark grey box). MSS (n = 40), MSI-L (n = 30), MSI-H (n = 14). Vertical bars represent *LATS1* fold ratio calibrated to the average C_t of MSS cases - ($\Delta\Delta C_t^{LATS1} = \Delta C_t^{LATS1, MSI} - \Delta C_t^{LATS1, MSS}$), error bars: SE.

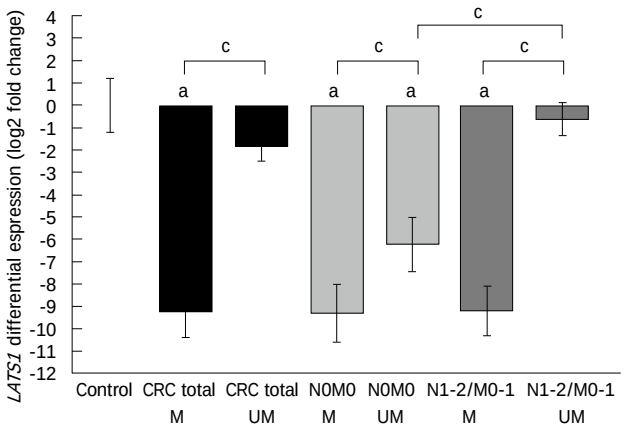

Figure 3 Large tumor suppressor 1 expression in colorectal cancer in relation to promoter methylation status. Comparison between large tumor suppressor 1 (*LATS1*) mRNA expression and epigenetic hypermethylation (M) or absence of hypermethylation (UM) of CpG islands located within the *LATS1* promoter region in a total of 44 colorectal cancer (CRC) cases (black vertical bars, n = 25 for M and n = 19 for UM). Vertical bars represent the *LATS1* fold ratio calibrated to the average C_t of control ($\Delta\Delta C_t^{LATS1} = \Delta C_t^{LATS1, sample} - \Delta C_t^{LATS1, control}$), error bars: SE. CRC cases were further divided into two subgroups: absence or presence of metastasis in lymph nodes/distant organs: N0M0: Light grey bars (n = 20; M: n = 9; UM: n = 11); N1-2/M0-1: Dark bars (n = 24; M: n = 16; UM: n = 8), respectively. $^aP < 0.05$ *vs* control group; $^cP < 0.05$ between subgroups (Mann-Whitney *U* test).

ated CRC tissue was much more pronounced in moderate-differentiated G2 cells ($P < 0.05$, Figure 4). Moreover, the *LATS1* ratio in G2 unmethylated cases was not statistically different from that in control healthy patients. Interestingly, G3 unmethylated cases showed much lower (ca. × 100) *LATS1* expression than G2 unmethylated biopsies ($P < 0.05$, Figure 4). Finally, we did not observe any statistically significant correlation between G grading and the *LATS1* mRNA level or hypermethylation of its promoter.

When we focused on MSI and the *LATS1* methylation status, we did not find any significant relationship because the statistical distribution of the results was very broad (Table 2, figure not shown). Most methylated cases (27/44) were considered MSS with no significant difference between methylated and unmethylated cases. MSI-L and MSI-H samples were also characterized as having relatively small differences in *LATS1* expression between methylated and unmethylated cases.

DISCUSSION

LATS1 is a tumor suppressor gene involved in several important mitotic processes, which are crucial in the development of CRC[7,44]. The most recent data suggest that the SWH pathway may play a very important role in CRC progression[45]. LATS1 is a key transducer of this pathway, and reduced expression of *LATS1* is connected with deregulation of SWH, thus activating the *YAP* oncogene[18]. Moreover, p53, a "genome guardian" protein, is indirectly regulated by LATS1[46]. MDM2, the regulator of p53 ubiquitination, is sequestered by native cellular LATS1, so that in the case of reduced *LATS1* expression, degradation of p53 cannot be triggered by MDM2[46]. Those observations suggest that studies on the role of *LATS1* in CRC should be intensified. Our investigation provides the first analysis of the *LATS1* expression profile in a relatively large group of CRC patients compared with 40 healthy persons as well as analysis of *LATS1* promoter hypermethylation as a putative quiescence factor for *LATS1* expression in CRC. The results of our quantitative study, which demonstrated decreased *LATS1* expression in 89% of CRC patients, are consistent with the decreased *LATS1* expression found in other tumors[20-22]. However, Bianchini *et al*[26] reported 3.11-fold increased expression of *LATS1* in 25 CRC patients compared with 13 non-cancerous adjacent tissue samples from the surgical margin. This discrepancy may be due to important methodological differences between the two studies. First, Bianchini *et al*[26] compared their CRC data to 13 non-cancerous adjacent tissue samples from the surgical margin, whereas in our study of 142 CRC (Dukes' stages A-D) patients, the histologically normal mucosa of 40 healthy controls was used as a reference sample. Second, Bianchini *et al*[26] used the microarray technique to generate expression profiles of 19200 different transcripts normalized to glyceraldehyde 3-phosphate dehydrogenase expression in only Dukes' B and C stage CRC patients. Seven transcripts are generated from *LATS1* (Ensembl database), however, Bianchini *et al*[26] did not specify the isoform they analyzed. Our QPCR assay was designed to amplify the functional isoform of *LAST1* that was also analyzed in other tumors[20-22]. Hence, our data cannot be directly compared with the contradictory results of Bianchini *et al*[26]. Moreover, we are not aware of any other reports suggesting increased *LATS1* expression in cancer. Immunohistochemical analysis of LATS1 protein expression in gastric cancer revealed lower expression

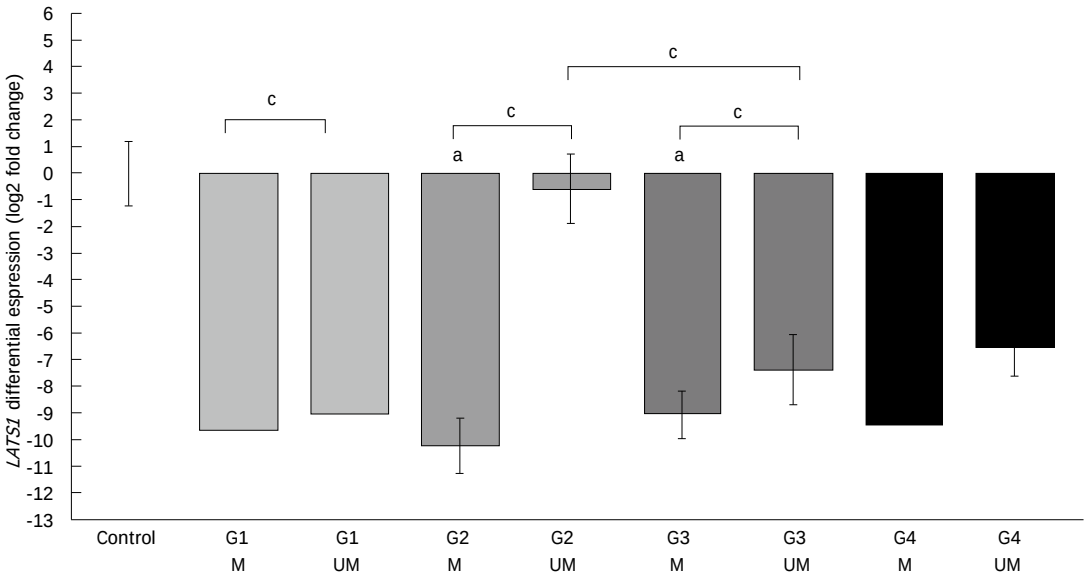

Figure 4 Methylation status of large tumor suppressor 1 in relation to the expression ratio and histological staging of cells. Forty-four colorectal cancer cases were classified according to histological examination: G1: Well-differentiated cells, *n* = 2 (light grey bars), G2: Moderately differentiated cells, *n* = 11 (grey bars), G3: Poorly differentiated cells, *n* = 28 (dark grey bars), G4: Undifferentiated cells, *n* = 3 (black bars). G1 epigenetic hypermethylation (M) (*n* = 1), G1 absence of hypermethylation (UM) (*n* = 1), G2 M (*n* = 3), G2 UM (*n* = 8), G3 M (*n* = 20), G3 UM (*n* = 8), G4 M (*n* = 1), G4 UM (*n* = 2). Vertical bars represent the large tumor suppressor 1 (*LATS1*) fold ratio calibrated to the average C_t of control ($\Delta\Delta C_t^{LATS1} = \Delta C_t^{LATS1, sample} - \Delta C_t^{LATS1, control}$), error bars: SE. $^aP < 0.05$ *vs* control group; $^cP < 0.05$ between subgroups (Mann-Whitney *U* test).

levels in 40 of 78 tumor lesions compared with normal gastric mucosa. The expression of LATS1 protein was significantly lower in gastric cancer with lymph node metastases than in cases without lymph node involvement[25]. Furthermore, in a group of 117 breast cancer patients, LATS1 mRNA was significantly decreased in the tumor tissue, and its decreased level was associated with a large tumor size, high lymph node metastasis rate, and poor prognosis[22]. In 30 astrocytoma cases, the level of LATS1 was 2-10 times lower as quantified by QPCR compared with 10 samples from normal brain tissue[21]. The most recent data showed that reduced expression of *LATS1* was correlated with the occurrence of metastatic glioma and poor survival of patients in a group of 17 cases[47]. Hence, decreased expression of *LATS1* in tumor tissue may suggest a suppressor role in CRC and other tumors.

MSI status has been regarded as one of the most important genetic markers and is strongly associated with molecular data, clinical findings, medical treatment, and patient outcome[48,49]. Our finding based on three BAT markers showed that more than half of the CRC patients had MSI tumors. Because we obtained samples from various clinics in different locations in Poland, our findings add to the observations by Smigiel *et al*[50] who observed MSI-L and MSI-H in 20% and 20.1% of cases, respectively, in a group of 143 CRC patients in the Lower Silesia region, which was not included in our analysis. The MSI phenotype may affect expression patterns of different proteins[51], and thus, we tried to estimate if decreased expression of *LATS1* was associated with MSI. Our findings excluded MSI-L and MSI-H as factors that may affect *LATS1* expression in the studied sample of CRC patients.

Inactivation of a typical tumor suppressor gene is generally induced by epigenetic factors such as mutation of one allele and/or loss of heterozygosity (LOH) of the other allele[52,53] or hypermethylation of CpG islands in the regulatory region of the gene[27-30]. Such factors may lead to a complete loss of gene function in cancer[54,55]. Expression of the *LATS1* transcript can be epigenetically decreased by hypermethylation of CpG islands located within the 5' upstream regulatory region of the gene[20-22]. Because *LATS1* was reduced in several malignancies, we decided to assess the hypermethylation status of the *LATS1* CpG island. Our study is the first report of the hypermethylation status of *LATS1* in CRC, showing an association between hypermethylation and decreased *LATS1* expression. *LATS1* hypermethylation was observed in 17/30 (56%) breast cancers and was associated with decreased *LATS1* expression; methylated cases showed a 3-fold decreased expression compared with unmethylated cases[22]. *LATS1* hypermethylation was found in 13/54 (24%) cases of head and neck cancer[24] and in 64% (56/88) of astrocytomas[21]. Moreover, in astrocytomas, the methylation status was associated with decreased *LATS1* expression[21]. A similar relationship between decreased *LATS1* expression and its hypermethylation was observed in our study in 57% of analyzed CRC cases. Interestingly, other known epigenetic factors do not seem to be involved in reduced *LATS1* expression in cancer. In a group of 25 breast cancers, LOH at 6q24-25.1 (*LATS1* locus) was found in only one case (4%), whereas no mutation was found and only two gene polymorphisms were observed. However, neither polymorphism caused amino acid substitution[31]. As further support that hypermethylation may be the major epigenetic factor in *LATS1* silencing, the expression of *LATS1* in the

hypermethylated cell lines U251 (an established glioma cell line) and SHG-44 (a human malignant glioma cell line) was restored by addition of 5-aza-deoxycytidine, and apoptosis of cancer cells results[21].

Decreased expression of *LATS1* that is associated with promoter hypermethylation may contribute to suppression of the SWH pathway[10,11,13,18]. This pathway is prone to deregulation because few proteins involved in signal transduction are both tumor suppressors and oncoproteins. Altered expression of *YAP*, *RASSF1A*, *LATS1*, and *MST2* in cancer cell lines leads to higher resistance of the cells to apoptosis[10,13,17,18]. Moreover, reduced expression of other genes that are not directly involved in the SWH pathway, such as WW and C2 domain containing 1 (*KIBRA*) and salvador homolog 1 (*SAV1*), may contribute to the quiescence of this pathway[11,16,56]. Such suppression of the SWH pathway is related to epithelial-to-mesenchymal transition features and poor prognosis in breast cancer[16].

In conclusion, this is the first study to show decreased expression of *LATS1* in CRC, confirming its tumor suppressor function and linking its downregulation to the epigenetic hypermethylation of the *LATS1* promoter region.

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COMMENTS

Background

Searching for new colorectal cancer (CRC) molecular markers is a very important objective, because CRC is one of the most common malignancies in the world and one of the most fatal of human neoplasms. The molecular mechanisms of CRC are still unknown, but deregulation of mitotic division as well as apoptosis resistance are clearly associated with CRC progression.

Research frontiers

Human large tumor suppressor 1 (*LATS1*) encodes a serine/threonine kinase, which mediates a tumor suppressor pathway called the Salvador-Warts-Hippo (SWH) pathway. Abnormal expression of *LATS1* was observed in some tumors, and its expression in CRC has not been analyzed quantitatively.

Innovations and breakthroughs

This is the first study of a large group of CRC patients that shows quantitatively reduced *LATS1* expression at the mRNA level. Decreased levels of *LATS1* were strongly associated with hypermethylation of its promoter, particularly in metastatic tumors.

Applications

With knowledge regarding the decreased expression of *LATS1* in CRC, focusing on its intracellular signaling pathways in CRC and the probable involvement of this gene in CRC pathogenesis as a molecular marker may be possible.

Terminology

LATS1 is a putative tumor suppressor gene that shows reduced expression in several malignancies. LATS1 is important in karyo- and cytokinesis and is part of the SWH pathway. Hypermethylation of the *LATS1* promoter is a common epigenetic factor responsible for downregulation and silencing of this gene.

Peer review

The authors collected and processed samples of CRC tumors and control colon biopsies from four collaborative clinics from four different regions of Poland. Molecular quantitative assays based on quantitative polymerase chain reaction revealed strongly reduced expression of *LATS1* in CRC tumors. Furthermore, this downregulation was strongly associated with the occurrence of hypermethylation of the *LATS1* promoter but not with microsatellite instability. This observation confirms the suppressor role of *LATS1* in carcinogenesis in this first study on a large group of CRC patients.

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BRIEF ARTICLE

Predictive findings for *Helicobacter pylori*-uninfected, -infected and -eradicated gastric mucosa: Validation study

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Abstract

AIM: To validate the usefulness of screening endoscopy findings for predicting *Helicobacter pylori* (*H. pylori*) infection status.

METHODS: *H. pylori* infection status was determined by histology, serology, and the urea breast test in 77 consecutive patients who underwent upper endoscopy. Based on the findings, patients were categorized as *H. pylori*-uninfected, -infected, or -eradicated cases. Using six photos of certain sites in the stomach per case, we determined the presence or absence of the following endoscopic findings: regular arrangement of collecting venules (RAC), linear erythema, hemorrhage, fundic gland polyp (FGP), atrophic change, rugal hyperplasia, edema, spotty erythema, exudate, xanthoma, and mottled patchy erythema (MPE). The diagnostic odds ratio (DOR) and inter-observer agreement (*Kappa* value) for these 11 endoscopic findings used in the determination of *H. pylori* infection status were calculated.

RESULTS: Of the 77 patients [32 men and 45 women; mean age (SD), 39.7 (13.4) years] assessed, 28 were *H. pylori* uninfected, 28 were infected, and 21 were eradicated. DOR values were significantly high (< 0.05) for the following *H. pylori* cases: uninfected cases with RAC (11.5), linear erythema (24.5), hemorrhage (4.1), and FGP (34.5); for infected cases with atrophic change (8.67), rugal hyperplasia (15.8), edema (14.2), spotty erythema (11.5), and exudate (3.52); and for eradicated cases with atrophic change (32.4) and MPE (103.0). *Kappa* values were excellent for FGP (0.93), good for RAC (0.63), hemorrhage (0.79), atrophic change (0.74), and MPE (0.75), moderate for linear erythema (0.51), rugal hyperplasia (0.49), edema (0.58), spotty erythema (0.47), and exudate (0.46), and poor for xanthoma (0.19).

CONCLUSION: The endoscopic findings of RAC, hemorrhage, FGP, atrophic change, and MPE will be useful for predicting *H. pylori* infection status.

Key words: Diagnostic odds ratio; Endoscopic finding; Eradication therapy; *Helicobacter pylori*; Inter-observer agreement

Core tip: To determine useful findings for predicting *Helicobacter pylori* (*H. pylori*)-uninfected, -infected,

or -eradicated cases, we evaluated following 11 endoscopic findings, regular arrangement of collecting venules (RAC), linear erythema, hemorrhage, fundic gland polyp (FGP), atrophic change, rugal hyperplasia, edema, spotty erythema, exudate, xanthoma, and mottled patchy erythema (MPE). Among these, RAC, hemorrhage, FGP, atrophic change, and MPE were found to be predictive findings for *H. pylori* infection status on screening endoscopy. The knowledge of these findings may contribute to the early detection of gastric cancer.

Watanabe K, Nagata N, Nakashima R, Furuhata E, Shimbo T, Kobayakawa M, Sakurai T, Imbe K, Niikura R, Yokoi C, Akiyama J, Uemura N. Predictive findings for *Helicobacter pylori*-uninfected, -infected and -eradicated gastric mucosa: Validation study. *World J Gastroenterol* 2013; 19(27): 4374-4379 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i27/4374.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i27.4374

INTRODUCTION

Gastric cancer remains the second leading cause of cancer death, accounting for 600000 deaths annually worldwide[1]. The incidence of gastric cancer is particularly high in Asia, especially in China, Japan and Korea where *Helicobacter pylori* (*H. pylori*) infection is highly prevalent. The risk of gastric cancer can differ depending on whether individuals are uninfected or infected with *H. pylori* or whether the infection has been eradicated[2-4]. It is therefore extremely important in the early detection of gastric cancer that *H. pylori* infection status is determined for each of these groups of individuals.

Endoscopy is an essential diagnostic tool for gastric cancer, enabling various findings induced by histological inflammation of the gastric mucosa to be detected. The development of histological gastritis is regarded as rare in *H. pylori*-uninfected cases, but it is usually noted in *H. pylori*-infected cases and improved by eradication therapy. This difference is reflected in the appearance of the gastric mucosa, which has been reported in the form of endoscopic findings in several studies[5-12]. However, the predictive value of the findings has not yet been validated.

In this study, we assessed, in a systematic manner, 11 endoscopic findings in dyspeptic patients and evaluated which of the findings were associated with *H. pylori*-uninfected, -infected, and -eradicated cases.

MATERIALS AND METHODS

Subjects

A total of 148 consecutive patients with dyspepsia who had undergone upper gastrointestinal endoscopy and had been strictly diagnosed with *H. pylori* infection between December 2008 and April 2009 at the National Center for Global Health and Medicine (NCGM) were identified from an endoscopic electronic database. The exclusion criteria applied were the use of non-steroidal anti-inflammatory drugs or anti-thrombogenic drugs and a history of gastric surgery, hemorrhagic disease, liver cirrhosis, renal failure, heart failure, or early or advanced gastric cancer, because these conditions could affect the mucosal appearance of the stomach[13-16]. After exclusion, 77 cases remained for analysis.

Written informed consent was obtained from all participants in accordance with the Declaration of Helsinki and its subsequent revision. The study protocol was approved by the Ethics Committee of NCGM (approval No. 811).

Diagnosis of H. pylori infection status

H. pylori infection status was evaluated by the presence of serum immunoglobulin G antibody against *H. pylori* (HM-CAP enzyme immunoassay, Enteric Products, Westbury, NY, United States), the [13C]-labeled urea breath test (13C-UBT, with a cut-off value of 2.5‰; Ubit, Otsuka Pharmaceuticals, Tokyo, Japan), and histological examination with toluidine blue staining of 3 endoscopic biopsy specimens taken from the greater curvature of the upper gastric body, angulus, and antrum, respectively. When all three methods yielded negative results, *H. pylori* infection status was considered "uninfected". When one or more of these methods yielded a positive result and there was no history of previous eradication therapy, *H. pylori* infection status was considered "infected". When histological examination and 13C-UBT yielded negative results and a history of eradication therapy was recorded, *H. pylori* infection status was considered "eradicated".

Endoscopic findings evaluated

All endoscopies were performed by well-trained endoscopists using a high resolution videoendoscope (GIF-260H, Olympus Co., Tokyo, Japan) with a pre-endoscopic oral solution containing dimethylpolysiloxane (Balgin Antifoaming Oral Solution 2%, Kaigen Co., Ltd., Osaka, Japan). In all cases, around 50-60 endoscopic images had been routinely recorded at fixed sites in the esophagus, stomach, and duodenum and saved to the electronic endoscopic database (Solemio ENDO, Olympus Co.). Six of the recorded images - specifically of the antrum, angulus, lesser and greater curvature of the lower body, greater curvature of the upper body, and cardia of the stomach - were used for analysis in each case.

The presence or absence of the following 11 distinctive endoscopic findings were evaluated (Figures 1 and 2): regular arrangement of collecting venules (RAC)[5], linear erythema[9], hemorrhage[9], fundic gland polyp (FGP)[10], atrophic change[6,7], severity of atrophy (open/closed)[6,7], rugal hyperplasia[8], edema[9] (which is visible as a thickened mucosal layer especially at the angulus and cardia), spotty erythema[9], exudate[9], xanthoma[11], and mottled patchy erythema (MPE)[12]. A well-experienced endoscopist (Kobayakawa M) assessed the findings, and to determine inter-observer agreement, another experi-

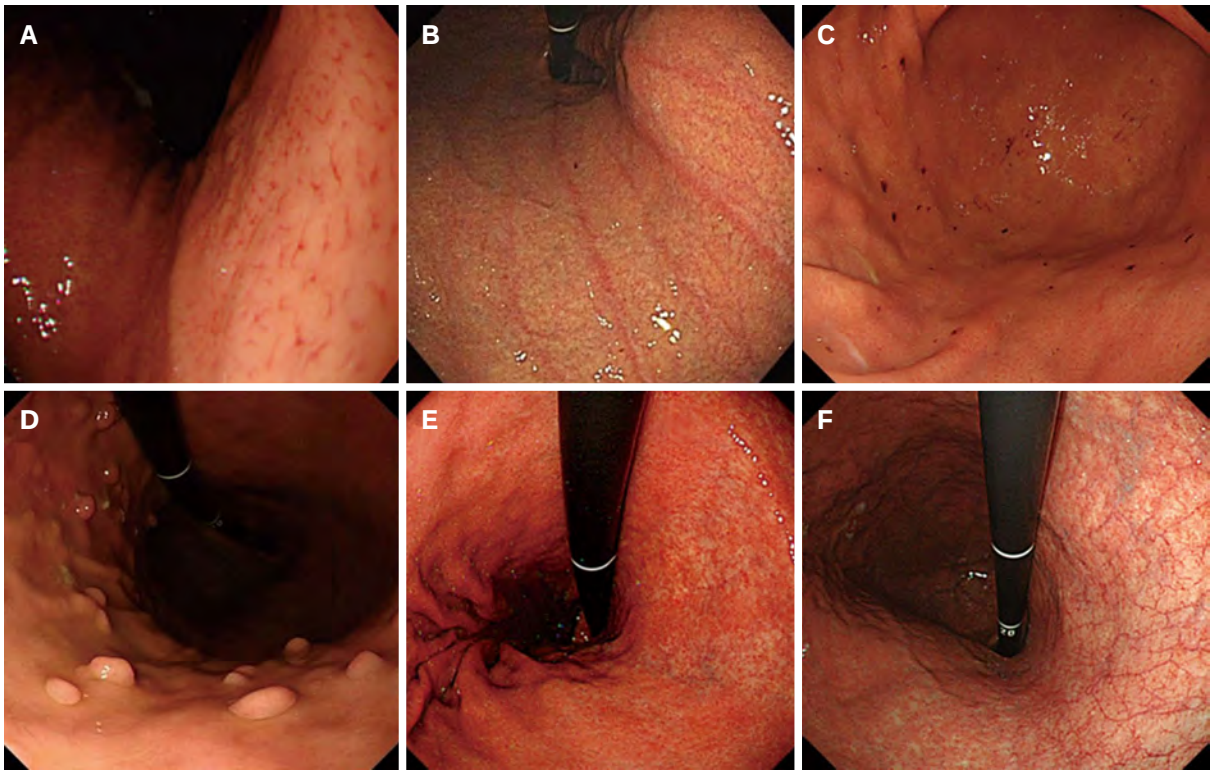

Figure 1 Five of the 11 endoscopic findings evaluated in this study. A: Regular arrangement of collecting venules in the body; B: Linear erythema in the lesser curvature of lower body; C: Hemorrhage in the lower body; D: Fundic gland polyp in the greater curvature of the body; E: Close type atrophic change in the lesser curvature of the body; F: Open type atrophic change over the lesser curvature of the body.

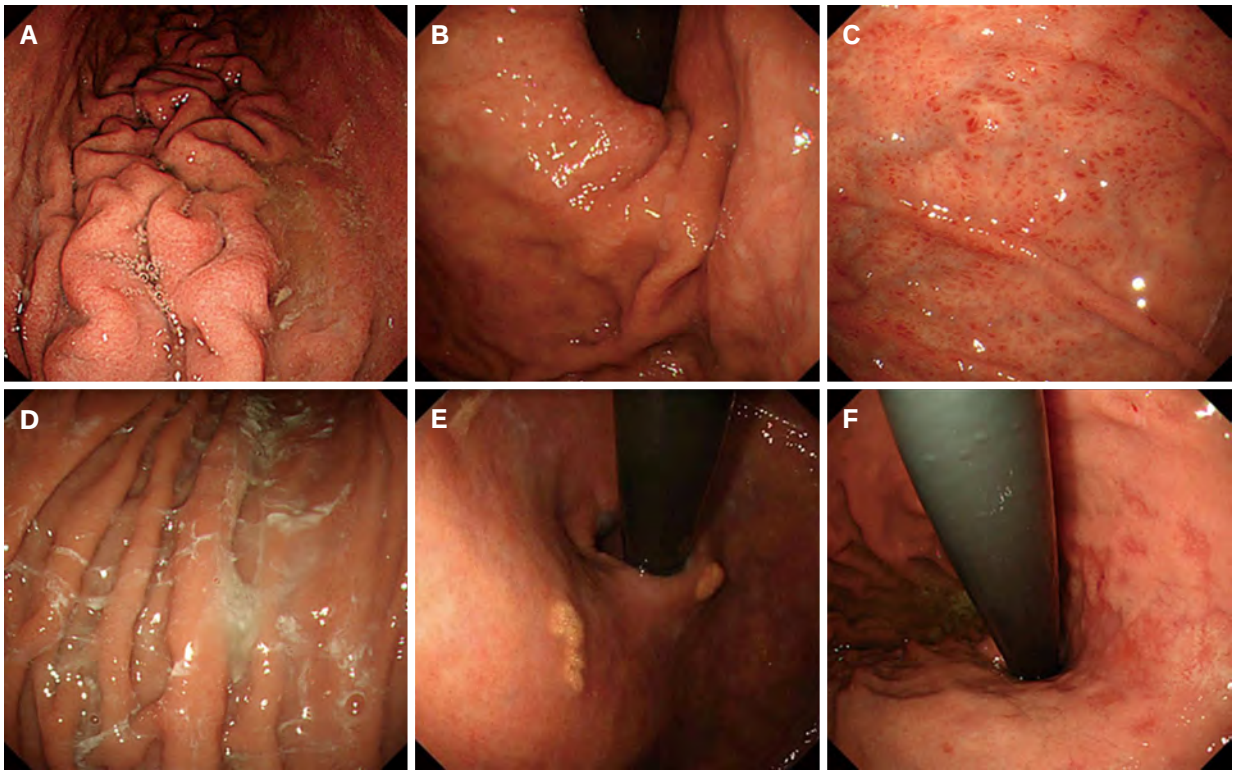

Figure 2 Remaining 6 of the 11 endoscopic findings evaluated. A: Rugal hyperplasia in the greater curvature of the middle body; B: Edema visible in the cardia; C: Spotty erythema in the greater curvature of the upper body; D: Exudate in the greater curvature of the upper body; E: Xanthoma in the upper body; F: Mottled patchy erythema in the lesser curvature of the body.

Table 1 Diagnostic odds ratios of endoscopic findings in the diagnosis of three groups of *Helicobacter pylori* infection status

Endoscopic finding	*H. pylori*		
	Uninfected	Infected	Eradicated
RAC	11.5 (3.73-35.1)	0.03 (0.00-0.14)[a]	1.65 (0.61-4.47)[a]
Linear erythema	24.5[1] (4.12-146)	0.05[1] (0.00-0.83)[a]	0.18 (0.00-1.20)[a]
Hemorrhage	4.11 (1.54-11.0)	0.03 (0.00-0.19)[a]	2.52 (0.92-6.95)[a]
Fundic gland polyp	34.5[1] (1.89-632)	0.10[1] (0.01-1.81)[a]	0.15[1] (0.01-2.81)[a]
Atrophic change	0.01 (0.00-0.06)	8.67[1] (2.11-35.6)[a]	32.4[1] (1.87-562)[a]
Rugal hyperplasia	0.02[1] (0.00-0.34)	15.8 (4.86-51.4)[a]	0.66 (0.22-2.03)
Edema	0.10 (0.03-0.31)	14.2 (4.52-44.1)[a]	0.66 (0.24-1.81)
Spotty erythema	0.09 (0.00-0.60)	11.5 (3.03-42.7)[a]	0.35 (0.00-1.54)
Exudate	0.27 (0.00-1.18)	3.52 (1.16-11.6)[a]	0.77 (0.21-2.93)
Xanthoma	0.86 (0.22-3.46)	2.45 (0.64-9.29)	0.30 (0.00-2.02)
Mottled patchy erythema	0.07[1] (0.00-1.17)	0.07[1] (0.00-1.17)	103[1] (5.64-1888)[a]

Values of odds ratios (95%CI) are shown. [1]Odds ratios were estimated using the substitution formula. 0.5 was added to all cell frequencies before calculation. [a]$P < 0.05$ *vs* uninfected. RAC: Regular arrangement of collecting venules; *H. pylori*: *Helicobacter pylori*.

enced endoscopist (Sakurai T) also assessed these findings. Both were blinded to clinical information in the cases examined.

Statistical analysis

To identify the predictive endoscopic findings for *H. pylori* infection status from among the 462 endoscopic images for the 77 patients, diagnostic odds ratios (DOR) for the 11 endoscopic findings in *H. pylori*-uninfected, -infected, and -eradicated cases were calculated. In addition, 95%CI were also estimated. DOR is defined as the positive likelihood ratio divided by the negative likelihood ratio. Positive likelihood ratio was calculated by sensitivity/1-specificity, and negative likelihood ratio was calculated by specificity/1-sensitivity[17].

The inter-observer agreement for each endoscopic finding among the two endoscopists was measured using *kappa* statistics. *Kappa* values (k) > 0.80 denoted excellent agreement, > 0.60-0.80 good, > 0.40-0.60 moderate, > 0.20-0.40 fair, and ≤ 0.20 poor[18]. Values of $P < 0.05$ were considered significant. All statistical analysis was performed using Stata version 10 software (StataCorp, Lakeway Drive College Station, TX, United States).

RESULTS

Patient characteristics

Of the 77 patients [32 men and 45 women; mean age (SD), 39.7 (13.4) years] assessed, 28 were *H. pylori* uninfected, 28 were infected, and 21 were eradicated.

Diagnostic odds ratio of endoscopic findings

The DOR for each endoscopic finding in the diagnosis of the three groups of *H. pylori* infection status are shown in Table 1. In cases diagnosed as *H. pylori* uninfected, RAC (11.5), linear erythema (24.5), hemorrhage (4.1), and FGP (34.5) had high DORs and were significantly associated ($P < 0.05$). In infected cases, atrophic change (8.67), rugal hyperplasia (15.8), edema (14.2), spotty erythema (11.5), and exudate (3.52) had high DORs and were significantly associated ($P < 0.05$). Lastly, in eradicated diagnosis, atrophic change (32.4) and MPE (103.0) had high DORs and were significantly associated ($P < 0.05$).

Open type atrophy was significantly ($P < 0.05$) more frequent on endoscopy in *H. pylori* infected patients (20/28, 71.4%) than in *H. pylori* eradicated patients (8/21, 38.1%).

Table 2 Inter-observer agreement for the 11 endoscopic findings evaluated

Endoscopic finding	*Kappa* value
RAC	0.63
Linear erythema	0.51
Hemorrhage	0.79
Fundic gland polyp	0.93
Atrophic change	0.74
Rugal hyperplasia	0.49
Edema	0.58
Spotty erythema	0.47
Exudate	0.46
Xanthoma	0.19
Mottled patchy erythema	0.75

RAC: Regular arrangement of collecting venules.

Inter-observer agreement for the endoscopic findings evaluated

The *kappa* value indicating agreement between the two endoscopists for each endoscopic finding is shown in Table 2.

DISCUSSION

This study identified several endoscopic findings that are clearly associated with uninfected, infected, and eradicated *H. pylori* infection. Some endoscopic findings have been previously reported to be correlated with *H. pylori* infection. Atrophic change was found to be associated with the infection in an aged group (OR = 9.8)[19] as well as in general[7,20]. Rugal hyperplasia was reported to be correlated with *H. pylori* infection[21]. Edema, with or without exudate, and spotty erythema were considered to be a result of mucosal inflammation, but these positive findings were not definitive[9]. Lastly, xanthoma, which refers to yellowish-white small nodules or plaques in the gastric mucosa, is considered to be related with *H. pylori* infection[11]. Among these findings, we clarified that all of them - atrophic change, rugal hyperplasia, edema, spotty erythema, and exudate - are valuable endoscopic findings of *H. pylori* infection.

In regard to the predictive findings related with *H. pylori* uninfected mucosa, RAC has been well studied and showed a positive association[5,22]. Fundic gland polyp is also considered to be a finding associated only with uninfected cases[10]. In the present study these endoscopic findings showed high odds ratios and thus support the results of earlier studies. While hemorrhage and linear er-

ythema were found to be associated with well-preserved gastric acid secretion, they were not clearly associated with *H. pylori* uninfected mucosa[9]. In the present study, however, these two findings had high odds ratios, suggesting they are valuable for predicting *H. pylori*-uninfected cases.

This study also investigated *H. pylori*-eradicated cases, because the preventive effect of *H. pylori* eradication therapy for gastric cancer has been reported[3,4] and therefore an increasing number of patients will likely receive *H. pylori* eradication therapy into the future[23,24]. Gastric cancer can, however, still occur in eradicated cases, but it is generally difficult to diagnose and few predictive endoscopic findings have been reported. Of those that have been suggested are the disappearance of rugal hyperplasia[8] and hyperplastic polyp[25], but as these findings need to be compared before and after eradication therapy, on their own it seems difficult to apply them to clinical use. Atrophic change, occurring as a result of *H. pylori* infection, is thought to remain after eradication therapy[26]. Moreover, in our previous study, MPE, which is recognized as a flat or slightly depressed reddish lesion that is distinguishable from the congested mucosa, emerged after *H. pylori* eradication therapy[12]. In the study too, atrophic change and MPE was highly predictive of *H. pylori* eradicated mucosa, suggesting that a combination of these findings is highly valuable in clinical practice.

In regard to the reproducibility of endoscopic image evaluation for these 11 findings, among the positive endoscopic findings found, RAC, hemorrhage, fundic gland polyp, atrophic change, and MPE all showed good inter-observer agreement, suggesting that these findings can be easily identified and will be generally useful. The other positive findings of edema, rugal hyperplasia, and spotty erythema showed moderate agreement, suggesting that they could also be suitable for general use.

There are several important strengths of this study, including that the diagnosis of *H. pylori* infection status was accurate, made on the basis of a combination of three different diagnostic tests to overcome any shortcomings of a single test. Moreover, DOR was used to estimate the diagnostic value: DOR is not influenced by prevalence rate, so the results are applicable in other populations with a different prevalence of *H. pylori* infection.

Nonetheless, this study has several limitations. First, the sample size of 77 patients is relatively small, and did not permit multivariate analysis to exclude confounders. Second, the population consists of relatively young patients, therefore cases in whom *H. pylori* is naturally eradicated without eradication therapy as a result of long-term course of severe atrophic gastritis, may not be included. Furthermore, photographic not video images were used for analysis and therefore the whole stomach was not observed, so some findings that may be present were not assessed.

In conclusion, the endoscopic findings associated with *H. pylori* infection status that are common and have good inter-observer agreement were clarified to be RAC, hemorrhage, fundic gland polyp, atrophic change, and MPE. These findings should be generally useful in clinical practice and contribute to the early detection of gastric cancer.

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COMMENTS

Background

The risk of gastric cancer can differ among *Helicobacter pylori* (*H. pylori*) uninfected, infected, and eradicated patients, therefore it is important to determine *H. pylori* infection status. Endoscopic prediction of *H. pylori* infection status can be extremely useful for early detection of gastric cancer, but the diagnostic value of *H. pylori* related endoscopic findings has not yet been validated.

Research frontiers

Some endoscopic findings were reported to be related with *H. pylori* uninfected, infected case, and little has been reported on eradicated case. The accuracy of these findings varies in each studies, and have not been well examined. In this study, the authors demonstrate the accuracy and reproducibility of endoscopic findings which have been previously reported to be correlated with *H. pylori* infection.

Innovations and breakthroughs

This is the first study which evaluate the value of predictive endoscopic findings separately for 3 groups of *H. pylori* infection status; uninfected, infected, and eradicated. Moreover, the authors used diagnostic odds ratio (DOR) to estimate the diagnostic value of endoscopic findings. DOR is not influenced by prevalence rate, so the results are applicable in other populations with a different prevalence of *H. pylori* infection.

Applications

The endoscopic findings associated with *H. pylori* infection status that are common and have good reproducibility were clarified to be regular arrangement of collecting venules, hemorrhage, fundic gland polyp, atrophic change, and mottled patchy erythema. These findings should be generally useful in clinical practice and contribute to the early detection of gastric cancer.

Terminology

DOR is defined as the positive likelihood ratio divided by the negative likelihood ratio. DOR is single indicator of diagnostic test, and higher value is indicative of better test performance, irrespective of prevalence rate.

Peer review

The authors showed endoscopic features of gastric mucosa according to *H. pylori* infection status. Although most endoscopists are usually aware of the correlation between endoscopic findings and *H. pylori* status, the simplification and clarification of the correlation by showing typical endoscopic findings and their diagnostic odds ratios may be worth publication.

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BRIEF ARTICLE

Effect of DA-9701 on gastric emptying in a mouse model: Assessment by ^{13}C-octanoic acid breath test

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Author contributions: Lim CH and Choi MG designed the study; Lim CH and Park H performed animal experiments; Lim CH and Park JM collected data, analyzed the results of the statistical analyses; Lim CH, Choi MG, and Baeg MK wrote and edited the paper, and approved the final version.

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Abstract

AIM: To evaluate the effects of DA-9701 on the gastric emptying of a solid meal using the ^{13}C-octanoic acid breath test in a mouse model.

METHODS: Male C57BL/6 mice aged > 8 wk and with body weights of 20-25 g were used in this study. The solid test meal consisted of 200 mg of egg yolk labeled with 1.5 L/g ^{13}C-octanoic acid. The mice were placed in a 130 mL chamber flushed with air at a flow speed of 200 mL/min. Breath samples were collected for 6 h. The half-emptying time and lag phase were calculated using a modified power exponential model. To assess the reproducibility of the ^{13}C-octanoic acid breath test, the breath test was performed two times at intervals of one week in ten mice without drug treatment. To assess the gastrokinetic effects of DA-9701, the breath test was performed three times in another twelve mice, with a randomized crossover sequence of three drug treatments: DA-9701 3 mg/kg, erythromycin 6 mg/kg, or saline. Each breath test was performed at an interval of one week.

RESULTS: Repeatedly measured half gastric emptying time of ten mice without drug treatment showed 0.856 of the intraclass correlation coefficient for the half gastric emptying time (P = 0.004). The mean cumulative excretion curve for the ^{13}C-octanoic acid breath test showed accelerated gastric emptying after DA-9701 treatment compared with the saline control (P = 0.028). The median half gastric emptying time after the DA-9701 treatment was significantly shorter than after the saline treatment [122.4 min (109.0-137.9 min) *vs* 134.5 min (128.4-167.0 min), respectively; P = 0.028] and similar to that after the erythromycin treatment [123.3 min (112.9-138.2 min)]. The lag phase, which was defined as the period taken to empty 15% of a meal, was significantly shorter after the DA-9701 treatment than after the saline treatment [48.1 min (44.6-57.1 min) *vs* 52.6 min (49.45-57.4 min), respectively; P = 0.049].

CONCLUSION: The novel prokinetic agent DA-9701 accelerated gastric emptying, assessed with repeated measurements in the same mouse using the ^{13}C-octanoic acid breath test. Our findings suggest that DA-9701 has therapeutic potential for the treatment of functional dyspepsia.

Key words: DA-9701; Gastric emptying; Prokinetic agent; Breath test; Functional dyspepsia

Core tip: DA-9701 is a newly formulated prokinetic agent obtained from extracts of Pharbitis Semen and Corydalis Tuber. The ^{13}C-octanoic acid breath test is a reliable and responsive method for measuring gastric

emptying in small laboratory animal. This technique can be performed repeatedly in the same animal and reflect exact pharmacological effects without sacrifice of the animal. This study demonstrated the gastrokinetic effects of DA-9701, using repeated ^{13}C-octanoic acid breath tests in the same animal. The gastrokinetic effect of DA-9701 could have therapeutic potential for the treatment of familial dysautonomia.

Lim CH, Choi MG, Park H, Baeg MK, Park JM. Effect of DA-9701 on gastric emptying in a mouse model: Assessment by ^{13}C-octanoic acid breath test. *World J Gastroenterol* 2013; 19(27): 4380-4385 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i27/4380.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i27.4380

INTRODUCTION

DA-9701 is a newly formulated prokinetic agent obtained from extracts of Pharbitis Semen and Corydalis Tuber. Both the seed of Pharbitis Semen and the root of Corydalis Tuber have been used in traditional Oriental medicine for the treatment of gastrointestinal symptoms. DA-9701 and its components accelerated gastric emptying and improved gastric accommodation in animal model[1-4]. A previous study demonstrated the gastrokinetic effect of DA-9701 in a rat model[1], using the method of Ozaki *et al*[5]. With this model, the animal must be killed to assess gastric emptying, and consequently, gastric emptying can only be measured at that one point in time. The disadvantage of this method is that it is impossible to assess gastric emptying repeatedly in the same animal. Therefore, the utility of the model is limited for evaluating pharmacologically induced gastric emptying because there is great intersubject variability.

Stable isotope breath tests are indirect noninvasive tests for measuring gastric emptying. Their advantages include the absence of a radiation hazard, ease of handling, and no requirement for positioning, unlike scintigraphic tests[6]. Breath tests offer an attractive method of measuring gastric emptying in small laboratory animals because the animal need not be killed, thus allowing repeated measurements to be made in the same animal. With the ^{13}C-octanoic acid breath test, gastric emptying can be assessed noninvasively, and the differences in gastric emptying induced in small laboratory animals by pharmacological agents can be quantified[7-11]. This test is a useful tool in the development of new prokinetic agents that modulate gastric emptying. The aim of this study was to evaluate the effects of DA-9701 on the gastric emptying of a solid meal using the ^{13}C-octanoic acid breath test in a mouse model.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice, aged > 8 wk and with body weights of 20-25 g, were used for the breath test. Ten mice were used to assess the reproducibility of the ^{13}C-octanoic acid breath test and another 12 mice were used to evaluate the effects of DA-9701. Sample sizes were calculated from the equation below by referring to a previous study using C57BL/6 mice[8]: $n = 2 + C (s/d)^2$, where s is SD, d is expected difference between two means, and C is constant. A sample size of 12 mice was suitable to identify, with a power of 0.8, the expected difference in half gastric emptying time of 50 min with an alpha significance level of 0.05. All the mice were housed in a room maintained at 21 ℃-23 ℃ on a 14/10 h light/dark cycle. The mice had continuous access to water and a standard commercial diet. All experiments were approved by the Institutional Animal Care and Use Committee of the Catholic University of Korea, Seoul, South Korea.

Test meal

The gastric emptying rates were assessed for a solid egg yolk meal. ^{13}C-Octanoic acid (Octanoic acid-1-^{13}C, 99 atom % ^{13}C, Sigma-Aldrich Co. LLC, St Louis, Missouri, United States) was added to raw yolk at a concentration of 1.5 μL/g. The yolk was homogenized and heated in water at 60 ℃ for 120 min. The test meal (200 mg) was given to each animal after an overnight fast with free access to water and was completely consumed by the mouse within 1 min in the breath test chamber.

Breath test protocol

To assess the gastric emptying rate using the ^{13}C-octanoic acid breath test with measurements made up to 360 min, the mice were fasted overnight[7,8]. They were then placed in a 130 mL gas-tight rubber-sealed chamber with room air. The flow rate of air was 200 mL/min, which was selected because this rate was sufficient to flush any residual CO_2 from the chamber between the sampling time points. Three-way valves were connected to the chamber for air inlet and outlet to allow sampling. To collect a breath sample, the airflow through the chamber was stopped for 4 min. At the end of the breath-accumulation period, 10 mL of breath was syringed from the chamber, and the airflow through the chamber was restored. Each mouse was maintained in the chamber for the whole 360 min sampling procedure. A baseline breath sample was taken before the test meal was consumed. Further breath samples were collected at 5 min intervals for the first 30 min, and at 15 min intervals thereafter until 360 min after the consumption of the test meal.

Reproducibility of the breath test

To assess the reproducibility of the ^{13}C-octanoic acid breath test, the breath test was performed two times at intervals of 1 wk in ten mice without drug treatment.

Drug treatment

This study was designed as a randomized crossover study. DA-9701 (3 mg/kg) was the test drug, erythromycin (6 mg/kg) the positive control, and normal saline the

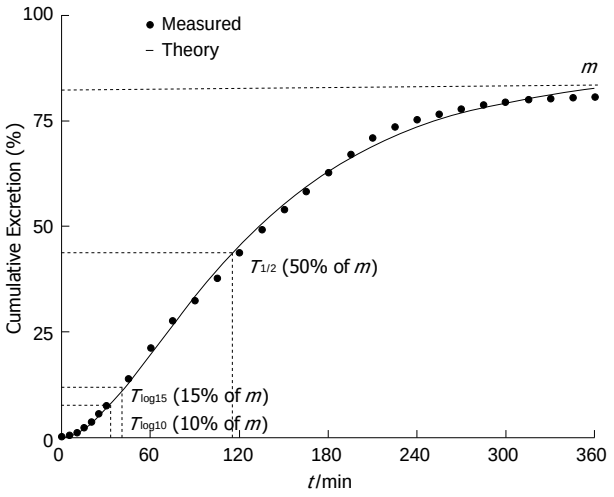

Figure 1 Typical excretion curve of the ^{13}C-octanoic acid breath test in the mouse model. *m*: The total cumulative ^{13}C recovery when time is infinite; $T_{1/2}$: Half gastric emptying time; T_{lag10}: Lag phase of 10 % emptying; T_{lag15}: Lag phase for 15% emptying.

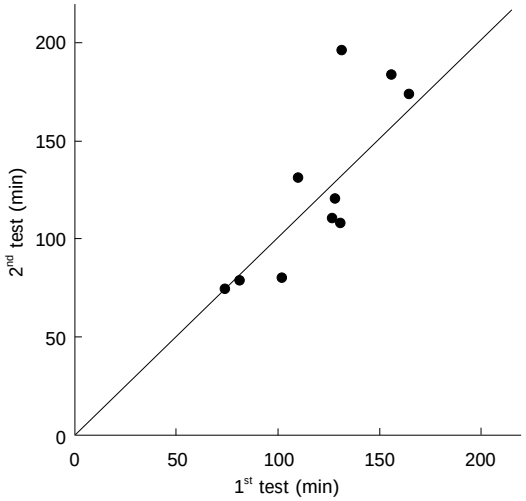

Figure 2 Reproducibility of the ^{13}C-octanoic acid breath test in ten mice.

placebo. There were six possible permutations of the drug treatment sequences, and two animals were allocated to each permutation. The test drug and the positive control were suspended in distilled water for their oral administration. A 10 mL/kg volume of DA-9701, erythromycin, or saline was given orally to each mouse by gavage 1 h before the breath test. The breath test was performed three times in each animal at intervals of 1 wk.

Data analysis

The $^{13}CO_2$ content of each breath sample was analyzed using a HeliView isotope ratio mass spectrometer (Medichems, Seoul, South Korea). The [^{13}C]/[^{12}C] ratio (δ) was expressed as parts per thoUnited Statesnd relative to the Pee Dee Belemnite calcium carbonate international primary standard. The gastric emptying rates were calculated from the resultant $^{13}CO_2$ excretion curves. The CO_2 production rate of the mice was assumed to be 40 mL/kg per minite based on normal values for the resting metabolic parameters measured in C57BL/6 mice[12]. The percentage $^{13}CO_2$ cumulative values were fitted using a modified power exponential model[13]: $y = m\ (1 - e^{-kt})^{\beta}$, where y is the cumulative percentage of $^{13}CO_2$ excretion in breath at time t (h), and m, k and β are estimated parameters. In this model, m is interpreted as the total cumulative ^{13}C recovery when the time is infinite. The cumulative $^{13}CO_2$ excretion cannot reach 100% because a substantial amount of the orally administered dose is fixed in the bicarbonate pool in the body. Because the cumulative excretion reaches a steady state, sampling up to 360 min allows the calculation of m, the theoretical recovery after infinite time. The half gastric emptying time ($T_{1/2}$), the lag phase for 10% emptying (T_{lag10}), and the lag phase for 15% emptying (T_{lag15}) in the breath test were calculated from this model as $T_{1/2} = (-1/k) \times \ln[1 - (0.5^{1/\beta})]$; $T_{lag10} = (-1/k) \times \ln[1 - (0.1^{1/\beta})]$; $T_{lag15} = (-1/k) \times \ln [1 - (0.15^{1/\beta})]$.

Statistical analysis

The results are expressed as medians and interquartile ranges. The reproducibility of breath test was evaluated using the intraclass correlation coefficient. A repeated measurements ANOVA followed by post hoc test at the point of every 60 min was performed for comparison of total cumulative excretion curve after drug treatment. Differences in the data were evaluated using the Wilcoxon signed-rank test for comparisons of the half gastric emptying times and lag phases after drug treatment. A difference was considered significant when the P value was less than 0.05.

RESULTS

A typical excretion curve for ^{13}C-octanoic acid breath test of a mouse is presented in Figure 1. Repeatedly measured half gastric emptying time of ten mice is presented Figure 2. The half gastric emptying times of the first and second breath tests were 127.5 min (96.7-137.6 min) and 115.4 (79.9-176.4 min). The half gastric emptying times of the first and second breath tests showed good correlation (intraclass correlation coefficient = 0.856, $P = 0.004$). The effects of the drug treatments on gastric emptying are shown in Figure 3. The cumulative excretion curve for ^{13}C-octanoic acid breath showed accelerated gastric emptying after treatment with DA-9701 compared with that after treatment with saline ($P = 0.028$). The effect of DA-9701 treatment on gastric emptying was similar to that of erythromycin.

The half gastric emptying times and lag phases are presented in Table 1. Both DA-9701 and erythromycin treatments induced significantly shorter half gastric emptying times compared with that induced with saline ($P = 0.028$ and $P = 0.049$, respectively). There was no significant difference in the half gastric emptying times of the DA-9701- and erythromycin-treated mice. There was no significant difference in T_{lag10} among the treatments. T_{lag15} was significantly shorter in mice treated with DA-9701

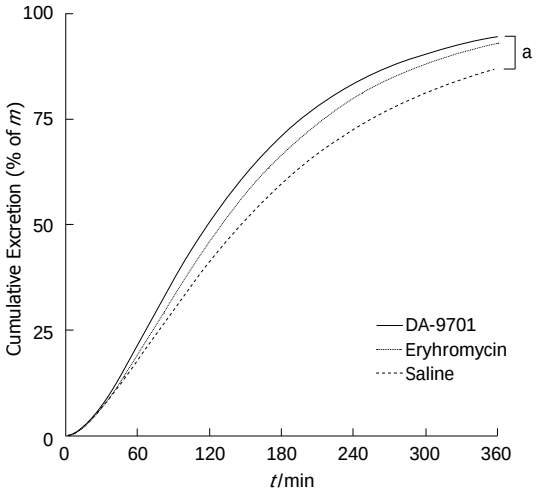

Figure 3 Mean excretion curve of the ^{13}C-octanoic acid breath test in mice treated with DA-9701, erythromycin, and saline. m: The total cumulative ^{13}C recovery when time is infinite. $^{a}P < 0.05$ between DA-9701 and saline.

Table 1 Half gastric empting time and lag phase measured by the ^{13}C-octanoic acid breath test in the mice treated with DA-9701, erythromycin, and saline

	DA-9701 (n = 12)	Erythromycin (n = 12)	Saline (n = 12)
$T_{1/2}$ (min)	122.4 (109.0-137.9)[1]	123.3 (112.9-138.2)[2]	134.5 (128.4-167.0)
T_{lag10} (min)	38.5 (33.2-46.4)	37.9 (35.8-41.4)	39.5 (35.2-45.3)
T_{lag15} (min)	48.1 (44.6-58.5)[2]	49.3 (46.5-53.3)	52.6 (48.9-57.6)

Data represent median with interquartile range in parentheses. [1]P = 0.028, [2]P = 0.049 *vs* saline treatment (Wilcoxon's signed ranks test). $T_{1/2}$: Half gastric emptying time; T_{lag10}: Lag phase for 10 % emptying; T_{lag15}: Lag phase for 15% emptying.

than in mice treated with saline (P = 0.049). The half gastric emptying time and T_{lag15} for each mouse treated with saline, DA-9701, or erythromycin is presented in Figure 4.

DISCUSSION

Dyspeptic symptoms are common problem in primary health care and gastroenterology practice. Although the precise pathophysiology of functional dyspepsia (FD) is not fully understood, disturbed gastric emptying is one of the important abnormalities in FD patients[14,15]. Current treatment approaches to FD include gastric acid suppression and gastroprokinetic drugs as primary treatment options[16]. However, no satisfactory therapeutic approach is currently available in clinical practice. There is an increasing need for the development of safe and effective prokinetic drugs without adverse effects[17-19].

We have demonstrated the effects of DA-9701 on gastric emptying, using repeated ^{13}C-octanoic acid breath tests in the same animal. DA-9701 shortened both the half emptying time and the lag phase, and these effects were similar to those of erythromycin. The half gastric emptying times achieved with the placebo and erythromycin in our model were similar to those reported in previous studies that

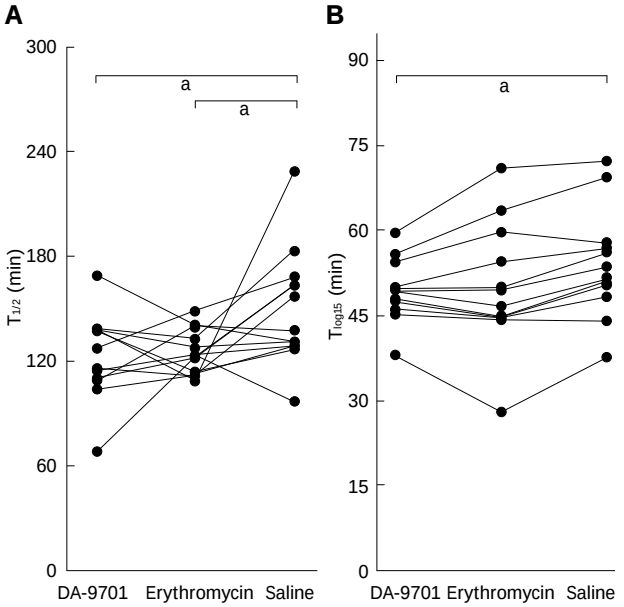

Figure 4 Half gastric emptying time and lag phase for 15% emptying measured by the ^{13}C-octanoic acid breath test in the each mouse treated with DA-9701, erythromycin, and saline. A: Half gastric emptying time ($T_{1/2}$); B: Lag phase for 15% emptying (T_{log15}). $^{a}P < 0.05$ *vs* saline.

used the same breath test[8]. We selected a dose of 3 mg/kg DA-9701 based on a previous study of the appropriate DA-9701 doses in a rat model, which showed dose-dependent effects in the range from 0.03 to 3 mg/kg[1]. Another experiment in which DA-9701 was administered at 3 mg/kg also showed accelerated gastric emptying in a model of delayed gastric emptying induced with apomorphine and cisplatin. DA-9701 and its components (corydaline and tetrahydroberberine) accelerated gastric emptying in a rat model when a method that requires the animals to be killed was used, although comparisons within the same animal were impossible[1-3]. We performed the repeated breath tests in each mouse after treatment with either DA-9701, the positive control, or the placebo, according to all possible permutations of the drug treatment sequence, and identified the prokinetic effects of DA-9701.

DA-9701 is a newly formulated prokinetic agent obtained from extracts of Pharbitis Semen and Corydalis Tuber. Both the seed of Pharbitis Semen and the root of Corydalis Tuber have been used in traditional Oriental medicine for the treatment of gastrointestinal symptoms. As well as accelerating gastric emptying, DA-9701 and its components improved gastric accommodation in a dog model with a barostat[1-4]. DA-9701 is composed of several isoquinoline alkaloid compounds, including corydaline, berberine, protopine, and palmatine[20-23]. Although the exact mechanism of its action has not been identified, tetrahydroberberine from DA-9701 has the properties of a D_2 receptor antagonist and a 5-HT_{1A} receptor agonist, with micromolar affinities for the dopamine D_2 and 5-HT_{1A} receptors[2]. DA-9701 significantly improved the symptoms of patients with FD, with efficacy similar to that of itopride and a comparable safety profile[24].

In this study, we have demonstrated the gastrokinetic

effect of DA-9701 using the ^{13}C-octanoic acid breath test in mice. A variety of techniques have been used to assess gastric emptying in small laboratory animals. Most techniques require the animal to be killed and the contents of the stomach and intestine to be quantified by measuring their radioactivity, counting the glass beads remaining, or measuring the concentration of a marker. The ^{13}C-octanoic acid breath test has been reported to be a reliable and responsive method for measuring gastric emptying in small laboratory animal[7,8,10,25], and has several advantages over other techniques. First, it allows repeated measurements to be made in the same animal, allowing exact pharmacological effects to be identified and minimizing the number of animals killed. Second, it does not require the animals to be handled or restrained, in contrast to scintigraphic tests. The results can also be expressed as rate curves and cumulative excretion curves. Therefore, the breath test can be used to determine the effects of physiological or pharmacological interventions on gastrointestinal motility, especially in developmental programs for new prokinetic drugs. However, to measure gastric emptying, the breath test requires a relatively long time and considerable effort to sample the animals' breath.

In conclusion, DA-9701 accelerates gastric emptying, and its effect is similar to that of erythromycin. The ^{13}C-octanoic acid breath test can be a useful tool to reflect physiological or pharmacological effects on gastric motility, especially in developmental programs for new prokinetic drugs. The gastrokinetic effect of DA-9701 could have therapeutic potential for the treatment of FD.

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COMMENTS

Background

Dyspeptic symptoms are common problem in primary health care and gastroenterology practice. Disturbed gastric emptying is one of the important abnormalities in functional dyspepsia (FD) patients. Gastroprokinetic drugs in one of the primary treatment options. There is an increasing need for the development of safe and effective prokinetic drugs without adverse effects

Innovations and breakthroughs

A previous study demonstrated the gastrokinetic effect of DA-9701 in a rat model using the method of Ozaki and Sukamoto. With this model, the animal must be killed to assess gastric emptying, and consequently, gastric emptying can only be measured at that one point in time. The disadvantage of this method is that it is impossible to assess gastric emptying repeatedly in the same animal. This study demonstrated the gastrokinetic effect of DA-9701 using repeated the ^{13}C-octanoic acid breath test in same animal with minimizing the number of animals killed.

Applications

The gastrokinetic effect of DA-9701 could have therapeutic potential for the treatment of FD. The ^{13}C-octanoic acid breath test can be a useful tool to reflect physiological or pharmacological effects on gastric motility, especially in developmental programs for new prokinetic drugs.

Terminology

^{13}C-octanoic acid breath test is one of stable isotope breath tests for measuring gastric emptying. The test meal containing ^{13}C-labelled substrates is retained in the stomach and emptied into the duodenum. The ^{13}C-labelled substrates are rapidly absorbed and transported to the liver. They are oxidized to $^{13}CO_2$ in the liver and excreted in the breath. Gastric emptying is the rate-limiting step that makes it possible to use the rate of $^{13}CO_2$ appearance in the breath approximate to the gastric emptying rate.

Peer review

This study examines the effects of DA-9701, a novel phyto-derived prokinetic agent, on gastric emptying of normal C57BL/6 mice. The authors used ^{13}C-octanoic acid breath test to investigate gastric emptying of three treatments (DA-9701, erythromycin *vs* saline, the latter used as placebo) with a randomized crossover design. This study is interesting and original in its methodological part because it presents a novel method to assess gastric emptying over time rather than evaluating a single moment after sacrificing laboratory animals. The authors precisely describe their experiments using the breath test technique, including a power calculation. Compared to saline, DA-9701 evoked a more rapid emptying similarly to erythromycin. The finding of a prokinetic effect of DA-9701 on gastric emptying is very promising in view of potential therapeutic options in FD.

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BRIEF ARTICLE

Therapeutic efficacy of baclofen in refractory gastroesophageal reflux-induced chronic cough

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Author contributions: Xu XH recruited the patients, performed experiments and statistical analysis of data, and wrote the manuscript; Yang ZM, Chen Q, Yu L, Liang SW and Lv HJ took part in the recruitment of patients and review of the manuscript; Qiu ZM designed and coordinated the program, and revised the manuscript.

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Abstract

AIM: To evaluate the efficacy and safety of baclofen for treatment of refractory gastroesophageal reflux-induced chronic cough (GERC) unresponsive to standard anti-reflux therapy.

METHODS: Sixteen patients with refractory GERC were given an 8-wk course of baclofen 20 mg three times a day as an add-on therapy to omeprazole. Changes in the cough symptom score, cough threshold to capsaicin, reflux symptom score and possible adverse effects were determined after treatment. The variables of multi-channel intraluminal impedance combined with pH monitoring were compared between responders and non-responders to baclofen.

RESULTS: Twelve of 16 patients completed treatment. Cough disappeared or improved in 56.3% (9/16) of patients, including 6 patients with acid reflux-induced cough (66.7%) and 3 patients with non-acid reflux-induced cough (33.3%). With baclofen treatment, the cough symptom score began to decrease at week 2, was clearly decreased at week 6 and reached a minimum at week 8. At the end of therapy, the lowest concentration of capsaicin required for induction of $\geqslant$ 2 and $\geqslant$ 5 coughs increased from 0.98 (1.46) to 1.95 (6.82) μmol/L (Z = -2.281, P = 0.024) and from 1.95 (7.31) to 7.8 (13.65) μmol/L (Z = -2.433, P = 0.014), respectively, and the reflux symptom score decreased from 8.0 ± 1.6 to 6.8 ± 0.8 (t = 2.454, P = 0.023). The number of acid reflux episodes was significantly lower in responders than in non-responders. The main adverse effects were somnolence, dizziness and fatigue.

CONCLUSION: Baclofen is a useful, but suboptimal treatment option for refractory GERC.

Key words: Baclofen; Cough; Esophageal pH monitoring; Gastroesophageal reflux; Proton pump inhibitors

Core tip: This study evaluated the efficacy and safety of baclofen in a cohort of patients with refractory gastroesophageal reflux-induced chronic cough (GERC) unresponsive to 8 wk of standard anti-reflux therapy consisting of omeprazole twice daily and domperidone. Baclofen is found to be a useful, but suboptimal treatment option for refractory GERC.

Xu XH, Yang ZM, Chen Q, Yu L, Liang SW, Lv HJ, Qiu ZM. Therapeutic efficacy of baclofen in refractory gastroesophageal reflux-induced chronic cough. *World J Gastroenterol* 2013; 19(27): 4386-4392 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i27/4386.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i27.4386

INTRODUCTION

Gastroesophageal reflux-induced chronic cough (GERC) is a clinical syndrome manifested predominantly by chronic cough caused by the backflow of gastric acid or other gastric contents into the esophagus[1]. Acid suppression by proton pump inhibitors alone or in combination with prokinetic agents is currently considered as the standard therapy for GERC, and can improve or eradicate reflux-related cough in the majority of patients, even though there is controversy on the therapeutic effects of these agents[2,3]. Unfortunately, some patients do not respond to standard therapy. Refractory GERC can be defined as cough due to reflux which does not respond to 8 wk of standard anti-reflux treatment with proton pump inhibitors alone twice daily before meals or in combination with prokinetic agents[4]. The management of refractory GERC remains a challenge.

Although anti-reflux surgery such as fundoplication may be an option and benefit some patients in this situation, its role has not been clearly defined and its application is restricted[5]. At present, intensified medical therapy is still the major treatment for refractory GERC. Baclofen, an inhibitor of transient lower esophageal sphincter relaxations (TLESRs)[6], can inhibit both acid and non-acid reflux and is now used in the treatment of refractory gastroesophageal reflux disease[7]. We previously showed in a case report that baclofen was helpful in resolving cough in patients with GERC resistant to standard anti-reflux medical therapy[4]. However, more clinical data are needed for the accurate assessment of its therapeutic efficacy.

Therefore, a prospective and open interventional clinical investigation was performed to evaluate the efficacy and safety of baclofen in a cohort of patients with refractory GERC.

MATERIALS AND METHODS

Patients

Sixteen patients with suspected refractory GERC were recruited from our respiratory clinic between October 2010 and November 2011, including 3 patients reported previously[4]. Their clinical characteristics are shown in Table 1. The inclusion criteria were as follows: (1) chronic cough with or without typical upper gastrointestinal symptoms such as regurgitation, heartburn and chest pain; (2) multichannel intraluminal impedance combined with pH monitoring (MII-pH) confirmed abnormal acid or non-acid reflux, as shown by a DeMeester score of ⩾ 14.72 and/or syndrome association probability (SAP) for acid or non-acid reflux of ⩾ 95%[8]; (3) cough fails to improve with 2 mo of standard anti-reflux treatment consisting of omeprazole 20 mg twice daily plus domperidone 10 mg three times a day; and (4) other causes of chronic cough such as upper airway cough syndrome, cough variant asthma and eosinophilic bronchitis were excluded.

This study was approved by the Ethics Committee of Tongji Hospital and was registered with the Chinese Clinical Trials Register (http://medresman.org) number ChiCTR-ONC-13003123. All patients gave informed consent before entering the study.

Table 1 Demographic characteristics of 16 patients with refractory gastroesophageal reflux-induced cough

Variables	Value
Sex (male/female)	9/7
Age (yr)	47.8 ± 11.6
Cough duration (mo)	36.0 (27.7)
FEV1 (% predictive value)	93.3 ± 8.3
FVC (% predictive value)	98.1 ± 11.1
FEV1/FVC (%)	80.3 ± 6.1
Cough symptom score	
Daytime	3 (0)
Nighttime	1 (1)
DeMeester score	33.1 ± 10.7
SAP (%)	
SAP for acid reflux	73.1 (28.2)
SAP for non-acid reflux	71.2 (37.6)

Data on cough duration, cough symptom score and SAP are presented as median (25%-75% interquartile) or mean ± SD. FEV1: Forced expiratory volume in one second; FVC: Forced vital capacity; SAP: Symptom association probability.

Cough severity was rated using the cough symptom score described by Hsu *et al*[9]. Cough sensitivity to inhaled capsaicin was determined according to a previously described method[10]. Cough threshold was defined as the lowest concentration of capsaicin required for the induction of ⩾ 2 (C2) and ⩾ 5 coughs (C5). Reflux-related symptoms were scored by a Chinese version of the gastroesophageal reflux diagnostic questionnaire (GerdQ) provided by the designer[11].

MII-pH was performed to evaluate the probability of GERC prior to the commencement of standard anti-reflux treatment as previously described by us[12]. Briefly, a 2.1-mm diameter combined MII-pH catheter with a six impedance channel sensor (K6011-E10632, MMS, Switzerland) and an antimony pH electrode (819100, MMS, The Netherlands) was inserted transnasally into the patient's esophagus, with the impedance channel sensor located at 3, 5, 7, 9, 15 and 17 cm above the manometrically located proximal border of the lower esophageal sphincter, and the pH electrode positioned at 5 cm above the proximal border of the lower esophageal sphincter. The catheter was connected to a portable data logger (Ohmega, MMS, The Netherlands) which recorded data from all seven channels (six impedance and one pH) with a frequency of 50 Hz over 24 h. Using appropriate software (Database soft, 8.7 version, Medical Measurement System BV, The Netherlands), the data were manually analyzed for reflux episodes, and classified as liquid, gas and mixed reflux on the basis of impedance values, or categorized as acidic (pH < 4.0), weakly acidic (pH 4.0-7.0) or weakly alkaline reflux (pH > 7.0). Combined with diary cards, SAP for acid or non-acid reflux was calculated respectively[13]. The DeMeester score was automatically calculated as described previously[14]. Furthermore, the number of reflux episodes, proximal reflux episodes, time of bolus exposure, time of bolus clearance and time

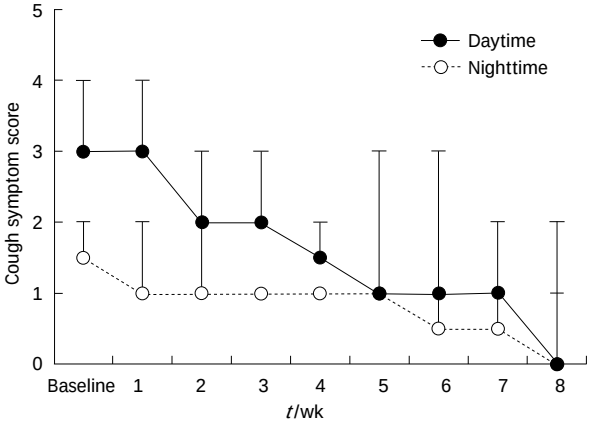

Figure 1 Changes in cough symptom score against the duration of treatment with baclofen. The data are presented as median (25%-75% interquartile).

of acid clearance were analyzed.

Procedures

Initial assessments included the collection of patients' general information and recording of the cough symptom score, reflux symptom score, cough sensitivity to capsaicin and study of MII-pH. The 16 patients with GERC resistant to standard anti-reflux treatment were then given an 8-wk course of baclofen 20 mg three times a day as an add-on therapy to omeprazole 20 mg twice a day, but discontinued domperidone. The patients received weekly follow-up. Responses to therapy, the cough symptom score and possible side effects were evaluated at each follow-up. When treatment was complete, reflux symptom score and cough sensitivity to capsaicin were repeated once more. Cough was considered controlled when it disappeared completely, improved when the cough symptom score decreased by one or more, and failed when the cough worsened or was not alleviated to a noticeable degree[15,16]. The patients who responded favorably to baclofen continued on this therapy for 1 mo and then the dose of baclofen was decreased by 10 mg every month with the lowest maintenance dose of 20 mg daily, while patients who did not improve with baclofen moved to the intensified acid suppression trials and given double dose of omeprazole (40 mg twice a day) for 4 wk and double dose of omeprazole combined with ranitidine 150 mg twice a day for 4 wk in a sequential manner. Refractory GERC was determined if the cough was controlled or improved by treatment with either baclofen, double dose of omeprazole or the combination of ranitidine with double dose of omeprazole.

Statistical analysis

Data with normal distribution were expressed as mean ± SD, or as median (25%-75% interquartile) if the distribution was skewed. A comparison of pre- and post-treatment was made using paired *t* tests for the data with normal distribution or with the Mann-Whitney *U* test for the data with skewed distribution. Software (SPSS version 17.0, Chicago, IL, United States) was used for statistical calculation. A *P* value of < 0.05 was considered significant.

RESULTS

Treatment efficacy

Of 16 patients, 4 withdrew from therapy due to intolerable and persistent nausea and diarrhea following the initiation of baclofen for a week ($n = 1$) and deterioration or no improvement in cough in the third week ($n = 3$). Among 12 patients who completed the treatment course of baclofen, cough was controlled in 7 patients (58.3%), improved in 2 patients (15.7%) and failed to improve in 3 patients (25.0%). The overall therapeutic efficacy of baclofen was 56.3% (9/16). Cough in patients responsive to baclofen was considered to be due to acid reflux in 6 (66.7%) and due to non-acid reflux in 3 (33.3%). In the remaining 7 patients who withdrew baclofen therapy ($n = 4$) or were resistant to treatment ($n = 3$), cough was resolved by subsequent therapies of double dose of omeprazole in 5 patients and double dose of omeprazole combined with ranitidine in 2 patients.

Changes in cough symptom score, cough threshold and reflux symptom score

With baclofen treatment, the cough symptom score was reduced at week 2, obviously decreased at week 6 and reached a minimum at week 8 (Figure 1). With the exception of 3 non-responders, baclofen treatment resulted in an increase in cough threshold to capsaicin in 9 patients. Cough threshold C2 increased from 0.98 (1.46) to 1.95 (6.82) μmol/L and C5 increased from 1.95 (7.31) to 7.8 (13.65) μmol/L (Figure 2). In contrast, the reflux symptom score reduced from 8.0 ± 1.6 to 6.8 ± 0.8 at the end of therapy (Figure 3).

Comparison of variables in MII-pH between responders and non-responders to baclofen

Most MII-pH variables were comparable between the responders and non-responders to baclofen treatment (Table 2). However, the number of acid reflux episodes was lower in the responders than in the non-responders ($Z = -2.277$, $P = 0.023$).

Adverse effects

The main adverse effects of baclofen were somnolence, dizziness and fatigue (Table 3). These adverse effects were usually tolerable and waned within 1-3 wk despite persistent somnolence and fatigue in 2 (12.5%) patients throughout the entire duration of treatment.

DISCUSSION

To date, the definition of refractory GERC remains to be elucidated. According to a widely accepted definition for refractory gastroesophageal disease, cough due to reflux can be considered refractory when the patient does not respond to 4-8 wk of treatment with proton pump inhibitors twice daily[17,18]. However, attention should be paid

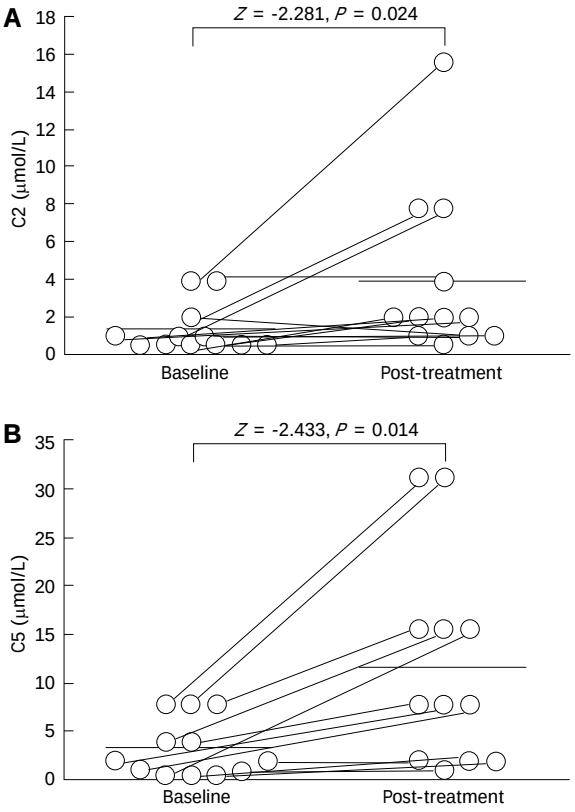

Figure 2 Changes in cough threshold to inhaled capsaicin after treatment with baclofen.

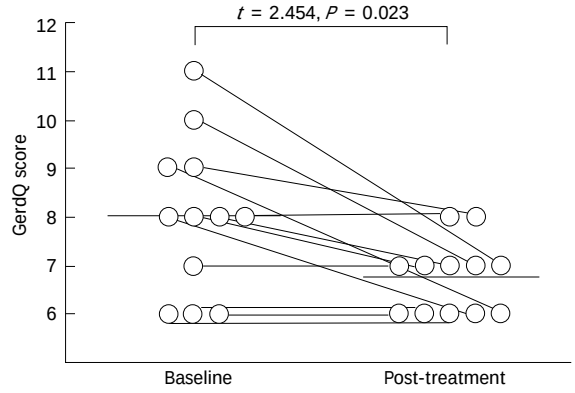

Figure 3 Changes in gastroesophageal reflux diagnostic questionnaire score after treatment with baclofen. GerdQ: Gastroesophageal reflux diagnostic questionnaire.

to the limitations of the diagnostic standard for refractory gastroesophageal reflux disease. Unlike regurgitation and heartburn, cough is an atypical symptom of gastroesophageal reflux disease and can be caused by a number of diseases other than GERC. A temporal cause-effect relationship between reflux and cough established by MII-pH, as indicated by the positive SAP, does not mean that reflux is the true cause of cough and must be verified by specific anti-reflux therapy[1,19]. In our cohort of patients, cough did not improve with a standard course of acid suppression therapy, but resolved after adjustment of the anti-reflux regimen. Therefore, the diagnosis

Table 2 Comparison of variables in multi-channel intraluminal impedance combined with pH monitoring between patients responsive and unresponsive to baclofen

Variables	Responsive (n = 9)	Unresponsive (n = 7)
DeMeester score	17.9 ± 5.1	45.0 ± 18.0
SAP (%)		
SAP for acid reflux	70.0 (28.2)	75.3 (56.0)
SAP for non-acid reflux	87.9 (35.0)	61.6 (59.1)
Acid reflux (n)	26.0 (31.1)	65.0 (51.0)[a]
Weakly acidic reflux (n)	22.8 (28.7)	22.0 (28.0)
Weakly alkaline reflux (n)	4.0 (22.0)	2.3 (21.7)
Gas reflux (n)	12.0 (51.0)	13.9 (10.0)
Liquid reflux (n)	26.0 (11.0)	24.0 (18.6)
Mixed reflux (n)	45.0 (43.0)	30.4 (33.7)
Proximal extent (n)	13.8 (12.6)	10.5 (11.5)
Bolus exposure (%)	1.1 (1.6)	1.3 (1.2)
Bolus clearance (s)	9.3 (9.1)	8.9 (6.2)

Except for the DeMeester score, the data are presented as median (25%-75% interquartile). [a]$P < 0.05$ *vs* responsive patients. SAP: Symptom association probability.

Table 3 Adverse effects in 16 patients during treatment with baclofen n (%)

Adverse effects	Frequency
Somnolence	5 (31.25)
Dizziness	2 (12.50)
Fatigue	3 (18.75)
Nausea	1 (6.25)
Diarrhea	1 (6.25)

of refractory GERC in these patients was confirmed.

TLESRs refer to periods of spontaneous (not preceded by a swallow) relaxation of the lower esophageal sphincter lasting 10-60 s[20]. TLESRs play a physiological role in venting air from the stomach, but are also the main mechanism for gastroesophageal reflux[21]. In patients with gastroesophageal reflux disease, TLESRs are usually more frequent and are twice as likely to be associated with a reflux event[22]. Proton pump inhibitors can increase the pH value and reduce the volume of the refluxate, and even decrease reflux episodes in combination with prokinetic agents, and thus improve or resolve the symptom in patients with GERC[23]. However, they essentially have no ability to rectify dysfunction in the lower esophageal sphincter and eliminate reflux episodes. In addition, proton pump inhibitors are often ineffective for non-acid (weakly acid or weakly alkaline) reflux[8]. This might explain why patients with GERC failed to respond to initial standard anti-reflux therapy. In this case, inhibitors of TLESRs may help to control the cough. Inhibitors of TLESRs previously under development include gamma-aminobutyric acid B receptor agonists, metabotropic glutamate receptor 5 antagonists and cannabinoid receptors agonists[21]. Inhibitors of TLESRs have primarily been utilized as an add-on treatment for patients with gastroesophageal reflux disease who failed proton pump inhibitors[7,18].

Baclofen is a selective agonist of the gamma-aminobutyric acid B receptor and can inhibit TLESRs by modification of the vagal reflex pathway[24]. There are several lines of evidence to show that baclofen can reduce the frequency of TLESRs, decrease reflux episodes[6,25,26], and reduce acid reflux-related symptoms by 72% and non-acid reflux-related symptoms by 21%[27]. Moreover, baclofen has direct antitussive activity and has been used for the treatment of refractory chronic cough of unknown cause[28,29]. Our results showed that baclofen, as an add-on therapy, reduced cough severity and cough sensitivity to capsaicin in 56.3% of patients with refractory chronic cough due to acid as well as non-acid reflux, which partially confirms our previous observations in a case report[4].

Our results also revealed that the therapeutic efficacy of baclofen was suboptimal, as more than 40% patients with refractory chronic cough due to acid reflux were resistant to baclofen, as characterized by significantly more acid reflux episodes in non-responders than in responders, and further augmented acid suppression eliminated the cough in patients who failed baclofen. This may be explained by the incomplete inhibition of TLESRs by baclofen. There is evidence that baclofen at the dose currently used only reduced the frequency of TLESRs by 40%-60% and decreased the reflux episodes by 43%[6,25,26]. Although studies in dogs have shown that baclofen can abolish TLESRs at higher doses[30], such high doses cannot be administered to humans due to side-effects. In addition, reflux may be secondary to the reduced pressure difference between the stomach and esophagus due to the lower baseline pressure of the lower esophageal sphincter, and may be unrelated to TLESRs[31]. Baclofen and the GABAB agonist lesogaberan have consistently been demonstrated to increase basal pressure of the lower esophageal sphincter in humans[32], which may contribute to the reduction in reflux episodes, but not the absence of reflux episodes. Residual reflux can continue to stimulate sensors with increased sensitivity located at the mucosa in the distal esophagus and cause cough through esophageal-tracheobronchial reflexes[33].

The main side-effects of baclofen emanating from the central nervous system limited its value in the treatment of refractory GERC[34]. Other adverse effects include dry mouth, nausea, vomiting, diarrhea and constipation. Our findings showed that drug-related somnolence, fatigue and dizziness, although common, waned within 3 wk and did not influence treatment in most patients. Considering that the dose of baclofen is gradually increased for the treatment of spasticity in clinical practice, perhaps a similar increase in the dose of baclofen from 5 to 20 mg may help improve tolerance in patients and reduce severe adverse effects[21].

There are several limitations in the present study. First, only a small number of patients with refractory GERC were recruited, and this may have limited the strength of the study. A recent multi-center survey showed that GERC is relatively rare in China[35], and patients with refractory GERC account for a small number. Therefore, it is difficult to enroll a large number of patients. Second, we were unable to directly evaluate the inhibitory efficacy of baclofen on acid or non-acid reflux as the patients refused to undergo a repeat invasive MII-pH study at the end of the treatment period. Moreover, it is difficult to exclude the contribution of the nonspecific non-reflux-related antitussive activity of baclofen. Nevertheless, the obvious parallel reduction in GerdQ suggests that the improvement in cough, at least in part, can be attributed to the blockade of abnormal reflux. Finally, the broad utility of baclofen in clinical practice can be criticized as it only resolves the cough in partial patients with refractory GERC. Nevertheless, considering the negative impact of chronic cough on patient's quality of life[36] and the difficulty in the management of refractory GERC, we believe that baclofen has its position in the treatment of refractory GERC, even though its effectiveness is limited.

In conclusion, baclofen may be useful for the treatment of refractory GERC. When a standard therapy for GERC fails, baclofen can at least be considered as a treatment option, even though its therapeutic efficacy is suboptimal.

COMMENTS

Background

Gastroesophageal reflux-induced chronic cough (GERC) is a special form of gastroesophageal reflux disease with a predominant chronic cough. Refractory GERC resistant to proton pump inhibitors alone twice daily or in combination with prokinetic agents is difficult to treat as there is currently no satisfactory therapy available.

Research frontiers

Baclofen, a selective agonist of the gamma-aminobutyric acid B receptor, can inhibit both acid and non-acid reflux by reducing the frequency of transient lower esophageal sphincter relaxations and is now used in the treatment of refractory gastroesophageal reflux disease. The authors previously showed in a case report that baclofen successfully resolved the cough in three patients with GERC as an add-on therapy to omeprazole. However, more clinical data are needed to assess its therapeutic efficacy.

Innovations and breakthroughs

In this prospective study involving 16 patients, the authors demonstrated that baclofen, as an add-on therapy to omeprazole, can eliminate or improve cough in 56.3% of patients with refractory GERC, and modify cough hypersensitivity to capsaicin. However, the study also revealed that the therapeutic efficacy of baclofen is suboptimal for the treatment of refractory GERC.

Applications

The study results suggest that baclofen may be considered as an option for the treatment of refractory GERC.

Terminology

Refractory GERC: Refractory GERC is defined as cough caused by reflux which persists despite 8 wk of standard anti-reflux treatment with the combination of proton pump inhibitors twice daily before meals with prokinetic agents.

Peer review

It is an interesting study and will add new information to the treatment of refractory cough due to reflux.

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BRIEF ARTICLE

Prevalence and risk factors of gallbladder polypoid lesions in Chinese petrochemical employees

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Abstract

AIM: To investigate the prevalence and risk factors of polypoid lesions of the gallbladder (PLGs) in petrochemical employees in Ningbo, Zhejiang Province, China.

METHODS: All active and retired employees aged 20-90 years (n = 11098) of a refinery and chemical plant in eastern China were requested to participate in a health survey. The participants were subjected to interview, physical examination, laboratory assessments and ultrasonography. All the participants were invited to have a physical examination after a face-to-face interview. Fasting blood samples were obtained from the antecubital vein, and the samples were used for the analysis of biochemical values. Abdominal ultrasonography was conducted.

RESULTS: A total of 10461 (7331 men and 3130 women) current and former petrochemical employees attended for screening. The overall prevalence of post-cholecystectomy, gallstones and PLGs was 0.9%, 5.2% and 7.4%, respectively. Compared with the increased prevalence of either gallstones or post-cholecystectomy in older persons, PLGs were more common in the middle-aged, peaking in those aged 40-59 years. Excluding the patients with gallstones, gallstones mixed with PLGs, or those who had undergone cholecystectomy, in the remaining 9828 participants, the prevalence of PLGs in men (8.9%) was significantly higher than that in women (5.5%, $P < 0.001$). The analyzed risk factors with increased OR for the development of PLGs were male gender (OR = 1.799, $P < 0.001$), age $\geqslant$ 30 years (OR = 2.699, $P < 0.001$) and hepatitis B surface antigen (HBsAg) positivity (OR = 1.374, $P = 0.006$).

CONCLUSION: PLGs are not rare among Chinese petrochemical employees. Male gender, HBsAg positivity, and middle age are risk factors for developing PLGs.

Key words: Prevalence; Risk factors; Polypoid lesions; Gallbladder; Chinese

Core tip: Polypoid lesions of the gallbladder (PLGs) are commonly encountered in clinical practice. This study investigated the prevalence and risk factors of PLGs in a cohort of petrochemical employees in the city of Ningbo, Zhejiang Province, China. It was demonstrated that 7.4% of the petrol-chemical employees studied had PLGs; and male, hepatitis B surface antigen positive and middle-aged employees, especially those aged

between 30 and 59 years, had higher risks for developing PLGs.

Mao YS, Mai YF, Li FJ, Zhang YM, Hu KM, Hong ZL, Zhu ZW. Prevalence and risk factors of gallbladder polypoid lesions in Chinese petrochemical employees. *World J Gastroenterol* 2013; 19(27): 4393-4399 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i27/4393.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i27.4393

INTRODUCTION

The widespread application and improved image quality of abdominal ultrasonography in modern clinical practice have led to an increase in the detection of abnormalities of the biliary tree, including polypoid lesions of the gallbladder (PLGs)[1,2]. The significance of PLGs is still poorly understood. In general, PLGs represent a heterogeneous group of changes in the gallbladder wall and include entities such as cholesterol polyps, inflammatory polyps, adenomas, leiomyomas and lipomas[3]. As a result, clinicians are ever more frequently confronted with the question of how to proceed in cases of incidentally discovered PLGs[3]; and patients with PLGs usually require repeated ultrasonography and follow-up[4], enduring a certain degree of anxiety.

The prevalence of PLGs is reported in the range 0.3%-9.5%, depending on the population studied and on the study design. Prevalence in Western studies falls in the range 1.0%-6.1%[5,6], which is lower than that reported in Southeast Asian populations, mainly in Japan, South Korea and Taiwan[7-10]. To date, only a few studies have investigated the prevalence of and risk factors for PLGs in the mainland of China. Yang *et al*[11] have reported that the prevalence of PLGs in Shanghai was 4.2%. Xu *et al*[12] have reported that the prevalence in Beijing was 6.9%. However, these two studies were retrospective analyses. To understand better the prevalence of and risk factors for PLGs in Chinese petrochemical employees in Zhejiang Province, a prospectively observational study of 10461 stable participants was conducted in 2009, which is expected to provide a comprehensive update to the results of the previous studies. This cohort of employees will be followed up in the next 5 years. All participants will be invited for annual reassessment, including similar questions, laboratory measurement, ultrasonography, and collection of biological material at baseline. In addition, the progression of PLGs will be observed prospectively.

MATERIALS AND METHODS

Study population

The study of PLGs was carried out as part of the petrochemical employees annual health check-up survey[13]. In 2009, a descriptive cross-sectional study was performed in a stable population of all employees of a refinery and chemical plant in the city of Ningbo, Zhejiang Province, in the eastern region of China. Chinese law requires state-owned companies to provide annual health check-ups for all employees. The study population included all regular employees with at least 1 mo service at the Zhenhai Refinery and Chemical Plant of the SINO-Petrol Chemical Company, in northeastern Ningbo between January 1 and December 31, 2009.

Invitation to participate was by letter and non-responders received one reminder. A research nurse interviewed subjects at Zhenhai Lianhua Hospital, the former Workers Hospital of the Zhenhai Refinery and Chemical Plant.

The present study was conducted in accordance with the Declaration of Helsinki, as amended in Scotland (2000). The protocol and statement of informed consent were approved by the Ethics Committee of Zhenhai Lianhua Hospital, Ningbo. Written informed consent was obtained from each participant.

Interview and physical examination

An appointment was made for each participant at the check-up center of Zhenhai Lianhua Hospital, where a face-to-face interview was conducted by a trained nurse to complete a two-page self-assessment questionnaire that included a gallbladder-related question: "Have you ever had gallbladder surgery?"

All individuals were invited to have a physical examination after the face-to-face interview. They were required to fast overnight. Body measurements were performed by a trained medical professional using a standardized protocol. Body weight and standing height were measured in light indoor clothing without shoes. Weight was measured to the nearest 0.1 kg using a calibrated hospital spring scale. Height and waist circumference (WC) were measured in centimeters. WC was measured between the lowest rib and the iliac crest, horizontally through the narrowest part of the torso. Body mass index (BMI) was then calculated as weight in kilograms divided by height in meters squared. Individuals were grouped into two categories based on BMI (< 25 and $\geqslant 25$ kg/m^2) and WC (< 90 and $\geqslant 90$ cm for men, and < 80 and $\geqslant 80$ cm for women)[14]. Blood pressure was measured using an automated sphygmomanometer with the subject in a sitting position. Systolic blood pressure and diastolic blood pressure were measured at the first and fifth Korotkoff phases, respectively.

Laboratory tests

Fasting blood samples were obtained from an antecubital vein, and the samples were used for the analysis of biochemical values. The values included triglyceride, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, uric acid, and fasting plasma glucose (FPG). All values were measured by an Olympus AU640 auto-analyzer (Olympus, Kobe, Japan) using standard methods. Hepatitis B surface antigen (HBsAg) was detected with a second-generation enzyme-linked immu-

Table 1 Population characteristics (n = 10461) n (%)

Characteristics	Male (n = 7331)	Female (n = 3130)
Age, mean ± SD (range) (yr)	46.0 ± 13.2 (21-87)	48.6 ± 12.4 (20-90)
Age distribution (yr)		
20-29	551 (7.5)	113 (3.6)
30-39	2221 (30.3)	736 (23.5)
40-49	1903 (26.0)	896 (28.6)
50-59	1506 (20.5)	776 (24.8)
60-69	621 (8.5)	374 (11.9)
⩾ 70	529 (7.2)	235 (7.5)
Smoking	2878 (39.3)	20 (0.6)
Alcohol consumption	1910 (26.1)	39 (1.2)
Concomitant condition		
Hypertension	3459 (47.2)	1093 (34.9)
Diabetes mellitus	368 (5.0)	162 (5.2)

nosorbent assay (VITROS ECi Immunodiagnostic System; Ortho Clinical Diagnostics, Inc., Johnson and Johnson, Raritan, NJ, United States). The reagents used were offered by the same company. The tests were undertaken strictly according to the instructions of the manufacturers.

Ultrasonography

All participants were asked to come to a morning examination after an overnight fast of ⩾ 8 h. Abdominal ultrasonography was conducted using a scanner equipped with a 2.0-5.0-MHz transducer (Voluson 730, GE Healthcare, Pittsburgh, PA, United States). The ultrasongraphers were unaware of the participants' clinical and laboratory characteristics. Gallstones were present if the gallbladder contained echoes that moved with gravity, except when the stones were large, a septum existed in the gallbladder, or there was an enclosed infundibulum. PLGs were diagnosed as immobile echoes protruding from inside the gallbladder wall into the lumen. The ultrasonic characteristics of PLGs included hyperechoic structures without acoustic shadow, unequivocal visualization in two planes (longitudinal and in cross-section), no change in position of the wall change secondary to change in subjects' position. In patients with multiple polyps, the size of the largest polyp was measured. Subjects with both gallstones and PLGs were classified into the gallstones group.

Statistical analysis

Data on quantitative characteristics were expressed as mean ± SD. Data on qualitative characteristics were expressed as percentage values or absolute numbers as indicated. The data analysis was performed with SPSS (version 11.5, SPSS Software, Chicago, IL, United States). For continuous variables, non-parametric tests (two-tailed Mann-Whitney U test) were used. Group differences between the numbers of subjects were analyzed using χ^2 test (normal data) and analysis of variance (continuous data). A multiple stepwise regression analysis (backward: Wald; cutoff for entry: 0.05, for removal: 0.10) was performed in order to evaluate independent relationships between sex, age and PLG. $P < 0.05$ was considered statistically significant.

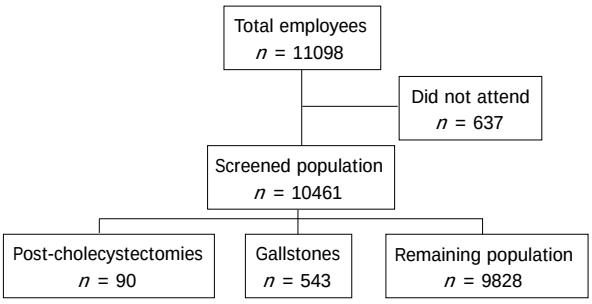

Figure 1 Recruitment flow chart.

RESULTS

Population studied

All of the 11098 active and retired employees were invited to participate in the study. There were 7760 (69.9%) men and 3338 (30.1%) women. We excluded 637 subjects for not attending the health check program. In all, 7331 (94.5%) of the invited men and 3130 (93.8%) of the women participated in the study. Figure 1 displays the recruitment flow chart. Demographic data on the 10461 attending individuals are shown in Table 1.

Prevalence of post-cholecystectomy, gallstones and PLGs

There were 90 (0.9%) patients who had undergone cholecystectomy. The overall prevalence of PLGs was 7.4% (777/10461), which was higher than that for gallstones, which was 5.2% (543/10461, $P < 0.001$). Nineteen subjects with both gallstone and PLG were classified into the group of gallstones. The prevalence was further stratified by age among post-cholecystectomy, gallstones and PLGs (Figure 2A). Compared with the increased prevalence of either gallstones or post-cholecystectomy in older persons, the PLGs were more common in the middle-aged, peaking in those aged 40-59 years. The difference between men and women was stratified with age (Table 2). The prevalence of gallstones and post-cholecystectomy in men was lower than that in women in all age groups. In contrast, the prevalence of PLGs in men was higher than that in women in all age groups. Overall, men had a significantly higher prevalence of PLGs compared to women (8.5% *vs* 5.0%, $P < 0.001$). In patients with PLGs, the proportion of large polypoid lesions (⩾ 10 mm) was small (14/777, 1.8%; 11 male, 3 female). This equated to a general population prevalence of larger PLGs of 0.1%. None of these 14 patients underwent cholecystectomy.

Characteristics of subjects according to PLG status

After excluding 543 patients with gallstones and 90 who underwent cholecystectomy, the remaining 9828 participants (93.9% of the total attending population) were further analyzed. Characteristics were compared between the group with PLGs and a control group. Controls were confirmed not to have gallstones, post-cholecystectomy or PLGs in our screening program. The control group consisted of 9051 subjects, including 6332 men and 2719

Table 2 Prevalence, age distribution and gender of patients with post-cholecystectomy, gallstones and polypoid lesions of the gallbladder in 10461 examinees *n* (%)

Age group (yr)	Examinees		Post-cholecystectomy			Gallstones			PLGs		
	Male	Female	Male	Female	*P* value	Male	Female	*P* value	Male	Female	*P* value
20-29	551	113	0 (0.0)	0 (0.0)	-	4 (0.7)	0 (0.0)	1.000	22 (4.0)	1 (0.9)	0.153
30-39	2221	736	4 (0.2)	6 (0.8)	0.019	40 (1.8)	22 (3.0)	0.051	201 (9.0)	31 (4.2)	< 0.001
40-49	1903	896	9 (0.5)	4 (0.4)	0.322	79 (4.2)	50 (5.6)	0.093	190 (10.0)	49 (5.5)	< 0.001
50-59	1506	776	6 (0.4)	18 (2.3)	< 0.001	94 (6.2)	53 (6.8)	0.588	142 (9.4)	53 (6.8)	0.035
60-69	621	374	11 (1.8)	11 (2.9)	0.223	58 (9.3)	42 (11.2)	0.337	38 (6.1)	15 (4.0)	0.151
⩾ 70	529	235	6 (1.1)	15 (6.4)	< 0.001	68 (12.9)	33 (14.0)	0.545	27 (5.1)	8 (3.4)	0.300
Total	7331	3130	36 (0.5)	54 (1.7)	< 0.001	343 (4.7)	200 (6.4)	< 0.001	620 (8.5)	157 (5.0)	< 0.001

PLG: Polypoid lesions of the gallbladders.

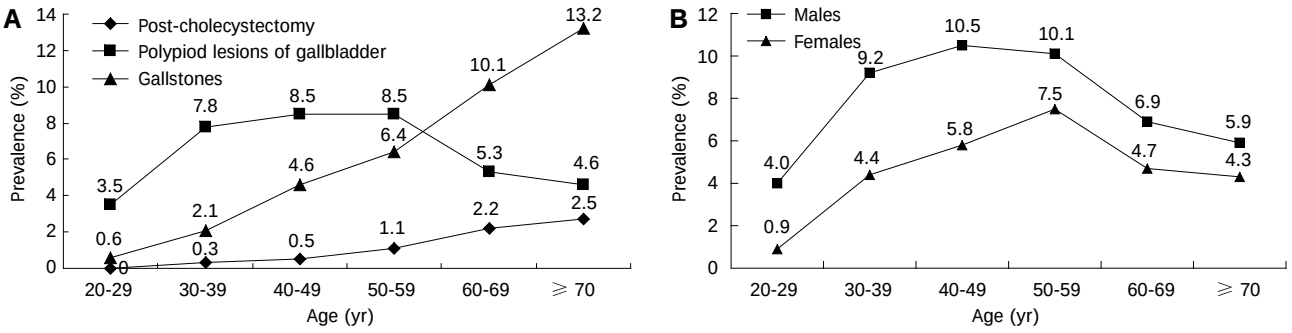

Figure 2 Prevalence of gallbladder diseases in different age groups and polypoid lesions of the gallbladder stratified by sex in Chinese petrochemical employees. A: Prevalence of post-cholecystectomy and gallstones increased with age, whereas polypoid lesions of the gallbladder (PLGs) were more common in the middle-aged people, peaking in those aged 40-59 years; B: Prevalence of PLGs stratified by sex in different age groups in the remaining 9828 Chinese petrochemical employees. Prevalence of PLGs in men was higher than in women in all age groups.

Table 3 Characteristics of the 9828 remaining subjects with and without polypoid lesions of the gallbladders

Variables	With PLG (*n* = 777)	Without PLG (*n* = 9051)	*P* value
Age (yr), ⩾ 30/< 30	754/23	8414/637	< 0.001
Sex, male/female	620/157	6332/2719	< 0.001
Smoking	245 (32.7)	2487 (27.5)	0.002
HBsAg positive	96 (12.4)	842 (9.3)	0.005
Alcohol consumption	166 (21.4)	1686 (18.4)	0.061
History of hypertension	140 (18.0)	1615 (17.8)	0.903
History of diabetes	27 (3.5)	428 (4.7)	0.110
Body mass index (kg/m^2)	23.2 ± 2.8	23.0 ± 3.0	0.384
Waist circumference (cm)	80.3 ± 8.4	80.0 ± 9.2	0.472
Systolic blood pressure (mmHg)	122.0 ± 14.8	122.2 ± 15.4	0.655
Diastolic blood pressure (mmHg)	77.7 ± 10.0	77.7 ± 9.9	0.992
Total cholesterol (mmol/L)	5.2 ± 1.0	5.3 ± 1.0	0.128
LDL cholesterol (mmol/L)	3.1 ± 0.8	3.1 ± 0.8	0.475
HDL cholesterol (mmol/L)	1.4 ± 0.4	1.5 ± 0.4	0.119
Triglycerides (mmol/L)	1.4 ± 1.0	1.5 ± 1.1	0.490
Fasting blood glucose (mmol/L)	4.8 ± 0.8	4.8 ± 0.8	0.773
Uric acid (μmol/L)	339.0 ± 77.1	336.3 ± 84.5	0.398

Data are expressed as absolute numbers (percentage) or mean ± SD. HBsAg: Hepatitis B surface antigen; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; PLG: Polypoid lesions of the gallbladders.

women (Table 3). Of the remaining 9828 subjects, the overall prevalence of PLGs was 7.9% (777/9828); 8.9% (620/6952) in men and 5.5% (157/2876) in women.

The differences in the prevalence of PLGs in the remaining population between men and women were investigated for every age decade (Figure 2B). The prevalence of PLGs in men was significantly higher in women ($P < 0.001$). In men, the prevalence was highest among those in their 40s (10.5%), followed by those in their 50s (10.1%), 30s (9.2%), 60s (6.9%), 70s or above (4.9%), and 20s (4.0%). In women, the prevalence was highest among those in their 50s (7.5%), followed by those in their 40s (5.8%), 60s (4.7%), 30s (4.4%), 70s or above (4.3%), and 20s (0.9%). In men, the overall difference in the age-dependent change in prevalence was found to be statistically significant ($\chi^2 = 31.994$, $P < 0.001$). In women, the overall difference in the age-dependent change in prevalence was also significant ($\chi^2 = 13.074$, $P = 0.023$). The highest prevalence (10.5%) of PLGs was found in male participants aged 40-49 years.

Logistic regression analysis of relevant risk factors for PLGs

The factors significantly associated with PLGs were male sex ($P < 0.001$), age ⩾ 30 years ($P < 0.001$), HBsAg positivity ($P = 0.005$) and cigarette smoking ($P = 0.002$) (Table 3). In order to identify the risk factors, we performed multivariate logistic regression analysis (backward stepping) (Table 4.) There was no significant difference between the PLG-positive and -negative groups for cigarette smoking (OR = 1.020, 95%CI: 0.854-1.220, $P = 0.823$). Male gender (OR = 1.799, 95%CI: 1.472-2.199, $P < 0.001$), age ⩾ 30 years (OR = 2.699, 95%CI:

Table 4 Multivariate logistic regression analysis for polypoid lesions of the gallbladders

Variables	*B*[1]	OR	95%CI	*P* value
Sex (male)	0.587	1.799	1.472-2.199	< 0.001
Age (yr)				
≥ 30	0.993	2.699	1.762-4.133	< 0.001
30-59	1.078	2.938	1.914-4.511	< 0.001
≥ 60	0.697	2.009	1.243-3.245	0.004
HBsAg (+)	0.318	1.374	1.097-1.721	0.006

[1]Regression coefficient. The dependent variable was the presence or absence of polypoid lesions of the gallbladders. The covariates include sex, age ≥ 30 years (divided into age 30-59 years and ≥ 60 years), body mass index ≥ 25.0 kg/m^2, systolic blood pressure ≥ 130 mmHg, diastolic blood pressure ≥ 85 mmHg, fasting blood glucose ≥ 5.6 mmol/L, triglyceride level ≥ 1.7 mmol/L, high-density lipoprotein cholesterol level ≥ 1.03 mmol/L in men or ≥ 1.29 mmol/L in women, and waist circumference ≥ 90 cm in men or ≥ 80 cm in women. HBsAg: Hepatitis B surface antigen.

1.762-4.133, $P < 0.001$) and HBsAg positivity (OR = 1.374, 95%CI: 1.097-1.721, $P = 0.006$) were still positively correlated with PLGs. However, the risk of PLGs was 2.938 times higher in middle-aged (30-59 years) subjects than in young persons, and old (≥ 60 years) people had a 2.009 times higher risk of PLGs than young people.

DISCUSSION

This large population-based survey of current and former petrochemical employees provides a unique opportunity to study the prevalence of gallbladder polyps and the distribution by age and sex in the eastern coastal region of China.

In this stable population, nearly 14% of men and 13% of women aged 20-90 years had gallbladder disorders, including post-cholecystectomy, gallstones and PLGs. Cholecystectomy had been performed in 0.5% of men and 1.7% of women. Gallstones were diagnosed in 4.7% of men and 6.4% of women. PLGs were diagnosed in 8.5% of men and 5.0% of women.

The prevalence of post-cholecystectomy (0.9%) was so low that only 90 participants underwent cholecystectomy. The prevalence of gallstones in this study (5.2%) is similar to that in previous sonographic screening studies conducted in Japan, Taiwan and Korea, where gallstones were identified in 3.6%-11.0% of the population[15-17]. The current study demonstrated a higher prevalence of gallstones in women than in men in all age groups and showed that prevalence increased with age, which is in agreement with previous studies[10].

The prevalence of PLGs was higher than that of gallstones in participants aged < 60 years, and lower than that of gallstones in participants aged ≥ 60 years in this study. These findings are comparable with other studies that have indicated that PLGs were prevalent in middle-aged patients, whereas prevalence of gallstones and cholecystectomy increased with age[18,19]. The true reason for diminishing the prevalence of PLGs with age cannot be clarified in our study. The reported risks for gallstones, such as age, large body size[20,21] and metabolic disorders[22] were not risk factors for development of PLGs in the present study. This difference in risk factors may have been due to the different pathogenesis of these common gallbladder diseases.

PLGs are not rare in Taiwan or in the mainland of China[10-12]. The present study indicated that petrochemical employees in Ningbo had a similar prevalence (7.4%) of PLGs, although a little lower than that in Taiwan (9.5%)[10], but higher than that in Shanghai (4.2%)[11] and Beijing (6.9%)[12]. After excluding 633 patients with gallstones or those who underwent cholecystectomy, in the remaining 9828 participants, the overall prevalence of PLGs was 7.9%; 8.9% in men and 5.5% in women, which was higher than that in Denmark (4.3%)[5], Japan (5.2% and 5.6%)[7,8] and Germany (6.1%)[23]. These differences in the prevalence of PLGs among the Chinese, Japanese, Danish and German people deserve further consideration.

The present study demonstrated that men had a higher prevalence of PLGs than women at all ages. The prevalence of PLGs was highest in middle-aged men in their 40s and 50s. In women, the prevalence of PLGs was highest in those in their 50s. These results were in accordance with a Japanese study[7]. In contrast, the prevalence of post-cholecystectomy or gallstones was higher in women than in men at all ages. However, this study did not identify the mechanisms mediating this sex-related risk.

Hepatitis B virus infection is endemic in China and the HBsAg-positive rate in the general population is approximately 10.0%[24]. The current study found that compared with the PLG-negative group, the PLG-positive group had a significantly higher incidence of HBsAg positivity. Logistic regression analysis showed that HBsAg positivity is a risk factor for PLGs. These results are in agreement with those of some previous studies in the Chinese population[10-12,25], but contrary to others[17,26]. The inconsistent findings may be related to the number of cases, sex ratio, ethnic difference and other factors. The pathophysiology of the association between PLGs and HBsAg positivity deserves further study.

The associations between PLG and sex, age, BMI, WC, blood pressure, serum uric acid, serum lipid level, history of hypertension, history of diabetes, cigarette smoking, and alcohol consumption were all tested independently. Male sex, age ≥ 30 years and cigarette smoking were statistically significant for the PLG group using the χ^2 test. Other variables were not significant by univariate analysis. In the multivariate analysis, male sex and age ≥ 30 years were still positively correlated with PLGs. This agreed with previous studies in which male sex was a major risk factor for PLGs. However, cigarette smoking was no longer associated with PLGs, which was in contrast with some previous studies. Okamoto *et al*[27] have reported that cigarette smoking is inversely related to PLGs in Japanese men. Significant effects of other demographic variables such as BMI, blood pressure, alcohol consumption, and history of hypertension and diabetes on the prevalence of PLGs were not identified

in the present study. Similarly, there was no relationship between PLGs and biochemical parameters such as FPG, lipid profile and uric acid.

The present study had some strengths and limitations. First, the employees were a stable population. The demographic characteristics of this group may not be completely generalizable, for example, there were substantially more men than in the general population, but that may be more representative than many other studies in the literature. Second, pathological data were not available for 777 subjects with PLGs, although a proportion (14/777, 1.8%) of the large-sized polyps are likely to develop neoplasms[4,28]. In general, polypoid lesions > 10 mm are an important predictor for malignancy. For small PLGs < 10 mm, it has been shown that most of these lesions are benign and remain so for several years[29,30]. Nevertheless, it is not possible in a screening study to establish the histopathological nature of polypoid lesions. Therefore, a longitudinal study with the same cohort is now underway to observe the progression of PLGs, especially for the 14 cases of larger PLGs.

In conclusion, this study demonstrated that 7.4% of the petrochemical employees had PLGs. Male sex and HBsAg positivity were associated with prevalence of PLGs; and age ≥ 30 years, especially 30-59 years, was positively associated with PLGs. Further research on the prevalence of and risk factors for PLGs is therefore necessary in the general population in the mainland of China.

COMMENTS

Background

The widespread application and improved image quality of abdominal ultrasonography in modern clinical practice have led to an increase in the detection of biliary tree abnormalities, including polypoid lesions of the gallbladder (PLGs). The reported prevalence of PLGs is 0.3%-9.5%, depending on the population studied and on the study design. To date, only a few studies have investigated the prevalence and risk factors for PLGs in China. This study investigated the prevalence of and risk factors for PLGs in a cohort of petrochemical employees in the city of Ningbo, China.

Research frontiers

This large population-based survey provided a unique opportunity to study the prevalence of gallbladder polyps and the distribution by age and sex in the eastern coastal region of China. This study demonstrated that men had a higher prevalence of PLGs than women at all ages. The prevalence of PLGs was highest in middle-aged men in their 40s and 50s. The demographic characteristics of this group may not be completely generalizable, for example, there were substantially more men than in the general population, but that may be more representative than many other studies in the literature.

Innovations and breakthroughs

The prevalence and risk factors of gallbladder polyps from 10461 subjects in eastern China were investigated. The results suggest that these populations do not have a low prevalence of PLGs, and middle-aged men or both sexes positive for hepatitis B surface antigen (HBsAg) have a high risk. These results provide an important reference for the prevention and treatment of gallbladder polyps.

Applications

To understand better the prevalence of and risk factors for PLGs in petrochemical employees in Zhejiang Province, China, a prospective observational study of 10461 stable participants was conducted in 2009, which is expected to provide a comprehensive update to the results of previous studies. The results provide an important evidence for the prevention and treatment of gallbladder polyps.

Terminology

PLGs represent a heterogeneous group of changes in the gallbladder wall and include entities such as cholesterol polyps, inflammatory polyps, adenomas, leiomyomas and lipomas.

Peer review

In this paper, the prevalence and risk factors of gallbladder polyps from 10461 subjects in eastern China were investigated. The results suggest that these populations do not have a low prevalence of PLGs, and middle-aged men or people of both sexes positive for HBsAg have a high risk. These results provide an important reference for the prevention and treatment of gallbladder polyps. Nevertheless, further clarification or revision in some places of the paper are needed.

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BRIEF ARTICLE

Transplantation *vs* resection for hepatocellular carcinoma with compensated liver function after downstaging therapy

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Abstract

AIM: Our study aimed to compare the results of liver transplantation (LT) and liver resection (LR) in patients with hepatocellular carcinoma (HCC) that met the Milan criteria after successful downstaging therapy.

METHODS: From February 2004 to August 2010, a consecutive series of 102 patients were diagnosed with advanced-stage HCC that met the modified UCSF down-staging protocol inclusion criteria. All of the patients accepted various down-staging therapies. The types and numbers of treatments were tailored to each patient according to the tumor characteristics, location, liver function and response. After various downstaging therapies, 66 patients had tumor characteristics that met the Milan criteria; 31 patients accepted LT in our center, and 35 patients accepted LR. The baseline characteristics, down-staging protocols, postoperative complications, overall survival and tumor free survival rate, and tumor recurrence rate were compared between the two groups. Kaplan-Meier analyses were used to estimate the long-term overall survival and tumor-free survival rate. Meanwhile, a Cox proportional hazards model was used for the multivariate analyses of overall survival and disease-free survival rate.

RESULTS: No significant difference was observed between the LT and LR groups with respect to the down-staging protocol, target tumor characteristics, and baseline patient characteristics. Fifteen patients suffered various complications after LT, and 8 patients had complications after LR. The overall complication rate for the LT group was 48.4%, which was significantly higher than the LR group (22.9%) (P = 0.031). The overall in-hospital mortality in hospital for the LT group was 12.9% *vs* 2.9% for the LR group (P = 0.172). The overall patient survival rates at 1-, 3- and 5-years were 87.1%, 80.6% and 77.4%, respectively, after LT and 91.4%, 77.1% and 68.6%, respectively, after LR (P = 0.498). The overall 1-, 3- and 5-year tumor recurrence-free rates were also comparable (P = 0.656). Poorer tumor differentiation (P = 0.041) and a higher post-downstage alpha-fetoprotein (AFP) level (> 400 ng/mL) (P = 0.015) were the two independent risk factors for tumor recurrence in the LT and LR patients who accepted successful down-staging therapy.

CONCLUSION: Due to the higher postoperative morbidity and similar survival and tumor recurrence-free rates, LR might offer better or similar outcome over LT, but a larger number and further randomized studies may be needed in the future for drawing any positive conclusions.

Key words: Liver; Resection; Transplantation; Downstage; Survival; Complication; Recurrence; Comparison

Core tip: We compared advanced-stage hepatocellular carcinoma (HCC) patients who underwent liver trans-

plantation (LT) or liver resection (LR) after successful downstaging therapy, and the recurrence rates and survival outcomes were similar, although the postoperative complication rate was higher for the LT group. The Milan criteria are one of the most strict and accepted criteria for HCC patients to determine eligibility for LT or LR. Therefore, our use of this selection criteria may make this study more ideal than others. Meanwhile, all of our patients accepted successful pre-operative downstaging therapy, the long waiting time can successful avoid the selective bias. So our comparison and results are more credible.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors and the fourth most common cause of mortality[1,2]. HCC is more common in northeast Asia due to the high prevalence of hepatitis B infection and in Western countries and Japan due to the high prevalence of hepatitis C infection[3,4]. HCC is difficult to manage compared to other malignancies due to the underlying liver cirrhosis caused by viral hepatitis. Fortunately, liver resection (LR) and liver transplantation (LT) are potentially curative treatments for early-stage HCC[5]. The most commonly accepted conditions for transplantation for HCC are the Milan criteria (early stage): a solitary tumor with a diameter < 5 cm or 2-3 cm tumors with the largest diameter < 3 cm and the absence of macroscopic vascular invasion or extrahepatic metastasis[6]. However, the lack of regular physical examination has led to more advanced HCCs at the time of diagnosis in developing countries, especially in China; thus, these advanced-stage HCC patients have lost the opportunity for an immediate cure, and downstaging therapy becomes the initial treatment option. Numerous loco-regional therapies, which serve as downstaging therapies, have been introduced for advanced-stage HCC patients: transarterial chemo-embolization (TACE), radiofrequency ablation (RFA), alcohol injection (EI), LR, and transarterial chemoinfusion (TACI) and sorafenib[7-10]. After successful downstaging therapy leading to a tumor that meets the Milan criteria for LT, another problem has emerged: which surgical method should be used, LR or LT? The choice of therapy has been debated for a long time. Considering the risk of recurrence and impaired liver function associated with cirrhosis, LT could be viewed as the optimal treatment for HCC because LT treats the tumor and the underlying liver disease[11]. However, this benefit may be offset by problems specifically related to transplantation: graft rejection, immunosuppression complications, recurrent viral hepatitis, increased mortality and a shortage of organ donors[6,12]. Since LT was introduced to HCC patients, the comparison of LR and LT has been constant[6,13-15]. Nevertheless, the optimal treatment strategy (LR or LT) for HCC patients who meet the Milan criteria after successful downstaging therapy has not been established. This study aimed to compare the outcomes of HR and LT in patients with HCC that met the Milan criteria after successful downstaging therapy.

MATERIALS AND METHODS

Patient characteristics

From February 2004 to August 2010, a consecutive series of 102 patients were diagnosed with advanced-stage HCC that met the modified UCSF down-staging protocol[7,16]. The eligibility criteria for down-staging were as follows: a single tumor with a diameter up to 8 cm, two to three tumors with individual diameters up to 5 cm and a total diameter up to 8 cm, and no vascular invasion by imaging criteria. The diagnosis of HCC was based on a serum hepatitis B virus (HBV) or heptatitis C virus (HCV) test, contrast-enhanced ultrasound, double-phase helical computed tomography (CT) scan, and a serum alpha-fetoprotein (AFP) level. A bone or a total body scan was used to identify possible metastasis. A biopsy was not routinely performed for every patient. All of the data were collected from the Chinese Liver Transplant Registry (http://www.cltr.org).

Downstaging protocol

The patients underwent various downstaging protocols. The type and number of treatments used were tailored to each patient according to tumor characteristics and response. Two local-regional therapies (TACE, RFA) were used for downstaging therapy. Patients who underwent accepted resection as a downstaging therapy were excluded from our study. The approach for RFA included percutaneous, laparoscopic and open techniques. The choice for the technique was individualized to a particular patient and based on local expertise. For tumors < 3 cm in size, RFA was recommended. As the tumor increases in size, the likelihood of incomplete treatment with RFA increases; thus, TACE or combination therapy was recommended for larger tumors[16].TACE was performed using standard techniques[17]. TACE was performed using 30 mg of mitomycin, 30 mg of adriamycin and 100 mg of cisplatinum mixed with lipiodol as the drug carrier. Then, embolization using permanent occlusive particles was performed. RFA was performed by using a "cool-tip" needle that contained an exposed 2- to 3-cm electrode and an internal water-cooling system (Radionics TM, Burlington, MA, United States); meanwhile, color-Doppler ultrasound was used as a guide for the percutaneous puncture or during intraoperative approach, and a laparoscopic ultrasonic probe was used during the laparoscopic cases.

Post-downstaging therapy evaluation and follow up

The response to downstaging therapy was evaluated by serum AFP once a month and contrast-enhanced CT bi-monthly in our center. Once the imaging examination indicated HCC characteristics that met the Milan criteria and the serum AFP was less than 500 ng/mL for patients with an initial AFP > 1000 ng/mL[8], LT or resection was offered to these patients. Resection was firstly considered for cirrhotic patients with well preserved liver function, LT was used if subtotal hepatic resection was not anatomically feasible and the living or deceased donor liver graft can be available, the patients with Child Class C were introduced to accept LT and excluded from our study. Additionally, these patients were advised to undergo a repeat bone scan and total body CT scan to identify any possible metastasis. After LT or LR, patients received bi-monthly follow up, and we retrospectively collected the data for these patients, comparing the baseline characteristics, postoperative complications, the 1-, 3-, and 5-years survival rates, and tumor recurrence-free rate. All of the LRs were open abdominal surgeries and regular liver lobectomy or segmentectomy. The detailed surgical procedure protocols and postoperative management for LR[18] and deceased donor LT (DDLT) have been presented in previous reports. No prisoner donors were used in our study. Living donor LT (LDLT) was performed after approval from the Ethics Committee of Sichuan University, and local authorization was obtained. All of the donations were voluntary and altruistic. We informed the donors and their families of the possible risks of donor hepatectomy. Written consent was provided by the donors for the storage of their information in the hospital database and its use for research. The inclusion and exclusion criteria[19] and surgical techniques[20] that were used for LT have been described previously. For the transplant recipients, tacrolimus or cyclosporine, mycophenolate and prednisone were used for the triple-drug immunosuppression regimen. The dose of tacrolimus and cyclosporine was adjusted based on the measured serum level. Prednisone was generally discontinued within 3 mo after transplantation[21]. Nucleoside analogues were used for all LR patients after LR if the HBV-DNA was positive pre-operation, hepatitis B hyper-immune globulin (HBIG) combined with nucleoside analogues were used for all LT patients post-transplantation.

Statistical analysis

The quantitative variables are expressed as the mean ± SD or median values with the range in parentheses, and qualitative variables were expressed as absolute numbers with the percentages in parentheses. Descriptive data for various patient characteristics were calculated separately for patients who received LT or LR. Continuous variables were compared using a nonparametric Wilcoxon test because some of the measurements did not follow a normal distribution. Categorical data were compared using a χ^2 test or Fisher's exact test, if necessary. Kaplan-Meier estimates of the long-term overall survival and tumor-free survival rates were calculated using the intention-to-treat principle and compared using the log-rank test. A Cox proportional hazards model was used for the multivariate analyses for survival and disease-free survival. The inclusion of variables into the final model was based on biological and statistical considerations. The statistical analyses were performed using the SAS statistical software package (version 9.1.3, SAS Institute, Inc, Cary, NC, United States), and a 2-sided P value < 0.5 was considered to be statistically significant.

Table 1 Downstaging treatments *n* (%)

	LT group	LR group
Number of treatments	31	35
TACE only	15 (48.4)	18 (51.4)
One time	5	7
Two times	8	7
Three times	2	4
RAF only	7 (22.6)	10 (28.6)
One time	5	6
Two time	2	4
TACE + RAF	4 (12.9)	3 (8.6)
TACE + TACE + RAF	3 (9.7)	2 (5.7)
TACE + RAF + TACE	2 (6.5)	2 (5.7)

TACE: Transarterial chemo-embolization; RFA: Radiofrequency ablation; LT: Liver transplantation; LR: Liver resection.

RESULTS

Downstaging protocols and results

TACE and RFA were performed for all advanced-stage HCC patients. After single or combined down-staging therapy, 66 patients (58.8%) showed successful down-staging. The details of these patients are shown in Table 1. In both groups, more patients underwent TACE than RAF as a single loco-regional therapy. Fewer patients in the LT group received only one kind of loco-regional therapy compared to the LR group (71% *vs* 80%, respectively), but this difference did not reach statistical significance (P = 0.396). Patients who received only TACE were more likely to undergo the therapy more than once, whereas patients who underwent RFA were more likely to undergo the therapy only once. Combination was performed for nine patients (29.0%) in the LT group and seven patients (20%) in the LR group (P = 0.396). There was no significant difference between the two groups for the type and number of down-staging treatments (P = 0.696).

Demographic data and tumor characteristics

Table 2 compares the characteristics of the 66 patients with successful downstaging who accepted LT (31 cases) or LR (35 cases). The two groups had similar demographic characteristics, and there were no significant differences in the number of tumors (P = 0.721), total tumor diameter (P = 0.376), tumor differentiation (P = 0.960) and the liver fibrosis degree scored by using the Ishak system (P = 0.069). Although the serum AFP level in the LT group (1425.4 ng/mL) was higher

Table 2 Baseline demographic and tumor characteristics in the two groups

	LT group (n = 31)	LR group (n = 35)	P value
Age (yr)	43.0 ± 8.2	45.5 ± 8.1	0.212
Gender (male:female)	20:11	20:15	0.544
Weight (kg)	68.6 ± 7.8	65.8 ± 10.6	0.241
Height (cm)	166.9 ± 8.6	164.6 ± 9.3	0.302
BMI (kg/m^2)	22.8 ± 1.8	23.2 ± 2.4	0.404
Underlying liver disease			0.901
HBV	29	33	
HCV	1	1	
HBV and HCV	1	1	
MELD score	8.6 ± 3.8	8.7 ± 4.7	0.866
Child score			0.617
A (5-6)	20	23	
B (7-9)	11	12	
C (≥ 10)	0	0	
Serum creatinine (μmol/L)	71.3 ± 24.1	64.3 ± 20.9	0.212
Active lesion number (Pre-/Post-downstage)			0.672/0.721
One target	13/16	15/19	
Two targets	10/9	14/11	
Three targets	8/6	6/5	
Total diameter of the tumors (cm)	6.8 ± 2.1/ 4.5 ± 1.7	6.7 ± 2.3/ 4.1 ± 1.9	0.786/0.376
AFP level (ng/mL)			
Pre-downstage	1425.4 ± 1512.6	1332.9 ± 1122.5	0.777
Post-downstage	218.0 ± 244.0	248.6 ± 267.6	0.631
Tumor differentiation			0.960
Well	16	19	
Moderate	7	6	
Poor	8	10	
Ishak score	4.6 ± 1.5	3.9 ± 1.3	0.069

LT: Liver transplantation; LR: Liver resection; HBV: Hepatitis B virus; HCV: Heptatitis C virus; AFP: Alpha-fetoprotein; BMI: Body mass index.

than in the LR group (1332.9 ng/mL) at baseline, after downstaging therapy, the reduction in AFP in the LT group was much greater than the LR group. However, no significant difference was observed for changes in AFP. Five patients (16.1%) prior to LT had a serum AFP greater than 400 ng/mL but a CT indicative of successful downstaging therapy, compared to eight patients (22.9%) in the LR group (P = 0.496).

Major postoperative complications and mortality

Complications developed in 15 patients after LT and 8 patients after LR, and the overall complication rate in the LT group (48.4%) was significantly higher than the LR group (22.9%) (P = 0.031). The overall mortality for the LT group was 12.9% *vs* 2.9% for the LR group (P = 0.172).This difference did not reach statistical significance (Table 3). One 42-year-old man was diagnosed with acute rejection by biopsy 1 mo after discharge, and he eventually died from this rejection. According to the Clavien scoring system for complications, the complication rates in the LT group for each grade were 16.1%, 9.7%, 6.5%, 3.2% and 12.9%, respectively, compared to 8.6%, 5.7%, 5.7%, 0.0% and 2.9% in the LR group, respectively (P = 0.026). The patients who died in hospital after LR or LT did not have any proof of HCC recurrence in the liver, and they were excluded from the recurrence rate calculation.

Table 3 Complications after liver transplantation or liver resection

Complications	LT group (n = 31)	LR group (n = 35)
Bile leakage	2 (Grade Ⅰ, Ⅰ)	1 (Grade Ⅰ)
Intra-abdominal bleeding	2 (Grade Ⅰ, Ⅴ)	2 (Grade Ⅱ, Ⅲ)
Wound infection	2 (Grade Ⅰ, Ⅰ)	1 (Grade Ⅰ)
Pleural effusion	1 (Grade Ⅲ)	2 (Grade Ⅰ, Ⅱ)
Respiratory failure	2 (Grade Ⅳ, Ⅴ)	1 (Grade Ⅴ)
Ileus	1 (Grade Ⅱ)	0
Hepatic artery thrombosis	1 (Grade Ⅱ)	0
Subphrenic abscess	1 (Grade Ⅲ)	1 (Grade Ⅲ)
Liver failure	1 (Grade Ⅴ)	0
Rejection	2 (Grade Ⅱ, Ⅴ)	0

Grade Ⅰ: Treated conservatively without any drugs; Grade Ⅱ: Treated with pharmacology; Grade Ⅲ: Intervention with anesthesia; Grade Ⅳ: Organ dysfunction; Grade Ⅴ: Death. LT: Liver transplantation; LR: Liver resection.

Survival and recurrence rates

The mean follow up was 3.6 ± 1.8 years for the LT group and 3.7 ± 1.6 years for the LR group (P = 0.838). The overall patient survival rates at 1-, 3- and 5-years were 87.1%, 80.6% and 77.4%, respectively, after LT and 91.4%, 77.1% and 68.6%, respectively, after LR (P = 0.498) (Figure 1A). The in-hospital deaths that occurred within one year are described above. One patient suffered a car accident and died seven months after tumor resection, and no tumor recurrence was observed during his follow up. Another 43-year-old man developed brain metastases and died 11 mo after LR. One year after the operation, the main cause of mortality was tumor recurrence, except for two LT patients. One recipient had a biliary stricture and underwent cholangioenterostomy, but the patient died from a lung infection three years after his living donor LT. Another 45-year-old woman died from chronic rejection four years after a deceased donor LT.

Three of 27 (11.1%) patients developed a recurrent tumor at a median of 1.8 years after LT, and 5 of 34 (14.7%) patients had a tumor recurrence at a median of 2.2 years after LR. A trend toward a longer time to recurrence after LR (2.2 ± 1.1 years) compared to LT (1.8 ± 1.4 years) was observed; however, this difference did not reach statistical significance (P = 0.664). The overall 1-, 3- and 5-year recurrence-free rates were 83.8%, 74.2%, and 67.7%, respectively, for the LT group and 88.6%, 74.3%, and 60.0%, respectively, for the LR group (P = 0.656) (Figure 1B). The most common site for tumor recurrence was the liver, followed by the lungs, lymph nodes, and rarely the bones and brain. There was no difference in the site of recurrence between the LT and LR groups (P = 0.872). In the LT group, 2 of 3 recipients had extrahepatic recurrences (lung and abdominal lymph node).

Table 4 Risk factors for tumor recurrence in the two groups

Factors	Odds	95%CI	*P* value
Tumor differentiation	2.225	1.365-3.882	0.041
Post-downstaging AFP level > 400 ng/mL	2.113	1.971-3.104	0.015

AFP: Alpha-fetoprotein.

In the LR group, of the five recurrences, 3 patients had an extrahepatic recurrence (lungs, abdominal lymph node and bones).

Risk factors for tumor recurrence

To clarify the prognostic factors for tumor recurrence in each group, multivariate data (*e.g.*, patient age, gender, weight, height, BMI, underlying liver disease, blood group, liver function, number of tumors, total diameter of the tumors, baseline AFP level, post-downstage AFP level, number of downstage therapies, the presence of satellite nodules, and tumor differentiation level, intrahepatic micrometastases and extrahepatic micrometastases) were analyzed and compared in each group using a stepwise, multivariate logistic regression analysis (step-down). The predictive factors for recurrence were similar in the two groups and were related to poorer tumor differentiation ($P = 0.041$) and a higher post-downstage AFP level (> 400 ng/mL) ($P = 0.015$) (Table 4). As a result, these two factors may become independent predictive factors for recurrence in LT and LR patients who underwent successful downstaging therapy.

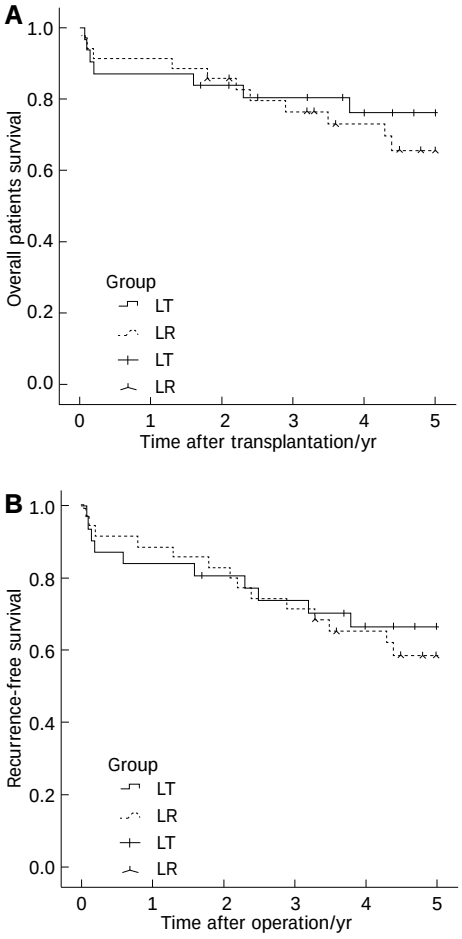

Figure 1 Comparison of liver transplantation and liver resection. A: The overall survival rates ($P = 0.498$); B: the recurrence-free rates ($P = 0.838$). LT: Liver transplantation; LR: Liver resection.

DISCUSSION

The majority of HCC patients are diagnosed at a late stage and therefore are not eligible for potentially curative treatment, such as resection or LT[22]. Ideal candidates based on the Milan criteria for LT or LR comprise fewer than 10% of the diagnosed HCC cases[23]. For patients with advanced HCC, LT yields a disappointing 5-year survival rate (18%-32%), largely due to tumor recurrence. Fortunately, disease down-staging using locoregional therapy may offer patients, who are not initially candidates, a chance to undergo a curative treatment, such as LT or LR[24]. TACE and RFA remain perhaps the most commonly used palliative treatments for unresectable HCC. However, to our knowledge, there has been no comparison of the outcomes between advanced HCC patients who underwent LR or LT after successful downstaging therapy (tumors that met the Milan criteria).

LT, including living donor LT and deceased LT, offers the theoretical advantage of removing the tumor and the organ at risk of developing future malignancy and is an established therapy for small, early-stage HCC in patients with cirrhosis[25]. The above are the greatest advantages of LT over resection, whereas resection is more easily and immediately available[26,27], effective[28-31], safer[32-34] and simpler[35]. Consequently, there is no consensus regarding the best surgical treatment for patients with well-compensated cirrhosis and early HCC that meets the Milan criteria[6], so LT should not be considered as the first choice for mild or even some cases of moderate cirrhosis because proper patient selection for resection may yield equal or better outcomes. Many comparisons have been made, but no consensus has been reached. In our study, we compared advanced-stage HCC patients who underwent LT or LR after successful downstaging therapy, and the recurrence rates and survival outcomes were similar, although the postoperative complication rate was higher for the LT group.

In our study, the complication rate after LT was much higher than after LR, but the in-hospital mortality was not significantly different between the two groups. Bellavance *et al*[36] reported the morbidities for patients whose tumor met the Milan criteria was 49% for the LR group and 65% for the LT group, which were both higher than in our study (22.9% for the LR group and 48.4% for the LT group). No difference was observed in the 1-year survival rates for the LR and LT patients in his study. However, the 5-year survival rate in his report was only 46% for the LR patients, which was lower than 66% in the LT group and much lower than the 68.6% observed in our study. The reported morbidity for early-stage HCC (within

the Milan criteria) ranged from 30% to 49% for LR patients and from 44% to 80%, and almost all of the reports demonstrated a higher morbidity for the LT group than for the LR group[6,37-39]. The higher morbidity in the LT group may be due to the longer operation time, more difficult operative procedure and the reconstruction of the hepatic vessel and bile duct. Another reason for the lower morbidity for the LR patients was that the resection avoided the risks associated with immunosuppression. These risks include toxicities (especially nephrotoxicity), infectious complications, and post-transplantation de novo neoplasms[6].

Although four LT patients died in the hospital from serious complications, only one patient died after LR. Although this result did not reach statistical significance, this trend for greater mortality in the LT group is well known. Of the 204 LR patients, no patient died in the hospital after LR, whereas the mortality rate was 3.4% for the LT group in Poon's report[28]. A similar conclusion was reached in Bigourdan *et al*[39], but in Margarit *et al*[40], the mortality for LR patients (5.6%) was higher than for LT patients (3.4%). A recent review of almost 60 cases of either LR or LT found that the mortality following transplantation was 60% higher than following resection (5%)[41]. Recipients who received an allogeneic liver graft, either full or partial size, all needed to take an immunosuppressant, such as tacrolimus, mycophenolate mofetil or steroids, and nephrotoxicity and immunosuppression may affect graft and patient survival[32,42]. In a series of 1000 liver transplant patients treated with tacrolimus immunosuppression, post-transplantation infection was the most common cause of death (34% of 360 deaths)[42].

As for long-term results, this study showed that LT and LR had similar recurrence-free survival and overall survival rates. Various overall and recurrence-free survival rates have been reported for patients undergoing LR or LT. Most of these results have shown that the rates of long-term survival and recurrence after transplantation are superior to those observed following resection[29,36,40,43,44]. Others have shown similar overall survival rates for the two groups and a higher recurrence-free survival rate for the LT group[40,45]. In a retrospective study, Otto *et al*[14] compared 50 patients who underwent LT and 52 patients who underwent LR and concluded that no significant difference was observed between the two groups for the 3-year survival and recurrence rates. In this study, tumor size was the only independent factor for recurrence. The notable difference among these reports may be caused by a superior eradication of gross or microscopic disease or by selection bias[14,36]. The use of too many different staging systems may be one cause of this bias because 18 different staging or scoring systems have been reported to be used. In our study, we used the Milan criteria as the selection criteria. The Milan criteria are one of the most strict and accepted criteria for HCC patients to determine eligibility for LT or LR. Therefore, the use of this selection criteria in our study may make this study more ideal than others. Meanwhile patients who are successfully down-staged and undergo operation may have a higher recurrence-free survival rate[46], and all of the patients accepted successful down-staging therapy, that is the reason for our higher tumor recurrence free survival rate. In our study, most of the patients in the LT and LR groups had their HCC recurrence within three years. Only two patients had tumor recurrence four years after LT or LR. These results were contrary to Lee's report[15], in which most recurrences happened within 2 years after LT and only rarely after that. Meanwhile, the post-operative antiviral therapies may also contribute to the good outcome after resection and LT in our study, it is because controlling viral replication halts disease progression and decreases the risk of tumor recurrence or developing new lesions[12]. However, the tumor recurrence rate after LR increased over time, and the long-term survival rates between the LR and LT groups differed significantly.

Many studies have reported predictors of prognosis based on univariate analyses combining LR and LT patients. Zhou *et al*[47] reported three factors were significant predictors of disease-free survival: microscopic venous invasion, tumor size-plus-number (> 4 cm *vs* ≤ 4 cm), and treatment (HR *vs* LT). However, his study included patients that did not meet the Milan criteria. AFP is a tumor marker that is expressed by HCC and is secreted into the serum of approximately 70% of patients with HCC. AFP have been widely used to diagnose[48] and monitor HCC. Previous studies have demonstrated that the baseline AFP level is a significant prognostic factor for various stages of HCC[49-51]. EA Pomfret[16] suggested that successful down-staging requires a significant decrease in the AFP, to < 500 ng/mL for patients with an initial AFP > 1000 ng/mL. For patients with an AFP > 500 ng/mL, the lack of evidence of a tumor by imaging, indicating either no cancer is present or there is a diffuse, small, and highly aggressive malignancy with a poor prognosis, is required. In the Bologna study[8], an AFP level ≥ 400 ng/mL was an exclusion criterion for LT after down-staging therapy. Many other reports have demonstrated that the change in AFP after LT or resection is valuable in predicting tumor recurrence for HCC patients[52,53]. In our study, another predictor for HCC recurrence and overall survival was the tumor differentiation level. In Imamura *et al*[54], an AFP ≥ 32 ng/mL was a risk factor for early tumor recurrence, and the gross tumor classification was a risk factor for late tumor recurrence after resection. Histological grade has been accepted as a significant predictor of the patient survival as showed in our study[28,55].

Our study does have some limitations, a randomized study would have been the best type of clinical study to resolve the debate regarding use of LT *vs* LR for HCC patients after successful downstaging therapies. This ideal study is indeed difficult to realize, if at all feasible, given the complex decision-making process involved in LT. In addition, we performed our analysis using only about 30 cases in each group, the total numbers presented in this series are low, however, all of the HCC patients in our

study were out of criteria for LR or LT at first, and all of them met the Milan criteria after successful downstaging therapies. So a larger multicenter study comparing an larger number of patients with HCC after successful downstaging therapies in both groups (LR and LT) would be ideal.

In conclusion, the present study shows that, the LT group had a significantly higher morbidity rate than the LR patients; however, the mortality rate did not differ between the two groups. There was no significant difference in the overall survival and HCC-free survival rates between the two groups. For the HCC patients who accepted successful downstaging therapies and be with compensated liver function (Child Class A or B), LR might offer better or similar outcome over LT; and that further randomized studies on a larger number of patients is warranted before drawing any conclusions.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors and the fourth most common cause of mortality. Fortunately, liver resection (LR) and liver transplantation (LT) are potentially curative treatments for early-stage HCC. The most commonly accepted conditions for transplantation for HCC are the Milan criteria (early stage). However, the lack of regular physical examination has led to more advanced HCCs at the time of diagnosis in developing countries, thus, these advanced-stage HCC patients have lost the opportunity for an immediate cure, and downstaging therapy becomes the initial treatment option. After successful downstaging therapy leading to a tumor that meets the Milan criteria for LT, another problem has emerged: which surgical method should be used, LR or LT? The choice of therapy has been debated for a long time.

Research frontiers

LR and LT are potentially curative treatments for early-stage HCC, all of them have advantages and disadvantages, considering the risk of recurrence and impaired liver function associated with cirrhosis, LT could be viewed as the optimal treatment for HCC because LT treats the tumor and the underlying liver disease. However, this benefit may be offset by problems specifically related to transplantation: graft rejection, immunosuppression complications, recurrent viral hepatitis, increased mortality and a shortage of organ donors.

Innovations and breakthroughs

There is no consensus regarding the best surgical treatment for patients with well-compensated cirrhosis and early HCC that meets the Milan criteria previously. Many comparisons have been made, but no consensus has been reached. In authors' study, authors compared advanced-stage HCC patients who underwent LT or LR after successful downstaging therapy, and the recurrence rates and survival outcomes were similar, although the postoperative complication rate was higher for the LT group. In authors' study, authors used the Milan criteria as the selection criteria. The Milan criteria are one of the most strict and accepted criteria for HCC patients to determine eligibility for LT or LR. Therefore, the use of this selection criteria in their study may make this study more ideal than others. Meanwhile, all of their patients accepted successful preoperative down-staging therapy, the long waiting time can successful avoid the selective bias. So authors' comparison and results are more credible.

Applications

This study result suggest that LT may not be considered as the primary treatment for patients with HCC that meets the Milan criteria after successful downstaging therapy.

Terminology

LT is a surgical method to cure end-stage liver disease, removing the liver with disease and implant one or part of new liver from donor; Down-staging therapy was a method to reduce the size or the number of tumor.

Peer review

It is interesting and important to investigate the advantages and risks of LT and LR in patients with advanced HCC that met the Milan criteria after successful downstaging therapy.

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CASE REPORT

Late post liver transplant protein losing enteropathy: Rare complication of incisional hernia

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Abstract

Development of oedema and hypoproteinaemia in a liver transplant recipient may be the first signs of graft dysfunction and should prompt a full assessment. We report the novel case of a patient who, years after liver transplantation developed a functional blind loop in an incisional hernia, which manifested as oedema and hypoproteinaemia secondary to protein losing enteropathy. After numerous investigations, the diagnosis was made by flurodeoxyglucose positron emmision tomography (FDG-PET) imaging. Surgical repair of the incisional hernia was followed several months later by resolution of the protein loss, and confirmed at a post operative FDG-PET scan at one year.

Key words: Protein losing enteropathies; Bacterial overgrowth; Hypoproteinaemia; Incarcerated hernia; Liver transplantation.

Core tip: This report presents a rare case of protein losing enteropathy as a result of bacterial overgrowth in the blind loop of an incisional hernia following liver transplantation. Surgical repair of the incisional hernia in this case brought about resolution of protein loss.

Evans JD, Perera MTPR, Pal CY, Neuberger J, Mirza DF. Late post liver transplant protein losing enteropathy: Rare complication of incisional hernia. *World J Gastroenterol* 2013; 19(27): 4409-4412 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i27/4409.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i27.4409

INTRODUCTION

Hypoproteinaemia and peripheral oedema are common manifestations of advanced liver disease. In most patients oedema resolves after transplantation with the return of synthetic function of the graft to full capacity, the timing of which may vary from days to several months in extreme cases. Reappearance of oedema in a transplant patient is a cause for concern and may be the first indication of graft failure, which could be the result of recurrent disease or chronic rejection. Chronic graft failure culminates in synthetic failure and in some cases ensuing portal hypertension, which may lead to enteropathy. With the long standing portal hypertension, oedema of the gastric or intestinal mucosa leads to impaired lymphatic circulation and absorption of proteins further augmenting hypoalbuminaemia[1]. Therefore in those presenting with low albumin and peripheral oedema graft failure is considered the first differential diagnosis, and in this light some of the simple but rare aetiological conditions may be overlooked.

Apart from advanced liver disease, aetiology of hypoalbuminaemia ranges from malnutrition and poor protein intake to increased protein loss from body due to various reasons. The renal and gastrointestinal tracts are common sites of protein loss. So called "blind loop

syndrome" refers to a segment of bowel that is bypassed in normal gastrointestinal functions, allowing overgrowth of pathogenic bacteria[2]. The case reported here illustrates a patient who presented many years after liver transplant with peripheral oedema and hypoalbuminaemia, the aetiology of which was traced to a "blind loop" located in an incisional hernia at the site of the transplant incision.

CASE REPORT

A 52-year-old female with hypertension and type Ⅱ diabetes underwent orthotopic liver transplantation for hepatitis C cirrhosis. This was followed two weeks later by early re-transplantation for hepatic artery thrombosis. Over the following ten years, she suffered a number of episodes of cholangitis and required percutaneous transhepatic cholangiography and balloon dilatation of a biliary stricture on two occasions. She also developed an incisional hernia adjacent to her liver transplant scar five years after her transplants, which was managed conservatively. She had stable liver graft function throughout, and was maintained on cyclosporine based immunosuppression regime. She had mild degree of renal impairment attributed to calcineurin inhibitors and her serum creatinine was 106 mmol/L.

Twelve years following her transplant and aged 64 years she developed abdominal bloating and peripheral oedema. Her serum albumin fell to 21 g/dL and total serum protein to 50 g/dL but her other liver function tests were unremarkable. Liver biopsy demonstrated a mild degree of inflammation and fibrosis. Oesophagastroduodenoscopy (OGD) revealed mild hypertensive gastropathy but in the absence of splenomegaly or thrombocytopenia portal hypertension was deemed an unlikely cause for her hypoproteinaemia. She had no evidence of excessive renal protein loss with a negative urine dipstick for protein, an albumin creatinine ratio of 2 and 24-h urinary protein of 126 mg. She had no diarrhoea or other symptoms or signs of inflammatory bowel disease. Duodenal biopsy demonstrated normal duodenal mucosa whilst on capsule endoscopy no significant pathology was found apart from scattered angioectasia throughout the jejunum and ileum and a suggested small polypoidal mass in caecum.

Over the two years that followed, she suffered repeated episodes of oedema, bloatedness and a drop in serum albumin to levels around 20 g/dL requiring multiple albumin infusions. These increased in frequency when she required 27 units of albumin (100 mL 20% HAS) over 2 mo, and was admitted for further evaluation of the cause of her protein losing enteropathy. All biochemical investigations including serum caeruloplasmin, faecal elastase, faecal alpha-1 antitrypsin and celiac screen was normal. Colonoscopy did not identify the possible polypoid lesion showed on capsule endoscopy. Repeat OGD revealed normal villi but a few scattered pearly white spots thought to represent lymphangiectasia. Pathological analysis of duodenal biopsies revealed normal villous architecture, no intraepithelial lymphocytes but a few distended lymphatic spaces.

It was suggested that she was likely to have a degree of bacterial overgrowth in loop of hernia but in absence of watery diarrhoea and vitamin deficiency, the chances of her symptoms being due to protein losing enteropathy was deemed unlikely. In the absence of any other definitive aetiology for continued protein loss she was subjected to a fluorodeoxyglucose computed tomography positron emission tomography (FDG CT-PET) scan which demonstrated increased uptake in the bowel in the region of her incisional hernia indicating increased metabolic activity or infection in the blind loop of the hernia (Figure 1). A month later she underwent uncomplicated repair of her incisional hernia. Her requirement for albumin infusions decreased over the following 9 mo and at present she has been 3 mo without an albumin infusion and is maintaining a steady serum albumin level of 34 g/dL and total protein of 60 g/dL with no symptoms. A repeat CT-PET scan one year post hernia repair has demonstrated no increased uptake in the bowel around the site of her previous incisional hernia (Figure 2).

DISCUSSION

Protein losing enteropathy (PLE) is a rare condition in which protein loss into the gastrointestinal tract results in hypoproteinaemia which is manifested as peripheral oedema, ascites and sometimes pleural or pericardial effusions. The causes of PLE are numerous but can be divided broadly into mucosal injury, either erosive or non-erosive, increased central venous pressure, mesenteric lymphatic obstruction or small intestinal bacterial overgrowth (SIBO), which was the cause in this case[1]. Abdominal hernia, internal or external, have been implicated in SIBO and PLE but only a very limited number of cases are reported in the literature[3,4]. The present case is the first reported of its kind, and the learning point lies on the diagnostic dilemma. Nevertheless systematic investigations helped localise the problem and FDG-PET scan was an invaluable diagnostic tool.

SIBO can result from a number of processes and may be considered as either functional or anatomical, and in some cases a combination of both. Functional causes include hypochlorhydria, dysmotility and some immunodeficiency syndromes[5]. Anatomical situations predisposing to SIBO include resection of the ileo-caecal valve or gastro-colic and jejuno-colic fistulae in which colonic bacteria can translocate to the small bowel, or in situations where a surgical "blind loop" is created by an end to side or roux-en-y anastomosis or as part of a Billroth Ⅱ gastrectomy[5,6]. A small bowel loop in an incarcerated hernia is similar to a functional blind loop in that patients often do not present with features of mechanical bowel obstruction and the classic features of

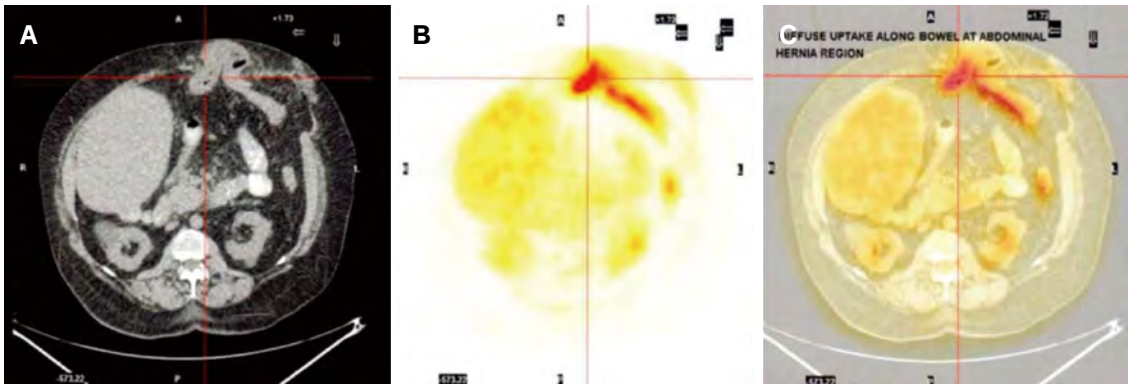

Figure 1 Computed tomography scan (A), flurodeoxyglucose positron emmision tomography (B) and combined computed tomography positron emission tomography image (C) across the site of incisional hernia. A: Incarcerated hernia with a loop of bowel; B: Confirms "hot spot" with increased flurodeoxyglucose uptake which corresponds with the centre of the incarcerated bowel loop within the hernia with some activity along the efferent loop distal to the hernia.

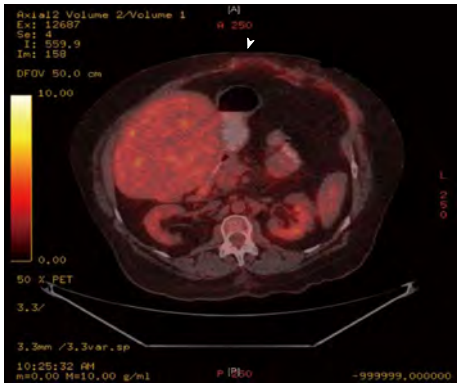

Figure 2 Computed tomography positron emission tomography image **12 mo following the incisional hernia repair.** The arrow heads points to the previous hernia site now repaired anatomically and the "hot spot" has now disappeared.

intestinal obstruction are not usually present. Decreased motility of the incarcerated segment however provides the home for bacterial overgrowth due to the loss of normal mechanical clearing action usually present in the bowel. Initially an overgrowth of intestinal type bacteria occurs but with longstanding functional obstruction colonic bacteria also may translocate to the site of the obstructed bowel[7]. The exact mechanism of protein loss in SIBO is by way of a combination of mechanisms including impaired digestion and absorption of proteins and increased secretion of protein rich fluid into the intestinal loop as a result of chronic ongoing mucosal inflammation[8]. It has been proposed that the immune system plays a role in the regulation of intestinal flora[9], and the induced immunosuppressed state in this patient may have been contributory however there is no documented evidence to prove this theory in circumstances such as these.

In summary, this is an extremely rare presentation of protein-losing enteropathy as a result of an incisional hernia following liver transplantation. Incisional hernia is a common surgical complication following liver transplantation, the incidence of which increases further with re-transplantation[10]. Impaired wound healing and loss of muscle mass in the immunosuppressed patient transplanted for end stage liver disease account for this higher incidence of incisional herniae, and the majority of these are managed conservatively if they remain asymptomatic or further surgery is contraindicated[11,12]. Bacterial overgrowth in an incarcerated segment of bowel leading to blind loop syndrome and hypoalbuminaemia may mimic some features of late liver graft failure and should be considered in such situations.

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CASE REPORT

Coincidence of active Crohn's disease and florid endometriosis in the terminal ileum: A case report

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Abstract

Crohn's disease (CD), a variant of chronic inflammatory bowel disease, frequently affects the terminal ileum and coecal region. The clinical symptoms are often subtle and depend on the inflammatory activity of disease. In women of child-bearing age, florid intestinal endometriosis can simulate CD. Moreover, current pathophysiological concepts include intestinal endometriosis as a putative founder lesion for consecutive CD establishment. The report summarizes clinical and histomorphological data of a 35-year-old woman with the rare coincidence of florid intestinal endometriosis and CD both affecting the terminal ileum. The patient was suffering over 10 years from strong abdominal disorders including constipation, diarrhea, weight loss, and diffuse abdominal pain. In magnetic resonance imaging-Sellink, strong inflammation and intestinal obstruction of the terminal ileum were found. The laparoscopy revealed further evidence for existence of an inflammatory disease like CD, but brownish spots on the peritoneum were found indicative for endometriosis. Surgical resection of the terminal ileum and the coecal segment was performed followed by histopathological investigations. In transmural sections of the terminal ileum, histomorphological features of florid endometriosis intermingled with florid CD was found. The diagnostic findings were substantiated with a panel of immunohistological stainings. In conclusion, the findings demonstrate that florid endometriosis persists in florid CD lesions and the putative link between intestinal endometriosis and CD is more complex than previously assumed.

Key words: Crohn's diseases; Intestinal endometriosis; Pathogenesis

Core tip: A 35-year-old woman with the rare constellation of a strong mixture of florid intestinal endometriosis and Crohn's disease in the terminal ileum is presented. Our findings suggest that there exist a putative link between the pathogenesis of both entities, which is more complex than previously assumed.

Kaemmerer E, Westerkamp M, Kasperk R, Niepmann G, Scherer A, Gassler N. Coincidence of active Crohn's disease and florid endometriosis in the terminal ileum: A case report. *World J Gastroenterol* 2013; 19(27): 4413-4417 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i27/4413.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i27.4413

INTRODUCTION

Crohn's disease (CD), a variant of chronic inflammatory bowel disease, is of unknown aetiology. Changes of the intestinal mucosal barrier are considered to play a role in

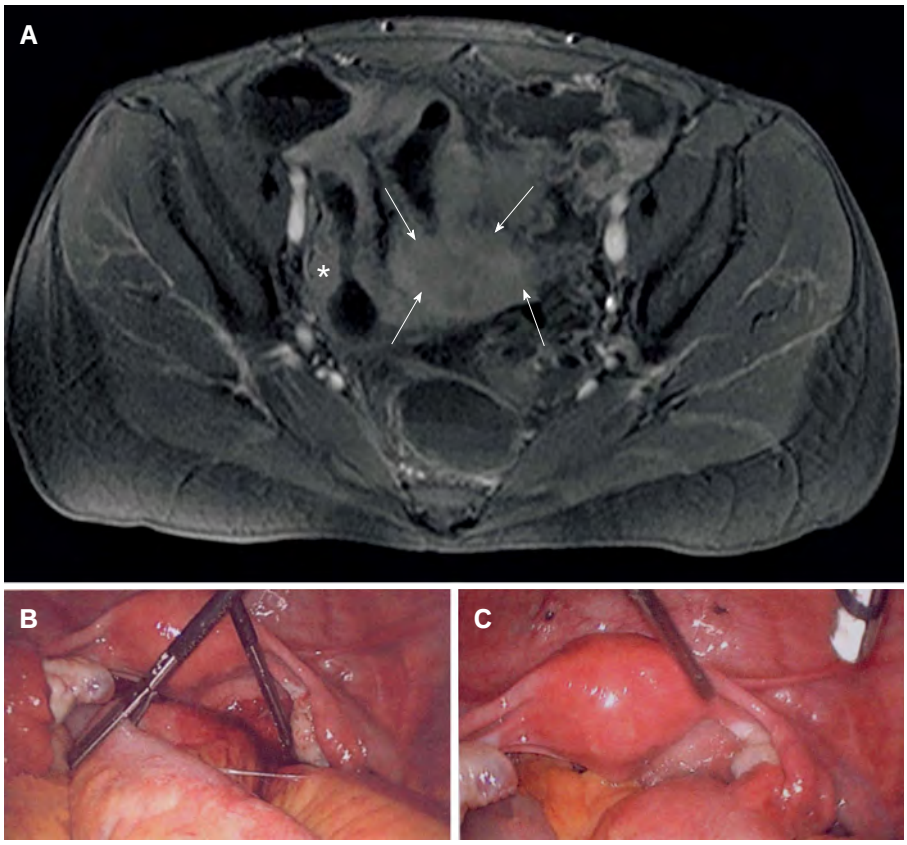

Figure 1 Radiological and surgical aspects of florid intestinal endometriosis and Crohn's disease coincidence. A: Magnetic resonance imaging-Sellink of the lower pelvis (axial T1 FS plus contrast agent). Bauhin's valve and coecum are marked with a asterisk. The arrows highlight an uncharacteristic tissue mass involving the bowel; B: Laparoscopically, adhesion of the distal small bowel to the pelvis wall; C: Brownish spots on the pelvis and bladder peritoneum are indicating for endometriosis.

the pathogenesis. Its incidence peaks in the 2nd and 3rd decades of life. CD most commonly affects the terminal ileum and coecal region, but may be found in any segment of the gastrointestinal tract. Clinical signs and symptoms of CD are often subtle and depend on the location and severity of the gastrointestinal involvement as well as inflammatory activity. CD related symptoms may include abdominal pain, severe diarrhea (sometimes with blood), weight loss and even malnutrition[1]. Growth retardation may occur in pediatric patients[2]. Other important symptoms which could be associated with CD are intestinal obstruction, perirectal or perianal abscesses, perforation and fistulas[3]. Because a plethora of non characteristic symptoms can be found in CD, a large list of differential diagnosis exists.

When the terminal ileum is affected in a woman of child-bearing age, endometriosis should be included in the differential diagnosis because this entity can simulate CD not only clinically[4]. Endometriosis is characterized by proliferation of endometrial tissue at extrauterine sites including ovaries, peritoneum and the intestinal tract. At present, the etiology of endometriosis is not fully understood. Its incidence peaks in the 3rd decade of life. About 5%-10% of women in menstruating age are affected[5]. Importantly, endometriosis affecting the intestinal tract is found in 3%-37% of women. Rectosigmoid, proximal/middle colon, appendix, and ileum are the most commonly affected sites. Similar to CD, the symptoms in intestinal endometriosis are not characteristic and include abdominal pain, (bloody) diarrhea, constipation, and segmental inflammation[6].

We present here the rare case of coincidence of active CD and florid endometriosis both affecting the terminal ileum of a 35-year-old woman.

CASE REPORT

The patient, a 35-year-old woman, was admitted to our surgical department first in August 2012. She was suffering from strong abdominal disorders over 10 years with constipation, diarrhea, and diffuse abdominal pain resulting in impaired nutrition and weight loss. The abdominal disorders were not related to the period, and dysmenorrhea was not given. Manifestation of CD was suggested years ago but not further substantiated or medical treated. The patient had a 12-year-old daughter, negative family history for chronic inflammatory bowel diseases and cancer. The last coloscopy in July 2012 showed a swelling of the intestinal mucosa of the ileocecal region. In biopsies, uncharacteristic moderate inflammation was found.

In order to clarify the obscure abdominal disorder, magnetic resonance imaging-Sellink was performed. Using this technique, strong inflammation and intestinal obstruction affecting a long part of the distal small intestinal bowel, Bauhin's valve, and coecum were found. In the lower pelvis, an uncharacteristic mass of tissue involving the bowel was additionally visible (Figure 1A). All results so far were unspecific but lead us to the estimated diagnosis of CD with severe intestinal obstruction and tumour like transformation. So we decided to perform laparoscopic intervention.

The laparoscopy revealed an inflammatory disease

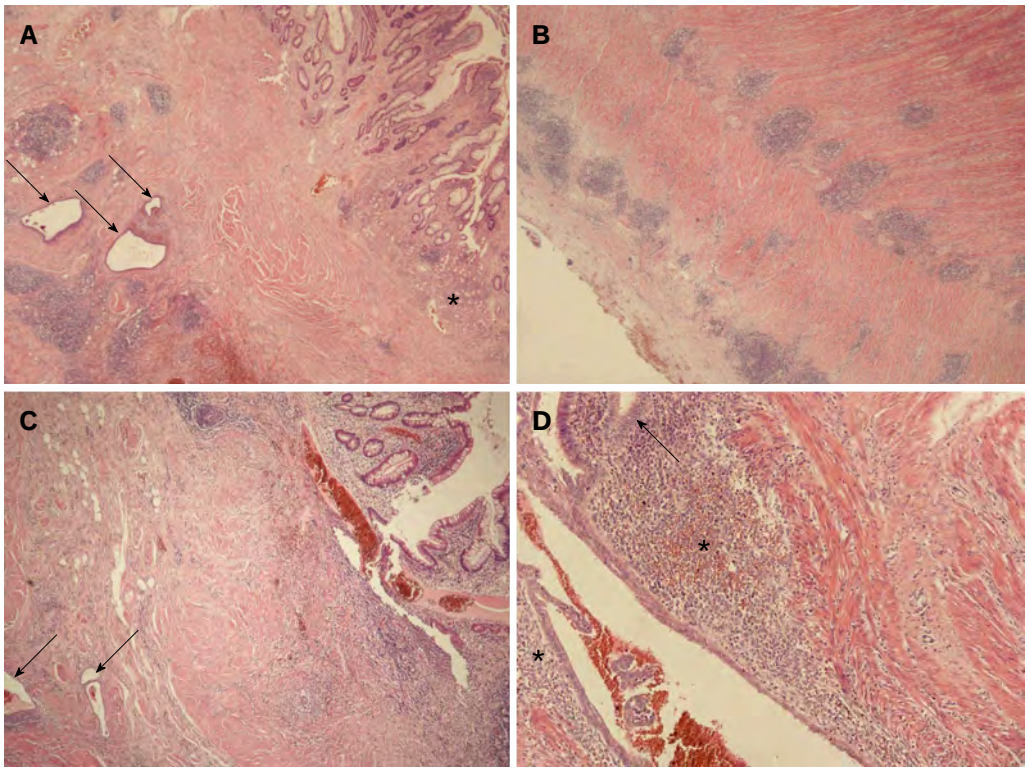

Figure 2 Coincidence of florid intestinal endometriosis and Crohn's disease in small intestinal wall. A: Complex inflammatory mucosal injury with establishment of pyloric gland metaplasia (asterisk). In deeper layers severe lymphoid aggregates (arrows) accompanied by endometrial glands exist (HE, × 10); B: Strong lymphoid hyperplasia partially affecting the prominent neuronal plexus, and serositis (HE, × 10); C: Florid aphthous mucosal lesion (HE, × 10), the arrows indicate endometriosis. D: Higher magnification of intestinal endometriosis(HE, × 100). Examples of epithelial proliferates are marked by an arrow. The cytogenic stroma is highlighted by asterisks. Note the intraglandular aggregates of erythrocytes.

affecting the distal small bowel with strong tumour like adhesion to the pelvis, but inconspicuous uterus and adnexa (Figure 1B). In addition, some brownish spots on the pelvis and bladder peritoneum were found that macroscopically appeared as endometriosis (Figure 1C). By laparoscopical intervention, it was impossible to free the intestinal segment. Consequently, a longitudinal laparotomie was performed. The tumour like inflamed small intestinal segment and the ileocoecal portion were resected. Both ureters were not affected by the inflammation. Following a gynaecological council and the fact that intraoperative frozen sections of the tumour-like lesion in the spatium rectovaginale did not demonstrate endometriosis, the inflamed mass in the lower pelvis was not resected. It was taken into account that resection of the tumour-like lesion, probably an additional manifestation of CD, was impossible without anterior rectum resection.

In the follow-up, the patient recovered quickly with one day observation on the intensive care unit. With the fast track concept the patient was quickly activated and within 4 d nutrition was increased to normal. Intestinal anastomosis (ileo-ascendostomia) and all wounds healed primarily and without any complications. Using anti-inflammatory and antibiotic therapies, the inflamed mass in the lower pelvis was diminished. The patient is now incorporated in our follow-up care system and will be systematically re-evaluated.

Pathologic studies and findings

The resected specimen was fixed in 10% formaldehyde for about 24 h followed by tissue preparation. Representative tissues were embedded in paraffin and sectioned. Tissue sections were stained with HE, sometimes with PAS. In addition, an extensive panel of immunohistochemical routine stains were performed using the DAKO Autostainer Link 48 system and commercially available primary antibodies and detection systems (all DAKO Deutschland, Hamburg, Germany). The primary antibodies included CD45 (dilution 1:2000), CD10 (dilution 1:50), K7 (dilution 1:1000), K20 (dilution 1:200), CDX2 (dilution 1:50), and progesterone receptor (dilution 1:350). With the exception of CDX2, all antigens were uncovered with pre-treatment at pH 6.1 as recommended by the supplier. In order to detect the CDX2 antigen, tissues slides were pre-treated at pH 9.0.

The surgical specimen includes a 32 cm small intestine with a 6 cm coecal segment, 4.5 cm appendix, and 1.5 cm mesenteric fatty tissue. High grade stenosis of the small intestine with wall thickening, several aphthous lesions, and destruction of Bauhin's valve were found. Macro-sections revealed diffuse fibrosis of the intestinal wall and aspects of serositis.

Tissue sections of the small intestinal bowel wall demonstrated a mixture of two histomorphological patterns (Figure 2). At first, a multifocal erosive-ulcerative, aph-

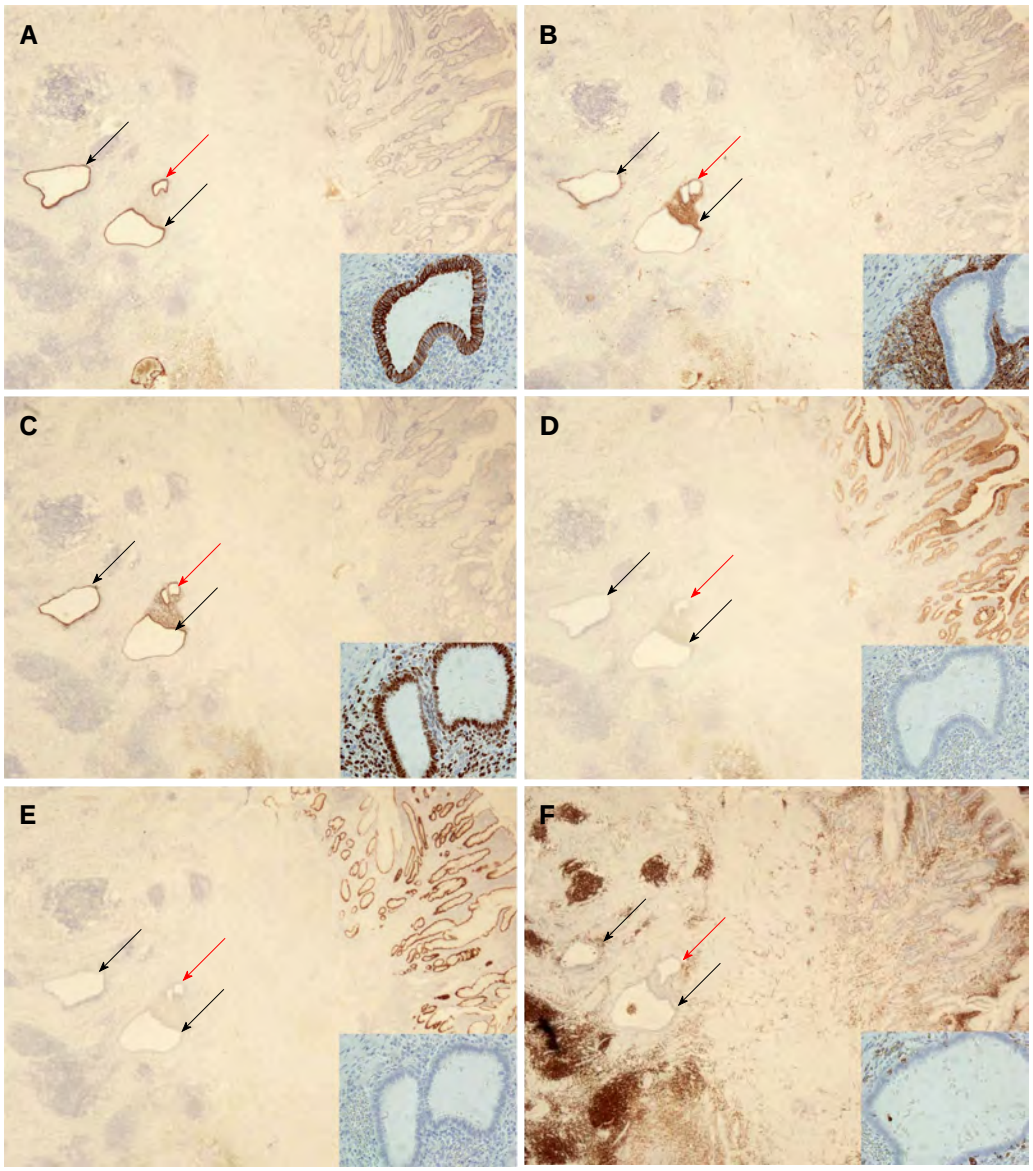

Figure 3 Immunohistochemical characterization of florid intestinal endometriosis and Crohn's disease. The intramural glandular structures (arrows in A-F) are positive for keratin 7 (A), CD10 (B), and progesterone receptor (C), but negative for keratin 20 (D) and CDX2 (E) which is vice versa to the mucosal staining; Strong anti-CD45 immunostaining illustrates severe lymphoid hyperplasia in CD (F). A-F: To illustrate immunostainings in detail always the intramural gland designated by a red arrow is shown in insets (original magnification: × 10; inset: × 250).

thous process with transmural lymphoid aggregates and chronic inflammation of serosal tissue layers was found. Moreover, pyloric gland metaplasia was frequently established in the mucosa layer. Despite missing granulomas, the morphological aspects were diagnostic for manifestation of CD (activity score Ⅲ). In addition to CD, a second histomorphological pattern was found. Several areas with gland forming epithelial cells adjacent to a cell rich matrix were distributed within the CD lesions, reflecting florid endometriosis in CD. The endometriosis glands were sometimes accompanied by few siderophages. The endometriosis was distributes throughout the small intestinal wall and preferentially found in the inner layer of the tunica muscularis. The basic histomorphological findings were further characterized by immunohistochemistry (Figure 3).

DISCUSSION

CD is an important variant of inflammatory bowel disease and it is assumed as a disorder with polyetiological background. Modern pathophysiological concepts include intestinal endometriosis as a putative risk factor to promote development of inflammatory bowel disease in particular CD. Following results from a Danish long-term study group in 2011 it is suggested that likelihood of CD in women with endometriosis is higher than in the control population[7]. In detail, the data reflects the situation that endometriosis gives a basis for the manifestation of CD and possible autoimmune triggered diseases even in the long term after the diagnosis of endometriosis. In this study the risk of CD manifestation was highest among women diagnosed with endometriosis below the

age of 25 years. The possible link between both diseases is substantiated by the following points: (1) both are inflammatory diseases; (2) the CD is preferentially found in women; and (3) both diseases are characterized by intermittent appearance. Common immunological features or an impact of endometriosis treatment with oral contraceptives on risk of CD must be also discussed as possible links[8,9]. In view with these studies, occurrence of florid CD mixed-up with florid small intestinal endometriosis as presented here is a rare constellation.

In contrast to CD, terminal ileal involvement from endometriosis is uncommon and occurs only in 1%-7% of all patients with intestinal endometriosis[10]. Patients with intestinal endometriosis of the terminal ileum are usually young nulliparous women who present with intermittent or persistent abdominal pain and possible obstruction. These features apply only partially to the case presented here.

The data presented here have some parallels to a Belgian case series[11]. In the Belgian study, clinical and histological findings from eight females with simultaneous histological diagnosis of intestinal endometriosis and CD were addressed. However, the strong mixture of active CD and florid intestinal endometrial deposits in terminal ileum tissues was not recorded. From this point of view, our case clearly demonstrates that both florid entities can mixed-up in the terminal ileum.

It could be speculated from our findings and the Belgian study that the sequence of events in manifestation of CD after endometriosis is high variable including persistence of intestinal endometriosis[11]. In addition, the strong mixture of the two histological patterns as demonstrated here raises the possibility that intestinal CD lesions are secondary involved by intestinal endometriosis. This hypothesis could be further substantiated by anamnestic data of our patient, where CD was assumed years ago, but (intestinal) endometriosis was never assumed.

Non-invasive diagnostic separation of intestinal endometriosis and CD raises several problems. This is due to the fact that both diseases may affect the bowel and may cause abdominal pain. They are associated with inflammation, tissue induration, wall thickening and structuring. In addition, both diseases have a known fairly long delay from onset to diagnosis. Immunological features of endometriosis, including serological alterations as raised cytokine levels, decreased cell apoptosis, B- and T-cell abnormalities are comparable to those seen in CD[12].

Finally, it has to be stressed that intestinal endometriosis and CD are important differential diagnoses in view of therapeutic procedures. Pharmaceutical therapies are completely different between intestinal endometriosis and CD. Concerning surgical intervention it should be stressed that intestinal segment resection could be therapeutic in intestinal endometriosis, whereas in CD patients resections are always symptomatic treatment.

Here we demonstrate the rare constellation of a strong mixture of florid intestinal endometriosis and CD in the terminal ileum of a 35-year-old patient. Our findings suggest that the putative link between the pathogenesis of both entities is more complex than previously assumed.

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CASE REPORT

Crohn's disease complicated by intestinal infection with methicillin-resistant *Staphylococcus aureus*

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Abstract

We report on a 24-year-old male patient with history of bloody diarrhea, abdominal pain and vomiting. Endoscopy revealed massive ulcerative discontinuous proctosigmoiditis with deep, sharply demarcated epithelial denudations and enterotoxigenic methicillin-resistant *Staphylococcus aureus* (MRSA) was detected in mucosal biopsies. After treatment with linezolide and steroids, a significant amelioration of colitis was detected and testing for MRSA became negative. In face of the case presented here, we suggest that in patients with refractory inflammatory bowel disease (IBD), microbiological assessment should be performed to detect a possible *Staphylococcus aureus* infection in order to initiate an antimicrobial treatment in addition to IBD-specific treatment.

Key words: Inflammatory bowel disease; Crohn's disease; Infectious colitis; *Staphylococcus aureus*; Methicillin-resistant *Staphylococcus aureus*

Core tip: The case presented here displays the complex situation of Crohn's disease aggravated by an intestinal bacterial infection, which is a commonly observed clinical scenario. However, the presence of enterotoxigenic methicillin-resistant *Staphylococcus aureus* (MRSA) in colonic mucosal biopsies is a very rare finding. Nevertheless, in face of the increasing prevalence of MRSA infections, clinicians should be aware of unusual opportunistic infections demanding a sophisticated antimicrobial screening and treatment to be combined with inflammatory bowel disease - specific medical therapy.

Bettenworth D, Nowacki TM, Friedrich A, Becker K, Wessling J, Heidemann J. Crohn's disease complicated by intestinal infection with methicillin-resistant *Staphylococcus aureus*. *World J Gastroenterol* 2013; 19(27): 4418-4421 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i27/4418.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i27.4418

INTRODUCTION

Patients with inflammatory bowel disease (IBD) are hospitalized more frequently compared to the general population. In addition, regular use of antibiotics and immunomodulating drugs further increase the patients risk to acquire antimicrobial resistant organisms. Recently, Nguyen *et al*[1]. demonstrated a 1.4-fold increased prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) colonisation of hospitalized IBD patients as compared

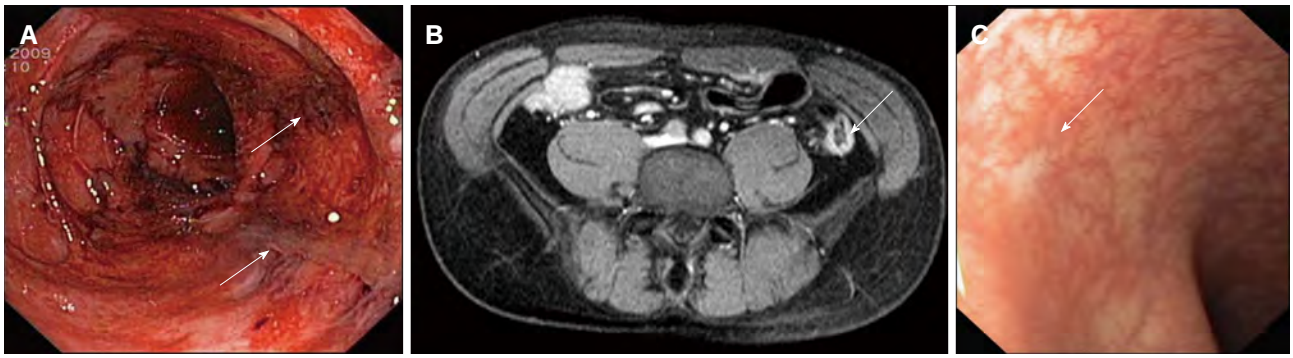

Figure 1 Massive colonic inflammation was detected by endoscopy and magnetic resonance imaging and ameliorated after antibiotic treatment. A: Sigmoidoscopy of the distal colon: Massive ulcerative proctosigmoiditis in a discontinuous pattern with deep and sharply delineated epithelial denudations (arrows) could be detected; B: Magnetic resonance imaging of the abdomen: contrast enhancement of the colonic wall in the area of the descending colon indicated inflammatory changes (arrow); C: Flexible sigmoidoscopy after ten days of antibiotic therapy with linezolid: Macroscopically, a near-total mucosal healing of colitis was detectable (arrow).

to general medical patients. This was associated with a seven-fold relative increase in in-hospital mortality. However, the clinical impact of intestinal MRSA infection on the course of IBD still remains unclear.

CASE REPORT

We report on a 24-year-old male Caucasian pig farmer who was transferred to our hospital with history of bloody diarrhea, abdominal cramping and vomiting for a 3-mo period. The preliminary diagnosis in the referring hospital was Crohn's disease (CD). However, the patient had not received neither immunosuppressive treatment nor steroid medication when he was admitted to our department. Physical examination provided no evidence of tenderness or a pathological abdominal mass. There were no signs of suspicious peripheral lymph nodes. Laboratory findings showed elevated values of inflammatory parameters, including elevated CRP (5 mg/dL) and leukocyte count (13.000/μL) as well as marked anemia (hemoglobin, 7.6 g/dL). Blood cultures for bacteria and polymerase chain reaction (PCR) results for cytomegalovirus (CMV), adenovirus, epstein-barr virus, herpes simplex virus type (HSV)-1, HSV-2 and varicella zoster virus were negative. Ultrasonographic examination of the abdomen revealed a thickened wall of the terminal ileum. On our ward, the patient collapsed due to anemia caused by rectal bleeding requiring blood transfusion. Esophago-gastro-duodenoscopy detected gastritis and a duodenal ulcer without evidence for *Helicobacter pylori* growth. Histological examination was without any signs of specific inflammation. To evaluate the severity of colonic inflammation, sigmoidoscopy was performed, revealing massive ulcerative proctosigmoiditis in a discontinuous pattern with deep, sharply delineated epithelial denudations (Figure 1A). Biopsies showed a massive infiltrate of inflammatory cells in the mucosa as well as in the submucosa resembling acute Crohn's colitis, however, granuloma formation was absent. CMV antigen and RNA was undetectable in mucosal biopsies. Furthermore, stool examination was negative for *Clostridium difficile*, *Salmonella spp.*, *Shigella spp.*, *Campylobacter spp.* and *Yersinia enterocolitica* as well as for helminth eggs and protozoan parasites, including *Giardia lamblia*. Therefore, the patient initially did not receive antimicrobial treatment. Magnetic resonance imaging of the abdomen showed inflammatory changes, predominantly in the area of the descending colon and the left colonic flexure (Figure 1B). Finally, microbiological assessment of four mucosal biopsies indicated growth of MRSA in all biopsies obtained. The MRSA was found to belong to *spa*-type t003. The strain was tested PCR-positive for *sed* encoding the staphylococcal enterotoxin D. Other pyrogenic toxin superantigen genes (*tst*, *sea*, *seb*, *sec* and *see*) as well as the exfoliative (epidermolytic) toxin encoding genes (*eta* and *etb*) and the genes encoding Panton-Valentine leukocidin (PVL) were tested negative applying sets of multiplex PCRs as previously described[2,3]. Remarkably, MRSA was found solely in perianal skin swabs, but not in swabs obtained from nostrils, scalp, axilla, and groin. We decided to commence antibiotic treatment with linezolid (600 mg *iv* bid). Additionally, steroid therapy with 100 mg prednisolone daily *iv* was continued. Furthermore, a decolonization therapy for MRSA was performed. Within days, the patient's clinical condition improved. After 10 d, a further sigmoidoscopy was performed revealing near-total mucosal healing of colitis. Histologically, moderate inflammatory infiltrations were found to be remaining. Diagnostic follow-up was conducted one month later. Meanwhile, the clinical symptoms had improved significantly. Abdominal pain and diarrhea were no longer present. Blood test results for blood count and CRP were normal. Esophago-gastro-duodenoscopy provided neither macroscopical nor histological evidence of inflammation. The duodenal ulcer was no longer detectable. Complete ileo-colonoscopy was performed displaying discrete pancolitis with mucosal friability and reduced vascular pattern. In the distal colon, multiple pseudopolypoid lesions were detectable, along with fibrin-coated ulcers as a correlate of inflammatory changes. The histological examination of colonic biopsies was again indicative of discrete discontinuous colitis resembling findings typical of CD. Microbiologic testing of

mucosal specimens and all skin swabs (including perianal) was now negative for MRSA. Again, no sign of CMV infection was found. Consecutively, the steroid medication was tapered.

Six weeks later, follow-up flexible sigmoidoscopy was performed. No acute inflammatory changes were detectable (Figure 1C). Histologically, mild signs of acute and chronic inflammation with interspersed crypt abscesses were found. At this time, the patient was on 5 mg prednisolone daily and had no complaints. Repeat MR enteroclysis was without signs of small intestinal IBD. Endoscopic follow-up 3 mo later again was showing complete mucosal healing. Histology was indicative of changes in the mucosa as well as a small hyperplasic polyp of the rectosigmoid colon. No signs of an acute flare-up occurred in a follow-up of further 12 mo. In addition, MRSA rescreening by nasal swabs applying polymerase chain reaction was performed and yielded negative results.

DISCUSSION

Staphylococcus aureus (*S. aureus*) is a leading cause of human bacterial infections worldwide aggravated by the continuing threat of multi-resistant strains as represented by the different clonal lineages of MRSA. It is estimated that 30% of healthy individuals are colonised with commensal *S. aureus* in their anterior nares that were found to represent the major source and an independent risk factor for subsequent nosocomial infections[4]. Inadequate antibiotic prescribing and poor adherence to infection control guidelines are the two main reasons for the development and spread of MRSA[5]. While the percentage of MRSA among *S. aureus* clinical isolates is believed to be 35%-70% in the United States[6], pan-European surveillance data on bloodstream infections showed marked variability in the proportion of MRSA ranging from less than 1% to more than 50%[7].

In the context of IBD, the presence of opportunistic infections and infections is of special interest, since infections were found to trigger an acute disease flare[8]. Moreover, the occurrence of opportunistic infections in patients with IBD has become a key safety issue especially with widespread use of immunosuppressive and immunomodulatory drugs[9]. Recently, it was shown that the use of any anti-inflammatory drugs such as corticosteroids, thiopurines, and anti-tumor necrosis factor alpha agents (*e.g.*, infliximab) is associated significantly with an increased risk of opportunistic infection in IBD patients[10]. In addition, the number of immunosuppressive agents combined appears to determine the individual risk of opportunistic infections[10].

Since the course of IBD is often chronically relapsing, IBD patients are frequently hospitalized and, thus, are at higher risk of hospital-associated infections[1]. Dysregulated barrier function of the intestinal surface epithelial lining is believed to represent a key factor for mucosal bacterial invasion. In active IBD, a disrupted epithelial barrier could therefore predispose for colonic *S. aureus* infections. In literature, only few reports on the possible correlation between IBD and MRSA infection are reported. One report published by Ishiyama *et al*[11] described an MRSA-associated diarrhea with positive stool cultures in patients after colorectal surgery. Recently, a first systematic analysis regarding this topic was performed by Nguyen *et al*[1]. It could be demonstrated that hospitalized IBD patients are at increased risk for MRSA infection as compared to non IBD-gastrointestinal and general medicine patients. Moreover, Nguyen *et al*[1] showed that the presence of MRSA is associated with seven-fold increased in-hospital mortality in the cohort of IBD patients. Further recognized risk factors for MRSA infection include bowel surgery, parenteral nutrition, and long hospitalization.

In our patient, the severe onset of the first acute flare of CD is striking and unusual. Endoscopically and histologically, massive inflammatory changes were present which resulted in relevant GI bleeding with anemia and hypotension. Furthermore, it is remarkable that microbiological testing for MRSA was completely negative except in specimens from colonic mucosa and perianal skin swabs. Lu *et al*[12] showed that *S. aureus* enterotoxins B might be associated with acute inflammatory response in mice. The detection of the enterotoxin D encoding gene in the patient's MRSA isolate makes a toxin-associated trigger of colitis plausible. It is likely that the presence of MRSA in our patient was no simple coincidence but a factor further worsening the disease course. This hypothesis is substantiated by the observation that the treatment with MRSA active antibiotic was accompanied by a rapid amelioration of colitis with mucosal healing.

Although the patient is working as a pig farmer, the MRSA subtype found in this patient belongs to a typical epidemic hospital-acquired MRSA clone and does not belong to the known livestock-associated clonal lineages (CC011/ST398). The relationship to the pig farming seems not to be epidemiologically relevant in this case and an earlier contact to healthcare seems more plausible as a source of colonization. In IBD patients, the risk of severe disease course may be increased. Yet, future studies are needed to define the putative connection between *S. aureus* and the course of IBD, the influence of the production of staphylococcal enterotoxins on IBD course, and if co-infection with (enterotoxigenic) *S. aureus* may be a predictor for severe disease course in IBD patients.

In case of refractory courses of IBD, microbiological assessment should be considered to rule out opportunistic *S.aureus* infection in order to initiate an antimicrobial treatment in addition to the IBD-related treatment.

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CASE REPORT

Rupture of a hepatic adenoma in a young woman after an abdominal trauma: A case report

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Abstract

Unlike hepatic haemorrhage following blunt abdominal trauma, spontaneous abdomen bleeding is rare, even in the presence of a hepatocellular adenoma (HA) or carcinoma. However, the diagnosis of a tumour underlying a haematoma after liver trauma is unusual, especially when it occurs more after two years after the accident. Here, we report a case of a ruptured HA due to blunt abdominal trauma. A 36-year-old woman was admitted to our hospital with sudden onset of upper abdominal pain. Her medical history revealed a blunt abdominal trauma two years prior. Initial abdominal computed tomography scan revealed a large haematoma measuring more than 16 cm in diameter in the right lobe of the liver. Magnetic resonance imaging showed haemorrhagic areas and some regions with hepatocyte hyperplasia, suggesting HA. The patient underwent right hepatic lobectomy, and a histopathological examination confirmed a diagnosis of HA. In conclusion, it is important to consider that abdominal trauma may hide old, asymptomatic and not previously detected injuries, as in the case reported.

Key words: Hepatic adenoma; Treatment; Hemoperitoneum; Trauma; Computed tomography

Core tip: This paper clarifies that surgical liver diseases should be evaluated by experts at specialized centers. In addition, experts should pay attention to unusual situations as reported. Asymptomatic liver tumors are more common than imagined, even when presented underlying other acute disease, such as blunt trauma.

Cotta-Pereira RL, Valente LF, De Paula DG, Eiras-Araújo AL, Iglesias AC. Rupture of a hepatic adenoma in a young woman after an abdominal trauma: A case report. *World J Gastroenterol* 2013; 19(27): 4422-4426 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i27/4422.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i27.4422

INTRODUCTION

Hepatocellular adenoma (HA) is rare, benign lesion occasionally found in young women with a long-term history of oral contraceptive use[1-3]. However, there are other predisposing factors, such anabolic androgenic steroids (AAS) use[4], diabetes mellitus, beta-thalassemia and glycogen storage disease[5-7].

The majority of patients with HA are asymptomatic, but the occurrence of large and multiple adenomas

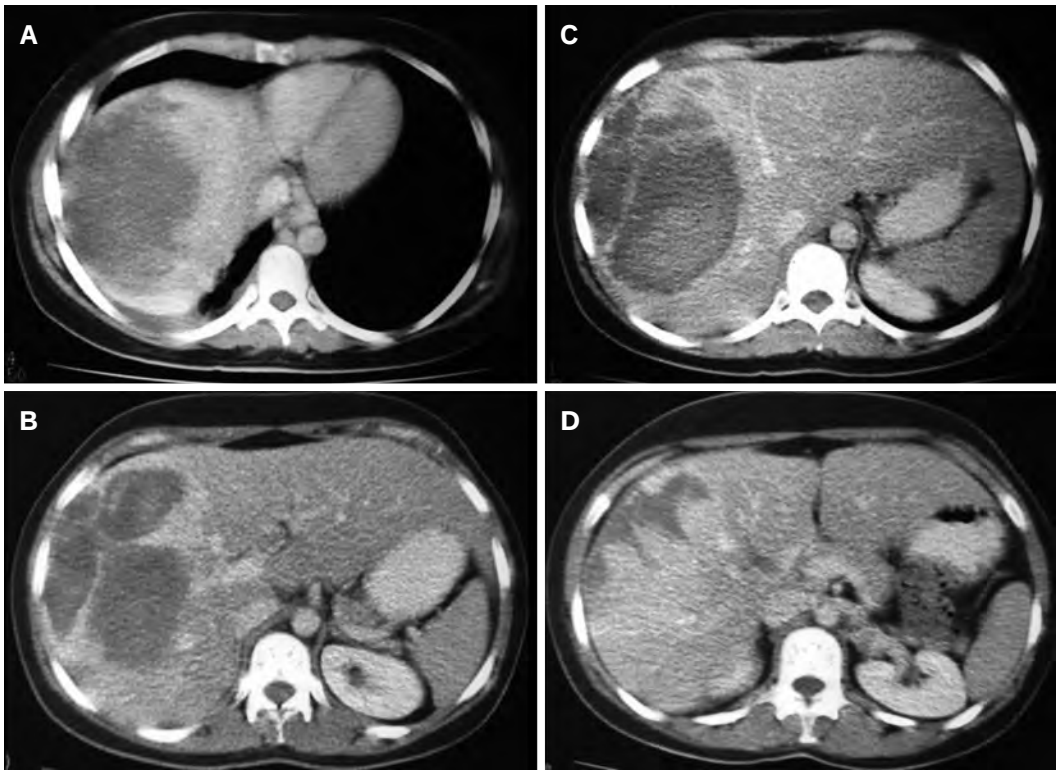

Figure 1 Unenhanced computed tomography scan showing liver injury with bleeding area, but also with areas of contrast enhancement. A-D: The existence of bleeding denotes a hepatic laceration, but the presence of vascularization reinforces the presence of a focal lesion.

is frequently associated with complications. The most important complications of HA are haemorrhage and malignant transformation into hepatocellular carcinoma (HCC), but the underlying pathophysiology is not fully known. Some data suggest that HA patients with beta-catenin mutations are more likely to undergo malignant transformation[8-10]. Symptomatic patients usually present with right upper quadrant pain secondary to HA bleeding, which can present as internal haemorrhage with necrotic changes (mostly observed in adenomas > 4 cm) or spontaneous rupture that causes subcapsular haematoma and possible haemoperitoneum[11].

In clinical practice, ultrasound (US), computed tomography (CT) and magnetic resonance imaging (MRI) are used to determine the diagnosis, but it is difficult to accurately distinguish between HA and other lesions, such as focal nodular hyperplasia (FNH). In such cases, a liver biopsy is sometimes necessary to establish a diagnosis[11,12]. It is important to emphasise that the presence of HA in trauma situations is quite rare[13].

CASE REPORT

A 36-year-old woman visited our hospital for evaluation of her abdominal discomfort and anaemia. Her past medical history revealed a fall down the stairs of her building two years prior. At that time, the patient was admitted to a tertiary hospital and underwent laboratory blood tests, US and CT (Figure 1). The diagnosis was haematoma after liver trauma, and a conservative treatment approach was proposed. No surgery or drainage was required. The patient was discharged after seven days and was referred for follow-up care. Two years after the accident, the patient had non-specific abdominal pain in the right hypochondrium and symptomatic anaemia. Upon physical examination of the patient at our hospital, tenderness in the upper right quadrant and a palpable mass were detected. CT revealed a large mass with areas of low attenuation in segments Ⅵ, Ⅶ and Ⅷ of the right lobe of the liver (Figure 2A). Laboratory exams revealed slight alterations of liver function (alanine aminotransferase: 132.30 IU/L, aspartate aminotransferase: 37.40 IU/L) and elevated alkaline phosphatase (20049 IU/L), but the levels of gamma-glutamyl transferase, total and fractionated bilirubin, cholinesterase, glycaemia and serum electrolytes were all within normal limits. The results of coagulation tests were entirely normal, as were the alpha-fetoprotein serum levels. Hepatitis virus markers, including hepatitis B and C, were negative.

A CT scan revealed a large, complex lesion in the right lobe of the liver with two distinct components. The upper component was moderately hyperdense with an attenuation coefficient of 52.4 UH and was not enhanced on a contrast-enhanced CT scan, suggesting liquefaction due to bleeding (Figure 2A and C). The lower component of the lesion was solid, markedly hypervascular and nourished via calibrous branches of the right hepatic artery (Figure 2B and D). There was no evidence of traumatic damage to the right thoracic-abdominal wall. The cutaneous, subcutaneous, muscular and osseous planes had

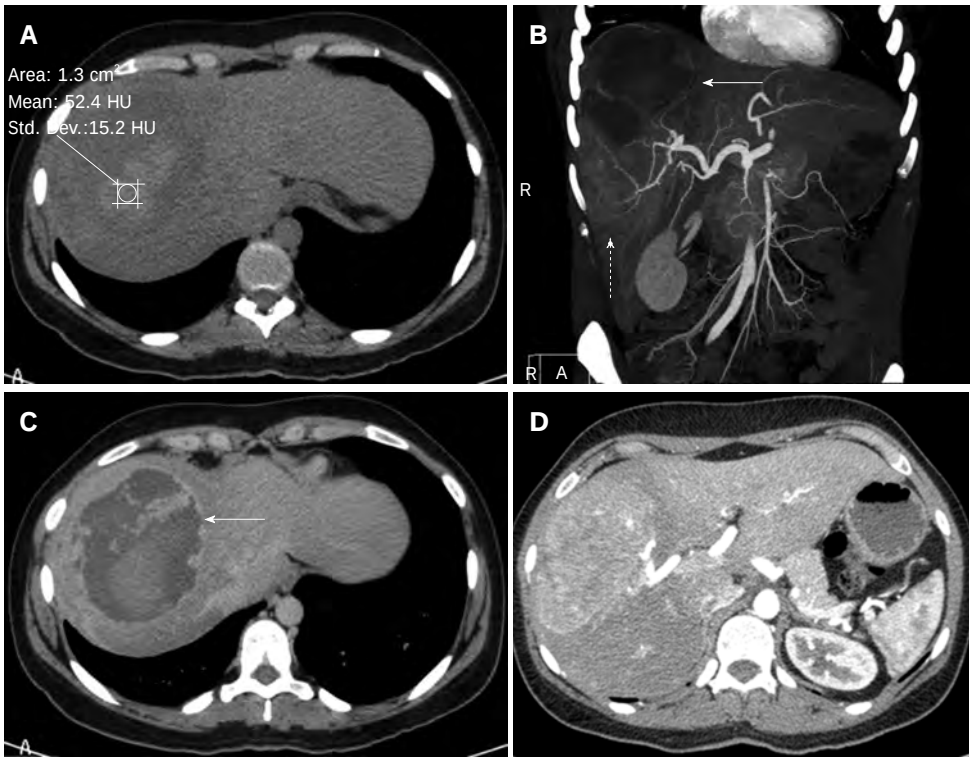

Figure 2 Computed tomography. A: Unenhanced computed tomography (CT) scan showing the upper component of the lesion with a relatively high density of 52.4 UH, suggestive of bleeding; B: Contrast-enhanced CT in the coronal plane showing both components of the lesion. The upper portion (arrow) has a low density due to the absence of impregnation. The lower component (dotted arrow) is solid and hypervascular with internal calibrous arteries; C: Contrast-enhanced CT. The haematic component illustrates a contrast-enhanced capsule (arrow); D: Contrast-enhanced CT. The study after contrast in the arterial phase at the level of the solid component exhibits an area with greater permeability than the liver, suggestive of hypervascularity.

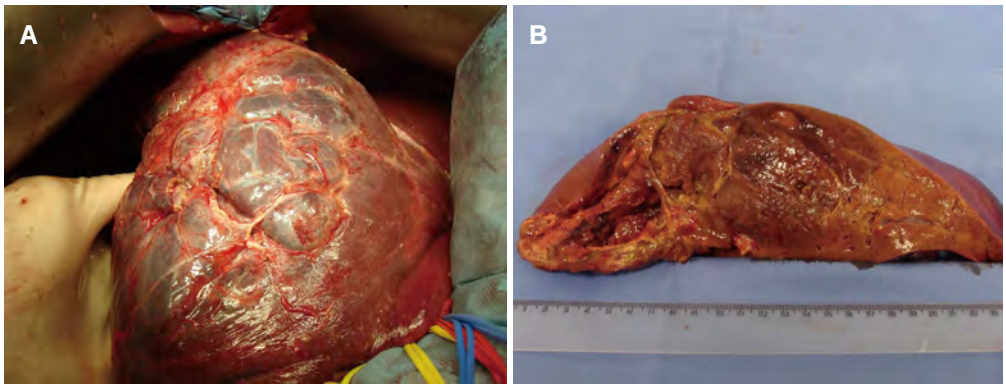

Figure 3 Entire right lobe of the liver. A: Intraoperative image of the lesion. Mobilised liver after ligament release, demonstrating the heterogeneous mass occupying almost the entire right lobe of the liver (segments Ⅵ, Ⅶ and Ⅷ), which was partially exophytic; B: Resected and sagittally cleaved surgical section at the transition between segments Ⅶ-Ⅵ and Ⅷ-Ⅴ. Note the adenomatous lesion with cavernomatous and haemorrhagic areas.

normal anatomy and densities. These features suggest the possibility of a pre-existing hepatic lesion associated with bleeding, which may or may not have been facilitated by the traumatic event. The CT images also showed signs of cirrhosis. These data, along with the age and sex of the patient, were suggestive of a diagnosis of bleeding HA.

We identified a heterogeneous mass with soft tissue density occupying almost the entire right lobe of the liver (Figure 3). The patient underwent right hepatectomy, and the surgical specimen was sent for pathological analysis. There were no postoperative complications, and the patient was discharged five days after surgery.

Histopathology examination revealed an HA with focal areas of haemorrhage (Figure 4). The lesion showed a heterogeneous pattern, indicating the existence of haemorrhagic areas in the tumour. The presence of a heterogeneous liver mass with internal haemorrhage and/or haemoperitoneum was suggestive of HA.

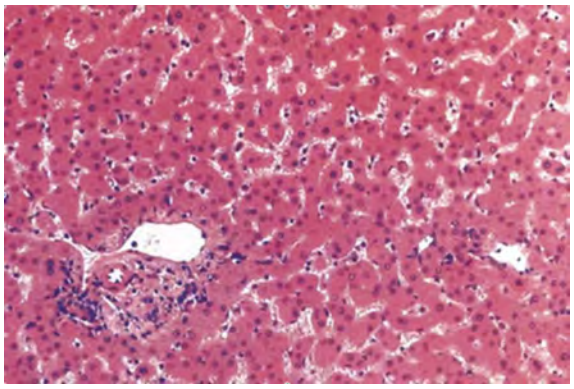

Figure 4 Histopathology showing the hepatocellular adenoma, a composed of cells that closely resemble normal hepatocytes, but in disorganized cords and with an abnormal lobular architecture (hematoxylin and eosin stain, × 200).

DISCUSSION

The treatment of blunt liver trauma has changed in the last several years, and conservative management is often prioritised over surgical treatment, even in patients with severe hepatic trauma[14,15]. The use of helical CT in the diagnosis and management of trauma injuries allows the option for nonsurgical treatments[15]. The American Association for the Surgery of Trauma (AAST) developed a CT-based liver injury grading system that allows physicians to select the best treatment option based on the imaging parameters[14-17]. Nonoperative treatment and detection of complications is based on clinical signs [*e.g.*, pain, meteorism, pulse, tension, urine, ventilation and biochemical tests (*e.g.*, haematocrit, haemoglobin and hepatic constants)]. This follow-up can take place in the intensive care unit and includes blood gas analysis and intra-abdominal pressure management[17]. Still, delayed laparotomies and late complications can occur, and the decreased rates of morbidity and mortality indicate that conservative options are better, even for patients with major liver trauma[14-19]. In our article, we point to an unusual event, which is the possibility of misdiagnosis following trauma.

Hepatic masses can exist prior to blunt abdominal trauma[20], and hepatic incidentalomas are not uncommon. As previously reported, the patient described herein already had a large adenoma when the trauma occurred, which resulted in rupture of the mass. Because she was seen at a centre that does not specialise in hepatic surgery, the correct diagnosis was not made, which could have been fatal[20]. The imaging diagnosis was a hepatic lesion (laceration/haematoma) due to trauma. Admittedly, adenomas may rupture spontaneously[13,18], and their propensity to haemorrhage is explained by their histological features.

Adenomas consist of large plates or cords of dilated sinusoids with poor connective tissue support. Classically, HA is a soft, well-demarcated tumour with little or no fibrous capsule and is composed of hepatocyte plates that are only mildly thickened and irregular. The tumour parenchyma is supplied by thin-walled arteries with minimal connective tissue[10-14,18]. Therefore, trauma to an adenoma most likely enhances their tendency to haemorrhage. Furthermore, it should be remembered that HA greater than 5 cm the indication for surgery should be given because of the high risk of tumor-related complications[21].

Currently, CT is the most frequently used modality in evaluating patients with liver injuries; it is a key element in the initial evaluation of haemodynamically stable patients who have suffered abdominal traumas[1-3]. Dramatic advances in multi-detector technology have significantly improved the accuracy of CT and dynamic contrast-enhanced CT, which can identify imaging characteristics of intrahepatic lesions, especially for rare changes caused by uncommon disease entities[1,4]. Emphasis should be laid on detecting the haemoperitoneum, then localising the source of bleeding and finally detecting the primary cause.

Spontaneous hepatic bleeding not related to trauma or anticoagulant therapy is a rare condition. This situation can be caused by underlying liver disease. Common causes are non-traumatic are HA or HCC. CT scans may be able to characterise and localise bleeding and to identify the underlying liver lesion[12]. Therefore, it is important to note that seemingly harmless blunt abdominal trauma may be very damaging when it is associated with an underlying hepatic lesion.

In conclusion, we note the importance of referring patients with liver trauma and possible hepatic disorders to specialised centres for the appropriate treatment.

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CASE REPORT

Intrahepatic cholangiocarcinoma diagnosed *via* endoscopic retrograde cholangiopancreatography with a short double-balloon enteroscope

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Abstract

Endoscopic retrograde cholangiopancreatography (ERCP) using a double-balloon enteroscope (DBE) in patients with bowel reconstruction due to a previous abdominal surgery is now widely accepted. In particular, a short DBE, which has a 2.8-mm working channel and 152-cm working length, is useful for ERCP because of its good rotational and straightening ability and the availability of various conventional ERCP accessories through the working channel. Herein we report a case of intrahepatic cholangiocarcinoma *via* ERCP with a short DBE. This is the first report in which the pre-cutting and the brush cytological examination were performed successfully under a DBE to diagnose intrahepatic cholangiocarcinoma pathologically. The short DBE allowed us to perform all diagnostic and therapeutic procedures accepted in conventional ERCP in patients with surgically altered anatomies.

Key words: Double-balloon enteroscope; Diagnosis; Endoscopic retrograde cholangiopancreatography; Intrahepatic cholangiocarcinoma; Cytology

Core tip: This is the first report in which the pathological diagnosis of intrahepatic cholangiocarcinoma could be made using the brush cytological examination *via* endoscopic retrograde cholangiopancreatography with a double-balloon enteroscope (DB-ERCP). In this paper, the methods of these procedures during DB-ERCP such as biliary cannulation, pre-cutting, and cytological examination are demonstrated in detail. Therefore, we believe that this paper must expand awareness of the utility of DB-ERCP for diagnosis of pancreatobiliary disease in patients with bowel reconstruction due to a previous abdominal surgery.

Ikeura T, Shimatani M, Takaoka M, Matsushita M, Miyoshi H, Kurishima A, Sumimoto K, Miyamoto S, Okazaki K. Intrahepatic cholangiocarcinoma diagnosed *via* endoscopic retrograde cholangiopancreatography with a short double-balloon enteroscope. *World J Gastroenterol* 2013; 19(27): 4427-4431 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i27/4427.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i27.4427

INTRODUCTION

Performing endoscopic retrograde cholangiopancreatography (ERCP) using a conventional duodenoscope in patients with bowel reconstruction due to a previous abdominal surgery is challenging because it is frequently

impossible to reach the papilla or the hepatico/choledochojejunal and pancreaticojejunal anastomosis owing to the insufficient scope length and unusual postsurgical conditions such as intestinal adhesions and anastomosis angulation. However, the use of a double-balloon enteroscope (DBE), which was originally developed for the management of small bowel diseases[1], has been shown to make it more feasible to perform ERCP in patients with bowel reconstruction[2-7]. Recently, ERCP using a DBE (DB-ERCP) is now widely accepted. In particular, a short DBE, which has a 2.8-mm working channel and 152-cm working length, is useful for ERCP because of its good rotational and straightening ability and the availability of various conventional ERCP accessories through the working channel. ERCP using a short DBE was reported to be useful and safe for diagnostic and therapeutic interventions such as sphincterotomy, stone extraction, and stent placement[8-11]. In this paper, we report a case of a post-surgical patient diagnosed as having intrahepatic cholangiocarcinoma *via* ERCP with a short DBE. We aimed to demonstrate the feasibility of successfully achieving access to the biliary duct by pre-cutting under a DBE and obtaining the pathological diagnosis by tissue sampling *via* DB-ERCP.

CASE REPORT

A 74-year-old man, who had undergone partial gastrectomy with gastrojejunostomy and Roux-en-Y reconstruction for gastric carcinoma five years before, presented to another hospital to evaluate a mass lesion in hepatic segment Ⅷ. A fluorine-18-fluorodeoxyglucose positron emission tomography scan revealed a pathological uptake pattern with a maximum standardized uptake value of 5.6 in the mass, suggestive of a malignant tumor. An ultrasound-guided percutaneous needle biopsy was performed to obtain a pathological diagnosis of the mass. However, the biopsy finding revealed no sign of malignancy. In addition, the patient developed biliary peritonitis after the liver biopsy and therefore required hospitalization and conservative treatment for approximately 1 mo. One month after discharge from the hospital, an ultrasound image revealed the mass lesion remained constant in size; however biliary stricture with upstream dilatation, which appeared to result from the mass lesion, was noted on magnetic resonance cholangiopancreatography (MRCP). Hence, he was referred to our hospital to undergo evaluation for malignancy by tissue sampling from the biliary stricture.

On admission, the patient was asymptomatic. The physical examination results were unremarkable. He had no history of alcohol abuse. The serum examination results indicated that the levels of the tumor markers and transaminase in the liver were within normal limits. T2-weighted magnetic resonance imaging showed a slightly hyperintense lesion around the dilated intrahepatic duct in segment Ⅷ (Figure 1A). On MRCP, a focal biliary stricture with upstream dilatation in the branch arising from the right anterior duct was observed (Figure 1B). The patient underwent ERCP with a short DBE (EC-450BI5; Fujifilm, Osaka, Japan) in the prone position with carbon dioxide (CO_2) insufflations. At first, a short DBE was carefully inserted into the blind loop and reached the papilla. Subsequently, standard biliary cannulation using a straight cannula (PR-10Q; Olympus, Tokyo, Japan) was attempted after the papilla was moved to the lower endoscopic field of view by keeping the overtube balloon inflated and rotating the enteroscope (Figure 2A), as described in a previous report[8]. However, we were unable to obtain cholangiography, but only pancreatography. Therefore, a 0.025-inch guide wire (Jagwire; Boston Scientific Japan, Tokyo, Japan) was inserted into the main pancreatic duct (Figure 3A), and the pre-cut technique was carried out with a wire-guided sphincterotome (Autotome RX, Boston Scientific, Japan). The incision was started toward the biliary direction and was stopped at the lower one-third of the ampullary mound (Figure 2B). After the incision, the orifice of the biliary duct became visible (Figure 2C), and selective biliary cannulation with a straight cannula was performed successfully (Figure 3B). After contrast enhancement of the biliary duct, a focal stricture in the branch of the right intrahepatic duct was identified under radiographic guidance (Figure 3C). A guide wire followed by a cannula was successfully passed through the stricture after several negotiations (Figure 3D). Subsequently, tissue sampling for cytological examination was performed at the level of the stricture using a brush device (Cytomax Ⅱ DLB 35-1.5; Cook, Osaka, Japan) introduced by a guide wire (Figure 3E). Finally, the nasobiliary drainage tube was placed at the distal side to collect the bile for cytological examination (Figure 3F). The day after the ERCP, a slight increase in the serum amylase level was observed, but the patient did not complain of abdominal pain. The result of the cytological examinations of the sample obtained by the brush and nasobiliary drainage tube was positive for malignancy. On the basis of the cytological finding, the patient underwent segmentectomy of the

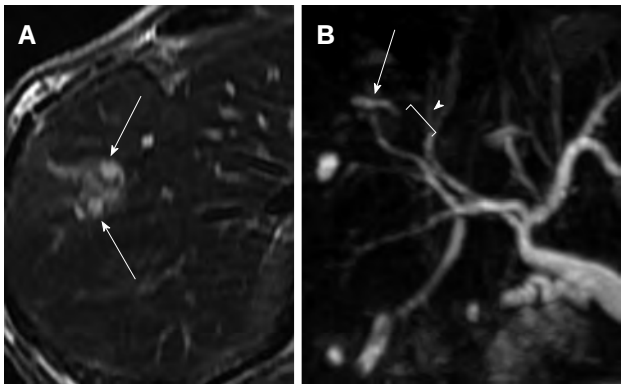

Figure 1 Magnetic resonance imaging. A: The T2-weighted magnetic resonance imaging shows a hyperintense lesion along the dilated intrahepatic duct (arrows); B: Magnetic resonance cholangiopancreatography shows a stricture of biliary duct (arrow) with the upstream dilatation (arrowhead).

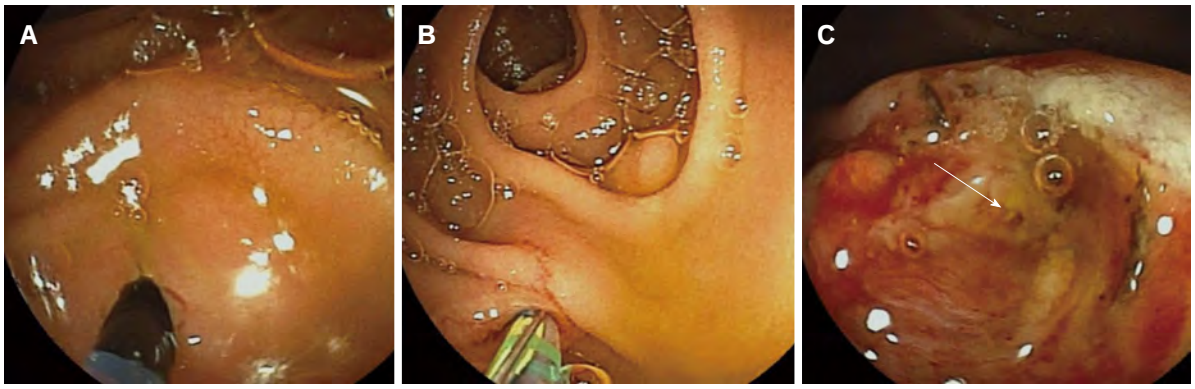

Figure 2 Endoscopic images during cannulation and transpancreatic sphincterotomy. A: Selective biliary cannulation was attempted in the position of the papilla moved to the lower endoscopic field of view; B: The wire-guided transpancreatic sphincterotomy was performed toward the biliary direction; C: The bile duct orifice was exposed after the transpancreatic sphincterotomy (arrow).

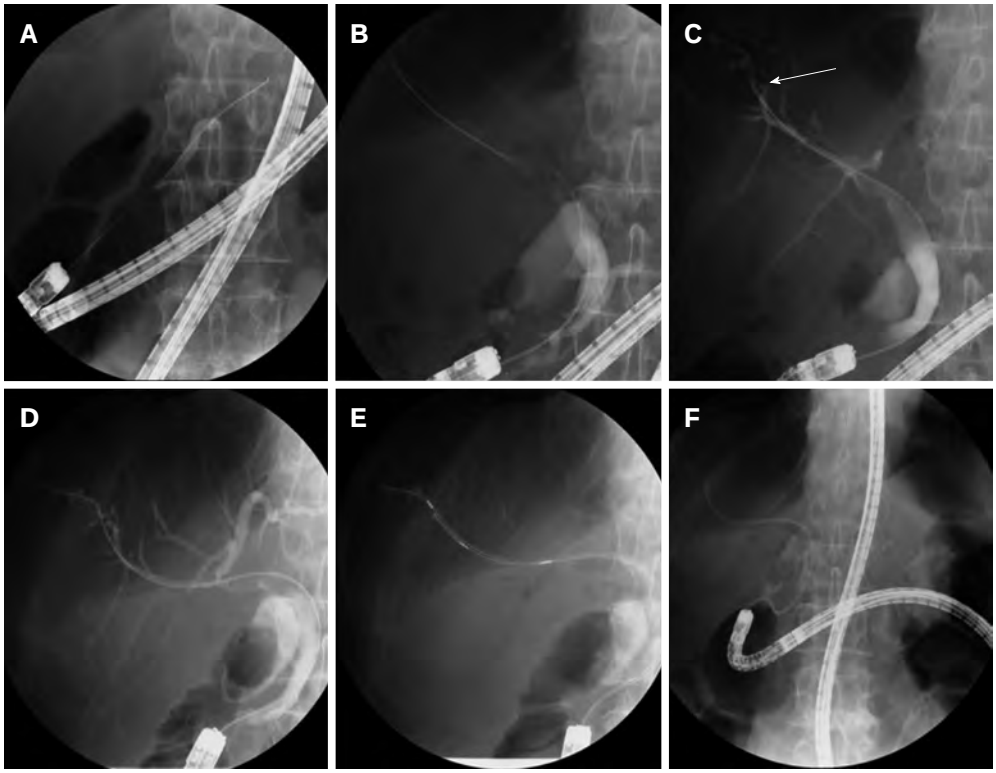

Figure 3 Endoscopic retrograde cholangiopancreatography. A: A guide wire was inserted deeply into the main pancreatic duct to perform the pre-cutting; B: After the pre-cutting, selective biliary cannulation was achieved; C: Cholangiography revealed a focal stricture in the branch of the right intrahepatic duct (arrow); D: A guide wire was passed through the biliary stricture; E: The brush cytological examination was carried out at the stricture; F: The placement of nasobiliary drainage tube was performed to collect the bile for cytology.

left lobe. The final pathological report indicated intrahepatic cholangiocarcinoma.

DISCUSSION

With the advent of DBEs, the endoscopic management of pancreaticobiliary diseases has become more feasible in patients with previous gastrointestinal surgery such as Billroth Ⅱ gastrojejunostomy, Roux-en-Y reconstruction, and Whipple's resection[2-6]. Accordingly, in these years, the number of published reports on the utility of DB-ERCP has been increasing. In most of these previous reports, a standard DBE (EN-450T5; Fujifilm)[2-6] and a single-balloon enteroscope[7] were used. However these enteroscopes have a limitation with regard to the availability of its accessories because of its 200-cm working length. In contrast, a short DBE has a 2.8-mm working channel and 152-cm working length, for which various diagnostic and therapeutic accessories used in conventional ERCP, including sphincterotome, balloon catheter, basket, biopsy forceps, brush, intraductal ultrasonic probe and biliary stent, are available without modification[8-11].

The use of a short DBE appears to not only widen the parameters of the procedures which we can perform in DB-ERCP, but also increase the success rate of the procedure owing to its good maneuverability. In fact, with a short DBE, we reported higher success rates in deep insertion [100/103 (97%)], cholangiography [98/100 (98%)], and therapeutic interventions [98/98 (100%)], compared with the previous reports[8].

Deep cannulation to the biliary duct is the most important step for successful ERCP. The failure rate for cannulation of the naive papilla in conventional ERCP is reported to be up to 10% of the ERCP procedures[12,13]. In general, this rate in DB-ERCP might be even higher because of the difficult location of the papilla and the instability of the manipulation of the enteroscope and cannula. In the case of a difficult biliary cannulation in conventional ERCP, the pre-cut techniques have been used as a widely accepted option to facilitate the biliary access[14,15]. The pre-cut techniques include needle knife sphincterotomy and wire assisted transpancreatic sphincterotomy. In contrast with conventional duodenoscopy, a DBE is forward viewing and has no elevator function, making the pre-cutting technically more challenging. From our experience, we recommend wire-guided transpancreatic sphincterotomy in performing the pre-cutting under a DBE, because the direction, depth, and length of incision are easier to control, compared with needle knife papillotomy performed freehand.

Although intrahepatic stricture frequently results from malignant disease such as cholangiocarcinoma and hepatocellular carcinoma, it is important to confirm the malignant cells histopathologically by the tissue sampling method, because an accurate diagnosis is crucial for the choice of an appropriate therapeutic strategy. In general, percutaneous needle biopsy is accepted for histological diagnosis when the mass causing the intrahepatic stricture can be detected on ultrasonography. However, the potential disadvantage of this technique is that complications such as pneumothorax, hemorrhage, biliary leakage, biliary peritonitis, and tumor seeding can occur[16,17]. In contrast, the transpapillary tissue sampling methods during ERCP include brush cytological examination, bile cytological examination, and biopsy. In particular, brush cytological examination is commonly used because it is relatively simple and safe. Although the specificity of this method for the diagnosis of cholangiocarcinoma is almost 100%, the sensitivity is by no means satisfactory, ranging from 44% to 81%[18]. In our institution, when brushing is performed, bile cytological examination is also performed *via* a nasobiliary drainage tube placed subsequently after brushing. We believe that such a combination of specimen collection methods enhances the cancer detection rate.

In conclusion, the short DBE allowed us to perform all diagnostic and therapeutic procedures accepted in conventional ERCP in patients with surgically altered anatomies.

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CASE REPORT

Hepatic adenoma mimicking a metastatic lesion on computed tomography-positron emission tomography scan

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Author contributions: Lim D and Lee SY wrote the manuscript and provided the images; Lim KH provided pathological expertise and the histological slide; Lee SY and Chan CY were involved in the management of the case and the critical review of the manuscript; all authors have read and approved the final manuscript.
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Abstract

Positron emission tomography (PET) using ^{18}F-fluorodeoxyglucose (^{18}F-FDG) is an imaging modality which reflects cellular glucose metabolism. Most malignant cells accumulate and trap ^{18}F-FDG, allowing the visualisation of increased uptake. It is hence widely used to differentiate malignant from benign lesions. "False positive" findings of hepatic lesions have been described in certain instances such as hepatic abscesses, but are rare in cases involving hepatocellular adenomas. To our knowledge, there have been only 7 reports in the English literature documenting PET-avid hepatocellular adenomas; 6 of the 7 reports were published in the last 3 years with the first report by Patel *et al*. We report the case of a 44-year-old Chinese female patient with a history of cervical adenocarcinoma, referred for a hepatic lesion noted on a surveillance computed tomography (CT) scan. A subsequent CT-PET performed showed a hypermetabolic lesion (standardized uptake value 7.9) in segment Ⅳb of the liver. After discussion at a multidisciplinary hepato-pancreato-biliary conference, the consensus was that of a metastatic lesion from her previous cervical adenocarcinoma, and a resection of the hepatic lesion was performed. Histology revealed features consistent with a hepatocyte nuclear factor-1 α inactivated steatotic hepatocellular adenoma.

Key words: Hepatic adenoma; Positron emission tomography; ^{18}F-fluorodeoxyglucose; Hepatocellular adenoma; Hepatocyte nuclear factor-1 α

Core tip: This case illustrates a unique example of a false-positive finding on the computed tomography-positron emission tomography (CT-PET) due to a hepatic adenoma mimicking a metastatic lesion in the liver. It serves to highlight that not all CT-PET findings are associated with malignancy. In equivocal cases where CT-PET findings are discordant with other imaging modalities and/or clinical/biochemical features, further evaluation with different imaging modalities or novel PET tracers may be considered. This is especially pertinent in this day and age, where the PET scan is widely used in clinical practise to differentiate benign from malignant lesions, as well as a modality in cancer surveillance and staging.

Lim D, Lee SY, Lim KH, Chan CY. Hepatic adenoma mimicking a metastatic lesion on computed tomography-positron emission tomography scan. *World J Gastroenterol* 2013; 19(27): 4432-4436 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i27/4432.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i27.4432

INTRODUCTION

Positron emission tomography (PET) using ^{18}F-fluorodeoxyglucose (^{18}F-FDG) is an imaging modality which reflects cellular glucose metabolism. In most malignant cells, the relatively low level of glucose-6-phosphate leads to accumulation and trapping of ^{18}F-FDG intracellularly, which in turns results in its visualization. As such, the PET scan is widely used for differentiating benign from malignant lesions, as well as a modality in cancer surveillance and staging. With regards to lesions in the liver, computed tomography (CT)-PET imaging classically shows increased uptake for moderately/poorly-differentiated hepatocellular carcinoma (HCC) and extra hepatic metastatic disease[1]. False-positive results have generally been described in hepatic abscesses, and false-negative results described in well-differentiated HCC[2].

Hepatocellular adenoma (HCA), also known as hepatic adenoma, is a benign tumor of the liver and the second most common benign liver neoplasm after focal nodular hyperplasia[3,4]. It occurs predominantly in women within their reproductive years with a male-to- female ratio of 1:8-10[5,6]. The estimated incidence is about 1 per million in women who have never used oral contraceptives (OCP), up to a significantly higher incidence of 30-40/million in long-term OCP users[3,4].

"False positive" findings in PET/CT are rare in HCA. To our knowledge, there have been only 7 reports in the English literature documenting PET-avid HCAs; 6 of the 7 reports were published in the last 3 years with the first report in 1997 by Patel *et al*[7-13].

CASE REPORT

A 44-year-old female patient, with a history of International Federation of Gynecology and Obstetrics stage IB2 cervical adenocarcinoma was referred to our hepato-pancreato-biliary (HPB) service for further management of a hepatic lesion noted on her surveillance CT scan (Figure 1). Her cervical adenocarcinoma was treated with a radical hysterectomy 6 mo prior to this presentation. The hepatic lesion measured 2.3 cm × 1.8 cm and was located in segment Ⅳb of the liver. A CT-PET was performed to further delineate the nature of the hepatic lesion and exclude extrahepatic metastases; the liver lesion appeared hypermetabolic with a standardized uptake value (SUV_{max}) of 7.9 (Figure 2). This was discussed at a multi-disciplinary HPB tumor conference and the consensus was that it was a solitary metastasis from the cervical adenocarcinoma without evidence of extrahepatic disease. In view of the patient's previous surgery and the need for a lymphadenectomy, a decision was made for open surgical resection of the liver lesion with a perihilar lymphadenectomy.

On presentation, the patient was asymptomatic and recovered well from her hysterectomy. Clinical examination was largely unremarkable. Laboratory results and tumor biomarkers were within normal ranges [liver function test, complete blood count, carcinoembryonic antigen, α-fetoprotein, carbohydrate antigen (CA) 19-9, CA125]. Her hepatitis B and C virology markers were negative. She had no family history of liver cancer or OCP usage.

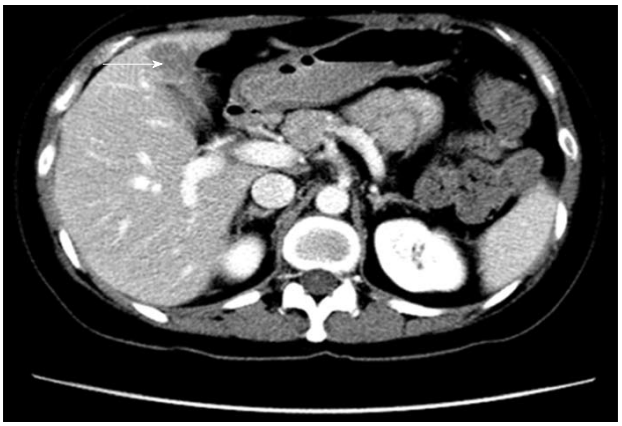

Figure 1 Surveillance computed tomography scan showing hypodense lesion in segment IVb of liver. Cross-sectional image of the hypodense lesion in segment IVb of the liver (arrow).

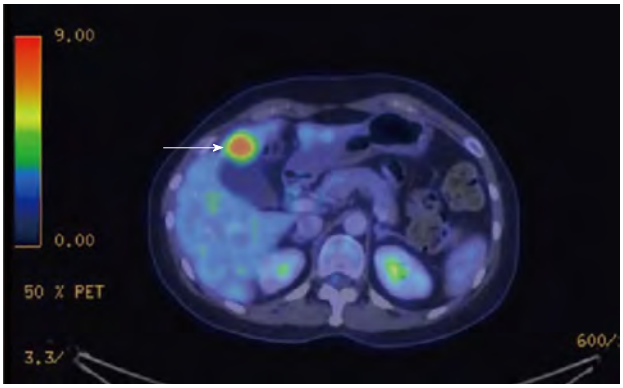

Figure 2 Computed tomography-positron emission tomography scan revealing the positron emission tomography avid lesion in segment Ⅳb of the liver. Cross-sectional image of the positron emission tomography avid lesion in segment Ⅳb of the liver (standardized uptake value 7.9) (arrow).

After exploration to exclude any peritoneal metastases, an open resection of this segment IVb hepatic lesion, cholecystectomy and perihilar lymphadenectomy was performed. A 3 cm × 2.5 cm segment IVb lesion was removed with clear margins. The patient recovered uneventfully.

Histology revealed a well demarcated lesion composed of bland uniform hepatocytes displaying clear vacuolated macrovesicular fat globules predominantly. No bile ducts, entrapped portal tracts or ecstatic vessels were identified. No central scar was seen. Reticulin staining showed normal trabeculae without expanded thickened areas. Glypican-3 was negative. Overall features were consistent with a hepatocyte nuclear factor-1 α (HNF-1α) steatotic adenoma (Figure 3). All lymph nodes excised were also negative for malignancy.

DISCUSSION

This case illustrates a unique example of a false-positive

finding on the CT-PET due to a HCA mimicking a metastatic lesion in the liver. Hepatic adenomas can be classified by their molecular phenotypes into four main groups: HNF-1α inactivation, β-catenin activating, inflammatory and unclassified[14,15]. The presence of the diffuse steatotic appearance and histological features in this case was consistent with a HNF-1α inactivated HCA. Radiological features of HNF-1α inactivated adenomas on Magnetic resonance imaging (MRI) include a diffuse signal dropout on T1-weighted chemical shift sequence, isosignal or slight hypersignal on T2-weighted images and moderate enhancement in the arterial phase, with no persistent enhancement in the portal venous and delayed phases. These appearances have been shown to have a high sensitivity and specificity for the diagnosis of steatotic adenomas[16,17].

There is an increasing importance in the role of FDG-PET in the evaluation of liver lesions to distinguish between benign and malignant. Its uses also include the surveillance, staging and monitoring of therapy for patients with cancer e.g. colorectal cancer. However, some of the limitations of PET/CT in evaluation of liver lesions are emerging[18,19]. The overall sensitivity of FDG PET/CT in detecting HCC is low with a reported range of 50%-65%, contributed mainly by the poor sensitivity in low-grade HCC[20,21].

The liver can have an unpredictable ^{18}F-FDG uptake[19]. Glucose-6-phosphatase enzyme and glucose transporter activity have a wide variation in HCC. As a further confounder, the liver is the major producer of non-dietary glucose and a major regulator of glucose homeostasis. This limitation of PET/CT in HCC handicaps the usefulness of PET/CT in the evaluation of HCA, as one of the major concerns of HCA is its malignant potential and it harboring a foci of HCC within[6].

This case highlights that not all "hot" hepatic lesions on the PET are associated with malignancy. Benign examples of high FDG activity in the liver include focal steatosis, hepatic adenomatosis (*e.g.*, inflammatory subtype) and infectious or inflammatory processes in the liver (*e.g.*, liver abscess, hepatic tuberculosis). These can consequently lead to "false-positive" PET results[10,19,22,23]. Due to the limitations of CT/PET as an imaging modality, it is not possible to distinguish a PET-avid HCA from a liver abscess or a hepatic tuberculoma solely from its features on CT/PET. Other information is required to make such a distinction, such as clinical history and laboratory investigations. For example, patients with liver abscess may have signs and symptoms of infection such as fever/chills and pain; liver function test may be mildly abnormal with leukocytosis and inflammatory makers such as C-reactive protein and erythrocyte sedimentation rate may be elevated. Patients with HCA will not have such signs/symptoms of infection but may have a history of OCP usage and tend to be females in the reproductive age group. There is no evidence in the literature to suggest that CT/PET alone can distinguish PET-avid HCA from a liver abscess/tuberculoma.

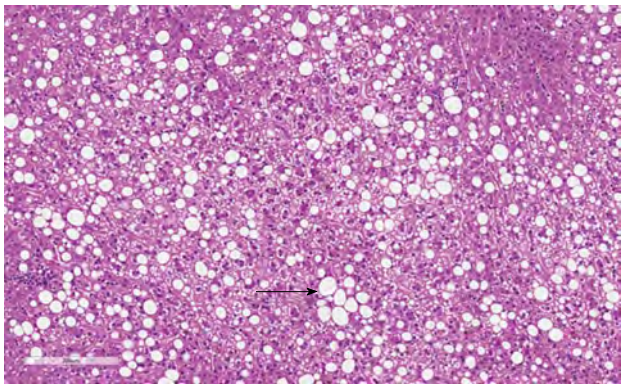

Figure 3 Representative histological slide of the hepatic adenoma. Medium power HE of the hepatic adenoma shows trabeculae with normal thickness composed of hepatocytes with moderate to severe steatotic changes (arrow) and bland uniform nuclei. No large cell or small cell change is identified. Notably, bile ducts, entrapped portal tracts or ecstatic vessels were absent. There is a circumscribed border without a thick capsule (HE, × 200).

Comparing our case to existing case reports of PET-avid HCAs in the literature, only 3 out of the 7 previous cases provided a detailed description of the histological findings[7-13]. Similar to our case, these 3 cases showed predominantly fatty change[8,9,11]. Of these 3 cases, only one report further classified the lesion as a HNF-1α hepatic adenoma[8].

Several hypotheses can explain the positive radiotracer uptake in such situations. In infections or inflammation, increased FDG uptake in lesions can be explained by the presence of inflammatory cells, which result in enhanced glucose metabolism or increased cell density[24-26]. However, pathological examination of the HCA in our patient revealed no evidence of inflammation. Also, none of the reported PET-avid HCAs in the literature included any of the inflammatory HCA subtype.

Radiotracer activity can increase at an early stage of malignant transformation and present as a "hot" lesion; activated β-catenin HCA has a higher malignant potential and may harbor small foci of HCC. In both our patient and the cases in the literature, none of the PET-avid HCAs were activated β-catenin mutated.

Focal fatty infiltration of the liver has been reported to be PET-avid. As a response to fat accumulation, a subacute inflammatory hepatic reaction with infiltration of activated Kupffer cells occur, resulting in a higher SUV_{max} than adjacent normal liver parenchymal[27,28]. Fat necrosis has been reported to have increased FDG uptake in benign lesions as well[29]. In the literature, 3 cases reported prominent fatty change but there was no mention of any inflammatory infiltrate (including our case), perhaps the fatty change itself was sufficient to evoke a PET-avid response without significant histological evidence of inflammatory infiltrate.

Lastly, hypervascularity of a HCA can induce an accumulation of glucose by the relative local increased blood flow to the lesion without a sufficient "washout". This hypothesis was suggested for HCA without any

evidence of inflammation, fatty content or malignant change[11].

In conclusion, hepatocellular adenomas are a heterogeneous group of liver benign neoplasms that are characterized by varied genetic and molecular abnormalities, pathology, tumor biology and radiological features. Positron emission tomography is increasingly utilized in cancer surveillance and work-up. This case highlights that "false-positives" can arise in case of benign liver lesions such as HCA. In equivocal cases where CT-PET findings are discordant with other imaging modalities, and/or clinical/biochemical features, further evaluation with different imaging modalities (*e.g.*, contrast enhanced sonography or MRI with liver-specific contrast) or novel PET tracers (*e.g.*, ^{11}C-acetate or ^{18}F-fluorocholine PET) should be considered[1,30-33].

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CASE REPORT

Hepatoid adenocarcinoma of the stomach: A report of three cases

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Abstract

Hepatoid adenocarcinoma of the stomach (HAS) is a rare form of gastric cancer that has unique clinicopathological features and an extremely poor prognosis. Here, we report on three patients with suspected gastric cancer who were referred to our hospital. Gastrointestinal fiberscopy on the three patients revealed two lesions in the antrum and a third lesion in the gastroesophageal junction. The alpha fetoprotein (AFP) serum levels were markedly elevated in all cases. At the time of diagnosis, two cases were advanced stages with lymph nodes and/or liver metastases. Two patients underwent exploratory laparotomy. A total gastrectomy was performed on the operable lesion, and an expanded gastrectomy was completed in the case with hepatic metastases. Histopathological analysis revealed that the tumors displayed two pathological changes: hepatoid-like foci and adenocarcinomatous. Furthermore, the tumor cells were immunohistochemically positive for AFP, alpha-1 antitrypsin, and alpha-1 antichymotrypsin. All three patients received chemotherapy. The follow-up duration ranged from 8-36 mo. Our experience and previous published studies have suggested that HAS is an aggressive type of adenocarcinoma. However, radical surgery and chemotherapy may positively impact clinical outcomes.

Key words: Hepatic adenocarcinoma; Gastric cancer; Alpha fetoprotein; Prognosis; Stomach

Core tip: Hepatoid adenocarcinoma of the stomach is a rare but important type of gastric cancer that has unique clinicopathological features and an extremely poor prognosis. We analyzed the relationship between clinicopathological features, treatment courses and prognosis using three case reports combined with previously published studies.

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INTRODUCTION

Hepatoid adenocarcinoma (HAC) is a rare type of extrahepatic tumor that has a morphological similarity to hepatocellular carcinoma (HCC)[1]. The first description of HAC was in the stomach, which is the most common location; however, HAC has been reported to develop in a variety of organs, such as gallbladder, lung, urinary bladder, esophagus, pancreas, peritoneum, Jejunum, colon, rectum, renal pelvis, ureter, ovaries, uterus and papilla of Vater[2-5]. Hepatoid adenocarcinoma of the stomach (HAS) is a relatively rare gastric carcinoma and

has an extremely poor diagnosis. Only a few cases have been previously reported. In this report, we describe three cases of HAS, and review the literature concerning its clinicopathological aspects.

CASE REPORT

Case 1

A 58-year-old Chinese male was admitted to our gastroenterology ward on February 12, 2010 with upper abdominal dull pain that had persisted for 1 mo. The patient had no relevant past medical history. The results of the physical examinations were unremarkable. A laboratory investigation showed that the patient's serum alpha fetoprotein (AFP) level was elevated to 5845 ng/mL. However, additional tumor markers were normal. Additionally, hepatitis B surface antigen and antibody and hepatitis C antibody were all negative. A gastroduodenoscopy revealed a gigantic mass with a central ulceration at the lesser curvature extending from the angle to the antrum of the stomach (Figure 1A). A gastric biopsy revealed poorly differentiated adenocarcinoma. Computed tomography (CT) scans of the abdomen and pelvis was performed and revealed thickening of the wall of the antrum, enlarged lymph nodes at the lesser curvature, multiple hepatic tumors in the bilateral lobes of the liver and tumor thrombus in the portal vein and its branches (Figure 2A and B). However, no cirrhotic change was observed in the liver. A diagnosis of AFP-producing gastric cancer with multiple liver metastases was made. The patient received systemic chemotherapies, including a regimen of four cycles of epirubicin-oxaliplatin-fluorouracil and a regimen of six cycles of oxaliplatin plus capecitabine. During the chemotherapy intermission, the liver metastases were treated with transcatheter arterial chemoembolization and CT-guided radiofrequency ablation. All treatments were provided by our gastric multidisciplinary team. These treatments resulted in partial remission (Figure 1B), and the patient's serum AFP levels decreased to 518.8 ng/mL. The CT scan showed that the patient's enlarged lymph nodes had decreased in size and that the liver metastatic foci were stable (Figure 2C and D). A distal gastrectomy with a lymph node dissection and a left lateral liver lobectomy was performed on June 11, 2011.

The distal gastrectomy resected a 2.0-cm gastric hepatoid adenocarcinoma at the lesser curvature, which infiltrated the muscularis propria (pT2) with no vascular invasion (V0). Additionally, a regional lymph node assessment revealed 16 lymph nodes with no cancer metastasis (pN0). However, there were several necrotic nodules in the left lateral lobe of the liver, but no cancer was detected. The histopathologic examination showed poorly differentiated adenocarcinoma with hepatoid features. The carcinoma was composed of polygonal tumor cells with abundant eosinophilic cytoplasm and round nuclei occasionally exhibiting obvious nucleoli with high mitotic activity. The tumor cells were arranged mainly in a trabecular pattern, whereas a smaller area of glandular formations was also observed (Figure 3A). Immunohistochemistry (IHC) revealed that the neoplastic cells were diffusely positive for alpha-1 antitrypsin (AAT), alpha-1 antichymotrypsin (ACT), cytokeratins 8, 18, 19 and carcinoembryonic antigen (CEA). Interestingly, many neoplastic cells showed cytoplasmic staining for AFP (Figure 4A). These IHC findings combined with the morphological features described above supported a diagnosis of HAS.

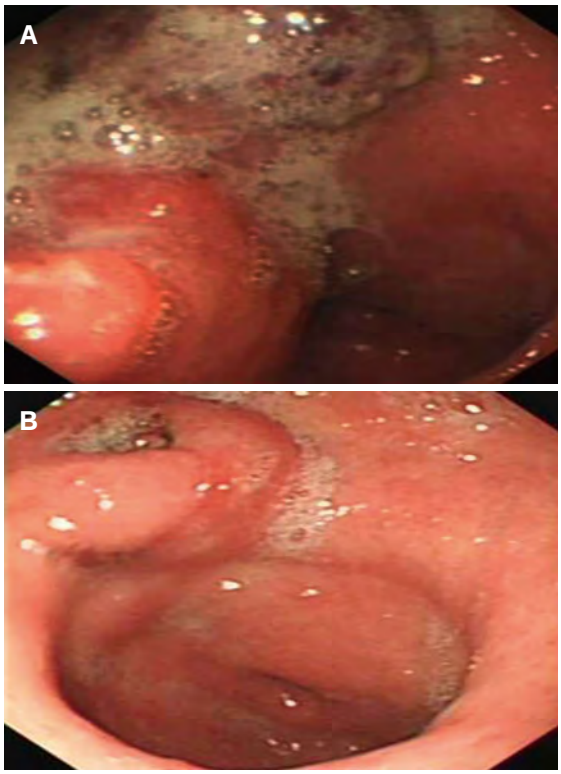

Figure 1 Endoscopic imaging of the gastric tumor. A: A gigantic mass with a central ulceration at the lesser curvature extending from the angle to antrum of the stomach; B: Partial remission of the gastric tumor after chemotherapy.

The patient recovered soon after surgery with no major complications. The total hospital stay for the surgical procedures was 15 d. Adjuvant chemotherapy with capecitabine was recommended, and the patient completed six cycles of chemotherapy. The patient's serum AFP levels returned to normal within one month post-surgery. No progress has been observed after reexamination with CT, and the serum AFP levels were stable at 20 mo post-surgery.

Case 2

A 54-year-old Chinese male presented to a local doctor with a one-month history of retrosternal pain. An endoscopy revealed an elevated tumor at the esophagogastric junction. Furthermore, a gastric biopsy discovered a poorly differentiated adenocarcinoma. The patient was referred to our hospital on March 3, 2011 for further examination. Upon physical examination, mild abdominal tenderness was detected under the xiphoid. A laboratory investigation showed that the patient's serum AFP levels were elevated to 858.1 ng/mL. Furthermore, the results

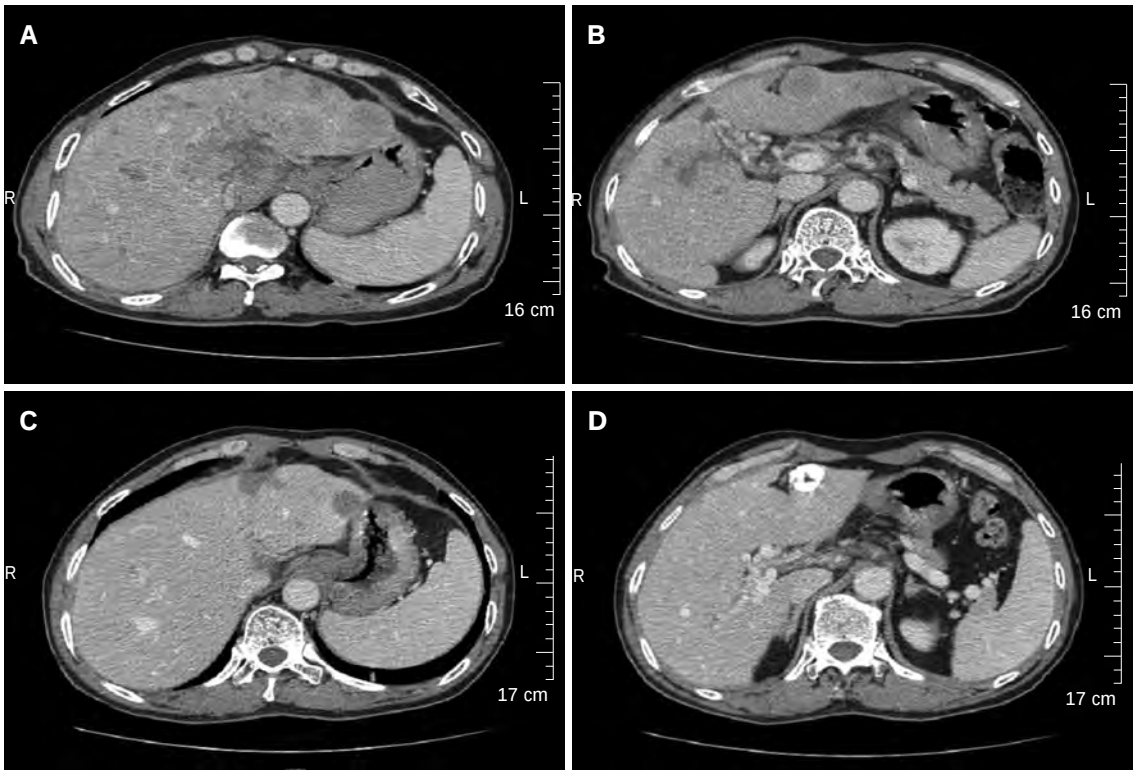

Figure 2 Computed tomography imaging of the gastric tumor and liver metastatic tumor. A and B: Computed tomography-scan revealing thickening of the wall of antrum, enlarged lymph nodes at the lesser curvature, multiple hepatic tumors in the bilateral lobes of the liver, tumor thrombus in the portal vein and its branches; C and D: After comprehensive therapies, enlarged lymph nodes had decreased in size and the liver metastatic foci were stable.

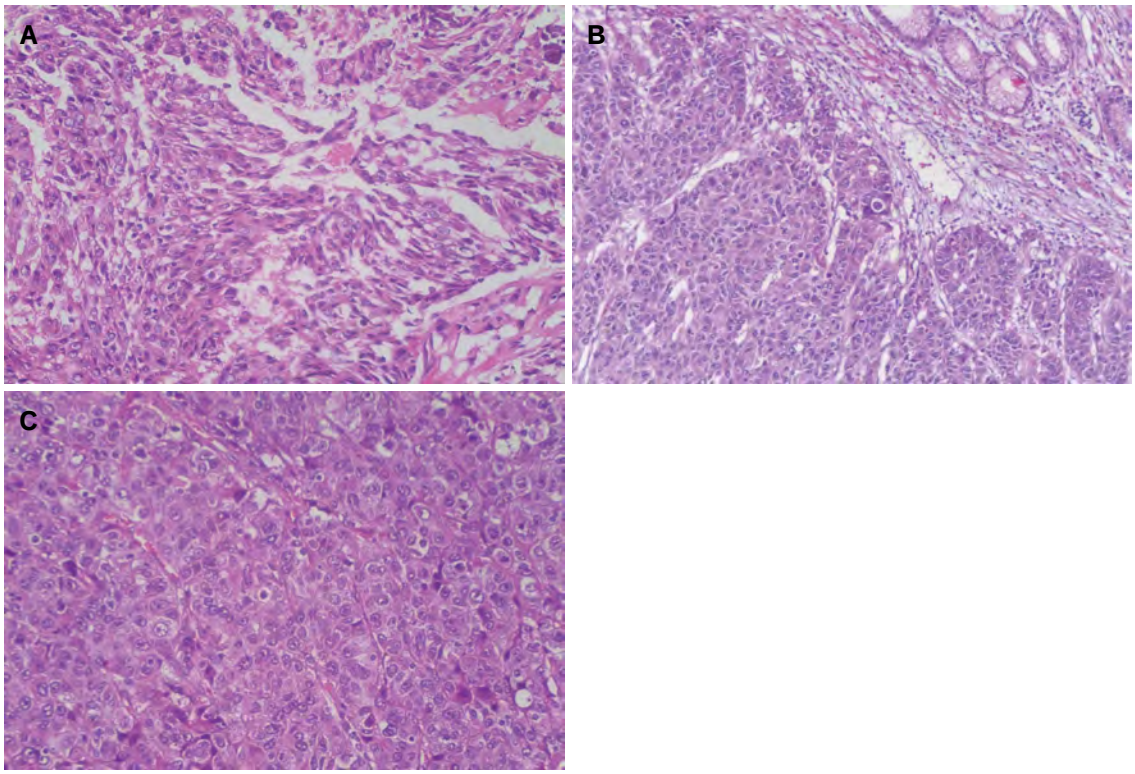

Figure 3 Presentations of hematoxylin and eosin stains: A: Tumor cells are arranged in a trabecular pattern (× 200); B: Tumor cells are arranged with cancer nests and adenoids (× 100); C: Tumor cells are featured with eosinophilic cytoplasm and round nuclei occasionally exhibiting obvious nucleoli (× 200).

of additional laboratory tests, including routine blood and liver function tests, were all within normal limits. In

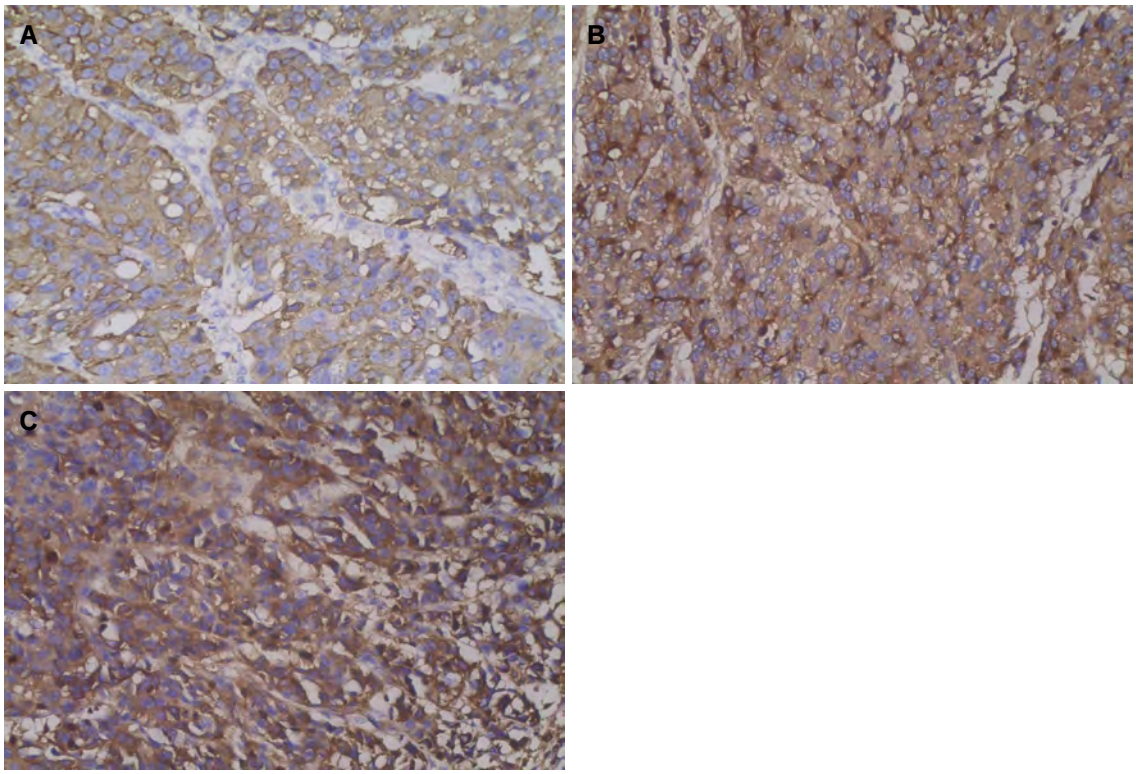

Figure 4 Presentations of immunohistochemical stains: A: Positively-stained alpha fetoprotein (× 200); B: Positively-stained alpha-1 antitrypsin (× 200); C: Positively-stained alpha-1 antichymotrypsin (× 200).

addition, hepatitis B surface antigen and antibody and hepatitis C antibody were all negative. Upper gastrointestinal radiological studies showed an irregular structure, filling defect and wall stiffness at the esophagogastric junction. A CT scan showed thickening of the wall of the esophagogastric junction; however, no metastasis was observed. AFP-producing gastric cancer was diagnosed, and a total gastrectomy with lymph node dissection was performed on March 12, 2011.

The surgically resected specimen showed an elevated tumor (4.0 cm in maximal diameter) at the gastroesophageal junction, with central ulceration and surface erosion. Light microscopic examination revealed that the tumor invasion was limited to the area from the mucosa to the proper muscle layer. The tumor was diagnosed as HAS based on the pathological and IHC findings (Figure 3B). However, no cancer metastasis was detected in the 23 lymph nodes examined. The tumor was diagnosed as stage IB (pT2N0M0) according to the American Joint Committee on Cancer guidelines[6].

Postoperatively, the patient underwent a regimen of six cycles of oxaliplatin plus fluorouracil adjuvant chemotherapy. The serum AFP levels decreased to 8.2 ng/mL within the first two months, but they increased again to 99.9 ng/mL at 5 mo post-surgery. Furthermore, the CT scan showed lung metastases. Therefore, a chemotherapy regimen with paclitaxel and capecitabine was administered. However, after two cycles of second-line chemotherapy, the serum AFP levels increased to 8431 ng/mL, and the lung metastases progressed. Therefore, the patient declined additional therapy and was discharged. A careful follow-up of this patient revealed growth of the lung metastases; the patient died 18 mo post-surgery.

Case 3

A 61-year-old woman was admitted to the clinic of our hospital on January 27, 2012 with epigastric pain that had persisted for one-month. The patient had no relevant medical history. A systematic review disclosed a weight loss of 5 kg in 1 mo but no hematemesis, hematochezia or melena. A physical examination revealed mild upper abdominal tenderness. A laboratory investigation showed a hematocrit of 27% with a hemoglobin concentration of 8.5 g/dL; the mean corpuscular volume was 70.8 fL, and the red cell distribution width was 18%. In addition, the AFP levels were > 50000 ng/mL. Additional tumor markers were normal. Furthermore, the hepatitis B and C panel was negative.

A gastroduodenoscopy revealed a large gastric antrum ulcer, and a CT scan showed wall thickening in the stomach, massive lymph node swelling around the lesser curvature of the stomach, head of pancreas, portal and splenic vein tumor thrombosis and multiple low density nodules in the spleen.

A histopathologic examination of the antral ulcer biopsy showed a moderately differentiated adenocarcinoma with hepatoid features (Figure 3C). The results from the IHC study revealed the expression of AFP, AAT and ACT in tumor cells (Figure 4B and C). The final diagnosis in this case was advanced stage HAS with lymph node

metastases. The patient underwent chemotherapy with oxaliplatin plus S-1. However, no remission was observed in the stomach tumor. The AFP levels were > 50000 ng/mL after four cycles of chemotherapy. Meanwhile, the patient had grade 4 myelosuppression and hepatic dysfunction (Child-Pugh class B). Therefore, further chemotherapy was terminated. The patient died 8 mo after admission.

DISCUSSION

HAC is a rare but important type of extrahepatic tumor that has a morphologic similarity to HCC[1]. HAC was first reported as an AFP-producing tumor in 1970[7]. Kodama *et al*[8] described two histological types of AFP-producing gastric carcinoma: the medullary type and the well-differentiated papillary or tubular type. The two types of gastric carcinomas were occasionally found to coexist in a single tumor. Ishikura *et al*[9,10] proposed the term "hepatoid adenocarcinoma of the stomach" for primary gastric carcinomas that are characterized by both hepatoid differentiation and the production of large amounts of AFP. The reported incidence of AFP producing gastric cancer includes 1.3%-15% of all gastric cancer cases[11]. Later, Ishikura *et al*[12] reported on primary AFP-negative gastric carcinomas with characteristic histologic features mimicking hepatocellular carcinoma. Moreover, Nagai *et al*[13] reported that 46% of HAS demonstrated negative AFP levels by IHC staining and that microscopically hepatoid features, regardless of AFP-staining, were more important for a positive prognosis. Currently, the diagnosis of HAS is not dependent on whether AFP is produced, but it is based on the characteristic histological features.

Similar to most hepatic cellular carcinoma, HAC can produce AFP, AAT and ACT. Furthermore, specific well-differentiated HAC can secret bile[14]. Hepatoid carcinoma has a tubular adenocarcinomatous component, but the relationship between the tubular adenocarcinomatous and the hepatoid component remains unclear. A recent analysis by Kumashiro *et al*[15] compared the cellular phenotypes of 23 cases of hepatoid adenocarcinoma of the stomach and showed tubular adenocarcinomatous components with 69 cases of non-hepatoid adenocarcinoma of the stomach. The results suggested that hepatoid adenocarcinoma was derived from an adenocarcinoma with an intestinal phenotype and that its hepatoid component was related to reduced CDX2 expression levels.

Hepatoid adenocarcinoma of the stomach frequently occurs in older people. The average patient age is 63.5 years, and the male-to-female ratio is 2.32:1. The most common location is the antrum (60.2%), followed by the cardia and fundus. Moreover, epigastric pain and generalized fatigue caused by anemia are the most common symptoms[11]. In most patients, metastases to the liver and/or lymph nodes are detected preoperatively[11].

The diagnosis of HAS depends on the recognition of the characteristic histological features[13]. Histopathologically, the tumor is composed of two closely related areas, including hepatoid-like foci and adenocarcinomatous. Tumor cells in the hepatoid foci resemble the morphology of HCC, and immunohistochemically can be positive for AFP, AAT and ACT. Additionally, polyclonal CEA staining shows a "canalicular" pattern, which is characteristic of hepatoid features. The adenocarcinomatous component may be well or poorly differentiated, often with clear cells and a papillary pattern[10,16]. In differential diagnosis, additional AFP-producing gastric tumors and metastasizing germ cell tumors should be excluded[17]. HCC should be excluded particularly when HAS metastasizes to the liver[18]. Generally, in HCC, neighboring cirrhotic lesions are found, and tumor cells are positive for Hep Par-1 antibody, a sensitive and specific immunohistochemical marker for hepatocyte differentiation, whereas in metastatic HAS Hep Par-1 is often negative, and neighboring cirrhotic lesions are rare[16]. A recent finding indicated that the immunostaining of the palate, lung and nasal epithelium carcinoma-associated protein could be used as a novel marker to distinguish HAS from primary HCC[19].

In terms of treatment, there are few data in the literature specifically pertaining to HAS. The disease is treated similarly to common gastric adenocarcinoma. Radical surgery is the optimal treatment for HAS and may effectively prolong survival. Additionally, the liver metastases should undergo simultaneous resections[20]. Adjuvant chemotherapy and radiotherapy should be given according to current gastric cancer indications, despite the fact that no specific data on the adjuvant treatment of HAS are available. For patients with metastasis, chemotherapy is the primary treatment modality[20]. It has been reported that a better prognosis might be associated with good performance and active treatment, including palliative gastrectomy and chemotherapy[21]. Palliative chemotherapy is recommended to all patients with advanced gastric cancer and good performance statuses[22], although there is no standard chemotherapy protocol for HAS.

According to the literature, the prognosis of HAS is poorer than that of ordinary gastric adenocarcinoma. Nagai *et al*[13] reported that hepatoid adenocarcinoma had a poorer prognosis compared with AFP-producing non-hepatoid adenocarcinoma. However, the reasons for the poor prognosis are not clearly understood. One possibility is that hepatoid adenocarcinoma produces AAT and/or ACT as well as AFP. AAT and ACT have immunosuppressive and protease-inhibitory properties that enhance invasiveness[11].

The three patients in our study had an average age of 57 years, and their serum AFP levels were elevated. Two patients were found in the advanced stage with lymph nodes and/or liver metastases at the time of diagnosis, and the third patient developed lung metastasis seven months postoperatively. Two patients underwent surgery. Although all three patients had received chemotherapies, their outcomes were not satisfactory. In the first case, the patient who underwent curative gastrectomy and adjuvant chemotherapy showed a relatively good prognosis.

In conclusion, HAS is a relatively rare tumor with

unique hepatoid features and a poor prognosis. Aggressive therapies, including surgery and chemotherapy, may yield good survival outcomes.

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Triple metachronous colon cancer

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Abstract

A 72-year-old male with an early stage "node-negative" sigmoid colon cancer developed 2 separate "node-negative" early stage colon cancers during a subsequent colonoscopy surveillance regimen, the first in the descending colon 7 years later, and the second in the cecum almost 14 years after the first cancer was resected. After the initial symptomatic cancer, all subsequent neoplastic disease, including malignant cancers were completely asymptomatic. This entity, multiple primary cancers, likely reflected the use of a colonoscopic surveillance regimen.

Key words: Colorectal cancer; Surveillance colonoscopy; Multiple primary cancer syndrome; Metachronous colorectal cancer

Core tip: Detection of increasing numbers of asymptomatic metachronous colon cancers may result from widening use of surveillance colonoscopy in patients with a previously treated early stage colon cancer.

Freeman HJ. Triple metachronous colon cancer. *World J Gastroenterol* 2013; 19(27): 4443-4444 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i27/4443.htm DOI: http://

TO THE EDITOR

An article recently published in the *World Journal of Gastroenterology* concerning a patient with quadruple malignancies involving different organ sites, but with survival over more than 20 years is rare (below 0.1%) and has special significance[1]. The authors suggest that the incidence of multiple primary cancers (MPC) is rising, possibly related to individual genetic susceptibility and other factors[1]. The patient described here below emphasizes that this rising incidence in MPC may also result from increasing use of surveillance colonoscopy regimens aimed at high risk groups, specifically those with early stage ("node-negative") colon cancer.

A 72-year-old otherwise healthy male developed constipation and rectal bleeding in June 1997. Subsequent colonoscopic evaluation revealed a 4 cm ulcerated mass in the distal sigmoid colon. Anterior resection revealed a moderately differentiated adenocarcinoma invading just through the muscularis propria (T3). Lymph nodes were negative for malignancy with no lymphovascular invasion. Subsequent annual colonoscopies showed small tubular adenomas that were resected. In addition, biopsies of the prior anastomosis were negative. In 2004, additional tubular adenomas were resected and a diminutive ulcerated sessile polypoid lesion in the rectum revealed severely dysplastic mucosa. Because of the high suspicion for an invasive malignancy, another laparotomy was done. The previous anastomosis was resected and revealed no evidence for malignancy and a further resection of the descending colon was done revealing a second infiltrative moderately differentiated colonic adenocarcinoma measuring 1 cm in diameter and extending into the muscularis propria (T2). Lymph nodes were negative for malignancy with no lymphvascular invasion. Subsequent colonoscopies in 2004, 2005 and 2006 revealed diminutive tubular adenomas that were resected. In 2010, a further colonoscopy revealed a single small tubulovillous adenoma in the ascending co-

lon that was removed. Another ulcerated polypoid lesion was detected in the cecum and biopsies confirmed a third moderately differentiated adenocarcinoma. Subsequent right hemicolectomy revealed extension through the muscularis propria with negative lymph nodes and no lymphovascular invasion (T3). In summary, despite 3 separate "node-negative" cancers, all treated by surgical resection alone, this patient remains well, now over 15 years. As a direct result of surveillance, the last 2 colon cancers were detected at an early and asymptomatic stage.

After complete resection of a colonic adenocarcinoma, colonoscopy guidelines recommend ongoing surveillance to exclude subsequent development of metachronous colon cancer[2]. A high intensity form of surveillance is often recommended for the initial 5 years[2]. In our experience, longer term colonoscopic surveillance after endoscopic removal of either malignant polyps[3] or more sessile early stage ("node-negative") colon cancers[4] have also yielded significant numbers of patients with metachronous colon cancers, a specific form of MPC[1], at an early asymptomatic stage of the disease. This experience underlines the importance of colonoscopy surveillance of high risk categories, particularly prior colonic cancer patients with "node-negative" disease. With the expectation that these highly-selected patients will survive for extended periods, colonoscopic surveillance *per se* can be expected to yield increasing numbers of patients with colonic metachronous MPC in the future.

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Word of caution before implementing ketotifen for gastrointestinal transit improvement

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Abstract

The therapeutic potential of long-term ketotifen in irritable bowel syndrome and postoperative ileus is currently under investigation. Ambiguous results of prolonged postoperative ketotifen use on gastrointestinal passage have been found. The current data point at a hampered gastrointestinal transit after prolonged postoperative ketotifen use in a rodent ileus induction model. Therefore, caution should be taken when administering ketotifen in the perioperative phase.

Key words: Ketotifen; Gastrointestinal transit; Postoperative ileus

Core tip: Prolonged postoperative ketotifen impairs gastrointestinal transit in a rodent ileus induction model.

Reisinger KW, de Haan JJ, Schreinemacher MH. Word of caution before implementing ketotifen for gastrointestinal transit improvement. *World J Gastroenterol* 2013; 19(27): 4445-4446 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i27/4445.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i27.4445

TO THE EDITOR

Postoperative ileus has a major impact on length of hospital stay after bowel resection[1]. Currently, the therapeutic potential of long-term ketotifen in postoperative ileus and irritable bowel syndrome is under investigation[2,3]. The underlying mechanism, however, remains unclear as ketotifen exerts both mast cell stabilizing and H1 receptor blocking effects. Furthermore, the long-term effects on gastrointestinal (GI) transit deserve definition. The and colleagues showed that ketotifen improved gastric emptying at 24 h follow-up; however continuing treatment up to 48 h postoperatively produced ambiguous results on GI passage[2]. Therefore, we performed a rat study to assess the influence of prolonged use of ketotifen on GI transit time at 5 d follow-up after ileus induction.

The same postoperative ileus induction model was used as previously described[4]. Male Wistar rats (250-300 g) were anesthetized using buprenorphine 0.1 mg/kg *sc* and anesthesia was maintained using 2.5% isoflurane. Subsequently, rats underwent a laparotomy *via* a midline abdominal incision under aseptic conditions. The small intestine was placed on moist gauze pads outside the abdomen, manipulated with moist cotton swab sticks for 5 min and kept moist at all times. After manipulation, the small intestine was placed back in the abdomen and the abdomen closed in 2 layers with continuous sutures. Rats (6 in each group) received either ketotifen in a high-dose (1 mg/kg) or low-dose (0.1 mg/kg), mast cell stabilizer cromoglicic acid (50 mg/kg) or vehicle (saline). The high dose of ketotifen is comparable to doses prescribed for humans. Cromoglicic acid prevents the release of mediators from mast cells through a non-H1/2-receptor pathway. Doses were administered twice daily in a volume of 1.5 mL *via*

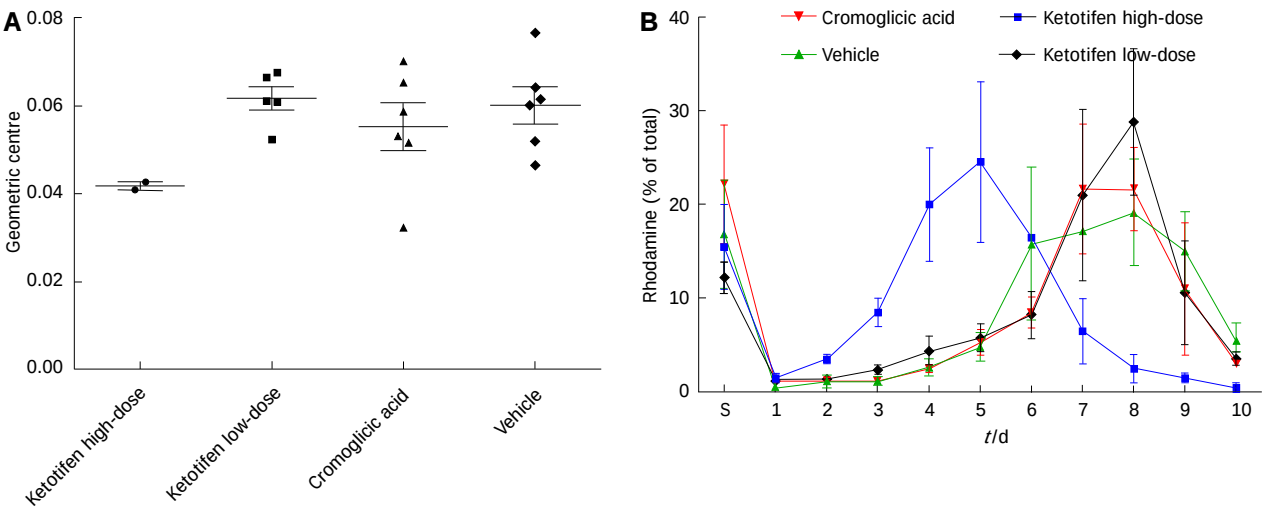

Figure 1 Geometric centre of recovered rhodamine, calculated as (Σ% fluorescence per segment × segment number)/100 (A), and amount of recovered rhodamine per bowel segment (ketotifen high-dose, *n* = 2; all other groups, *n* = 6) (B).

oral gavage starting at 2 d preoperatively until sacrifice. GI transit time was measured at the time of sacrifice (5 d postoperatively, by cervical dislocation after anesthesia with 4% isoflurane) by evaluating the GI distribution of rhodamine-B-labeled dextran (Sigma-Aldrich, St. Louis, MO, United States). Rhodamine [200 μL of 6.25 mg/mL in phosphate buffered saline (PBS)] was administered *via* oral gavage. One hour after administration the animals were sacrificed, the small bowel divided in 10 equal parts (part 1: beginning at jejunum, part 10: ending at the transition of ileum to coecum) and resected together with the stomach. A fluorescence reader was used to quantify the rhodamine-containing gut content in the supernatant after vigorous mixing and centrifuging of the gastric and bowel contents in 2 mL PBS. A histogram of fluorescence distribution per segment (% of total recovered rhodamine) was plotted for transit analysis and expressed as geometric center for statistical analysis. Geometric centers were calculated for each animal as (Σ% fluorescence per segment × segment number)/100.

In the high-dose ketotifen group, 4 out of 6 rats died before reaching 5 d follow-up with an extremely distended stomach at necropsy. The geometric centers of the surviving animals were also markedly lower than the other groups, but numbers ($n = 2$) were too low to allow for statistical analysis (Figure 1). However, GI transit times in the low-dose group were comparable with the control group ($P = 0.66$, Mann-Whitney U test) implying that the beneficial ketotifen effects after postoperative ileus are dose-dependent and probably restricted to the very early postoperative period, *i.e.*, less than 5 d. GI transit in the cromoglicic acid group was comparable to control ($P = 0.70$, Mann-Whitney U test). These results suggest that the effects of ketotifen on GI transit may indeed not, or not fully, depend on mast cell stabilization but rather a H1 receptor pathway.

As stated earlier by The *et al*[2], caution should be taken when administering ketotifen in the perioperative phase as prolonged postoperative treatment may have an inhibitory effect on enteric smooth muscle contraction. Indeed, the current data point at a hampered GI transit after prolonged postoperative ketotifen use. A careful treatment regimen as proposed by de Jonge *et al*[5], *i.e.*, preoperative treatment only, is therefore mandatory.

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EDITORIAL

Interplay of autophagy and innate immunity in Crohn's disease: A key immunobiologic feature

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Abstract

Crohn's disease representing a clinical phenotype of inflammatory bowel disease is a polygenic immune disorder with complex multifactor etiology. Recent genome-wide association studies of susceptibility loci have highlighted on the importance of the autophagy pathway, which previously had not been implicated in disease pathology. Autophagy represents an evolutionarily highly conserved multi-step process of cellular self-digestion due to sequestration of excessive, damaged, or aged proteins and intracellular organelles in double-membranous vesicles of autophagosomes, terminally self-digested in lysosomes. Autophagy is deeply involved in regulation of cell development and differentiation, survival and senescence, and it also fundamentally affects the inflammatory pathways, as well as the innate and adaptive arms of immune responses. Autophagy is mainly activated due to sensors of the innate immunity, *i.e.*, by pattern recognition receptor signaling. The interplay of genes regulating immune functions is strongly influenced by the environment, especially gut resident microbiota. The basic challenge for intestinal immune recognition is the requirement of a simultaneous delicate balance between tolerance and responsiveness towards microbes. On the basis of autophagy-related risk genetic polymorphisms (*ATG16L1*, *IRGM*, *NOD2*, *XBP1*) impaired sensing and handling of intracellular bacteria by innate immunity, closely interrelated with the autophagic and unfolded protein pathways seem to be the most relevant immunobiologic events. Autophagy is now widely considered as a key regulator mechanism with the capacity to integrate several aspects of Crohn's disease pathogenesis. In this review, recent advances in the exciting crosstalk of susceptibility coding variants-related autophagy and innate immunity are discussed.

Key words: Crohn's disease; Innate immunity; Autophagy genes; Autophagy; Gut microbiota

Core tip: In case of Crohn's disease, on the basis of autophagy-related risk genetic polymorphisms impaired sensing and responding of intracellular bacteria by innate immunity, closely interrelated with the autophagic and unfolded protein pathways seem to be the most relevant immunobiologic events. Autophagy represents a key regulator mechanism with the capacity to integrate several aspects of Crohn's disease pathogenesis.

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis, the main clinical phenotypes of (idiopathic, relapsing-remitting) inflammatory bowel disease (IBD) are systemic disorders affect-

ing the gastrointestinal-tract with frequent extraintestinal manifestations and associated autoimmune conditions[1]. IBD is considered as a polygenic immune disorder with complex multifactor etiology. Generally, IBD is arising in susceptible individuals in whom upon environmental triggers a sustained disturbed, deleterious mucosal immune reaction is provoked towards commensal microbiota[2]. In chronic inflammatory conditions, when organs with large epithelial surfaces are affected, like in IBD the epithelial barrier function is critical for the disease onset. Since the epithelium is densely inhabited by a resident microbial flora the role of native immunity is particularly appreciated in recognizing and distinguishing commensal enteric bacteria from the invading ones, and thus, in maintaining tolerance and homeostasis[2]. Subsequently, the chronic unrestrained inflammatory response that occurs in IBD is mainly driven by a disintegrated host immune regulatory network. In IBD development the host genetic susceptibility represents an important etiologic factor. In CD the genetic component is strongly indicated by familial aggregation, and further, by an approx. Twenty-six-fold greater population-based sibling risk, and an approximately 30%-35% of concordance rate in monozygotic twins[3,4]. The introduction of genome-wide association studies (GWAS) has yielded an expansion in studying the genetic basis of IBD. Nowadays more than 70 loci are associated with CD[5]. Further, in CD pathogenesis GWAS highlighted on certain earlier not really suspected biological pathways, such as autophagy. In polygenic diseases functional variants of single genes could be identified. Indeed, many of the recently identified genetic risk loci in CD are related to various cell types and pathways, suggesting the involvement of fairly different aspects of host immune responses in the IBD phenotype. Missing heritability in CD cannot be simply explained by genetic alterations[2]. Moreover, the fact of the worldwide considerable increase in disease incidence and prevalance emphasizes the importance of additional, environmental and epigenetic contributions[6,7]. The interplay of genes regulating immune functions is strongly affected by the environment, especially gut resident microbiota. On the basis of genetic alterations in CD impaired sensing and handling of intracellular bacteria by the innate immunity, that is closely interrelated with the autophagic and unfolded protein pathways seem to be the most relevant pathophysiologic features[8].

AUTOPHAGY MACHINERY

Besides the proteasomal degradation pathway autophagy represents an additional, evolutionarily highly conserved multi-step process of cellular self-digestion due to sequestration of excessive, damaged, or aged proteins and intracellular organelles in double-membranous vesicles of autophagosomes, terminally self-digested in lysosomes[9]. Autophagy is deeply implicated in the regulation of numerous physiologic functions including cell development and differentiation, survival and senescence, and it also affects fundamentally the inflammatory process, and the innate and adaptive arms of immune responses[10]. On a basal level intact autophagy serves constantly and constitutively as a critical adaptive and surveillance mechanism in maintaining cellular homeostasis[11]. Nevertheless, autophagy is inducible in response to different cellular metabolic stress conditions, such as nutrient and growth factor deprivation in order to preserve cell viability. Further, autophagy is upregulated in cases of protein aggregation and accumulation of misfolded proteins, *i.e.,* when the structural remodeling is mandatory. In respect of innate immunity, however, autophagy plays an essential role during infections by degrading intracellular pathogens[10,12]. Different types of autophagy according to the route of delivery to lysosomes and the main physiological functions have been characterized, such as macro- and micro-autophagy, and chaperon-mediated autophagy[11]. Upon specific targeted degradation of cytosolic aggregated proteins, lipids, and organelles (ribosomes, nucleosomes, peroxisomes, mitochondria, endoplasmic reticulum), selective forms of autophagy (aggregophagy, lipophagy, ribophagy, nucleophagy, pexophagy, mitophagy, reticulophagy) can further be classified[9,11]. In addition, elimination of intracellularly infective pathogens represents another selective form of autophagy, namely xenophagy (Figure 1). Xenophagy can be considered as a substantial element of the innate immune system. Generally (macro)autophagy refers to cytoplasmatic bulk, non-selective degradation of subcellular constituents. Within this complex catabolic pathway tightly regulated by a limited number of autophagy genes (*ATGs*) various morphologic stages of the assembly process are distinguishable[12]. It is initiated with the phagophore (isolation membrane) formation around different molecules or particles to be sequestred, and is followed by elongation and maturation into the autophagosome, leading finally to fusion with lysosomes[9,12]. Subsequently, the phagophore is controlled by *Beclin1 (ATG6)* and *ATG14* genes, and both the inhibitory class1 canonical phosphatidylinositol 3'-kinase/AKT (PI3K/AKT) mammalian target of rapamycin (mTOR) and the promoting c-Jun N-terminal kinases (JNK1) pathways[13,14]. The intricate formation of autophagosome is regulated mainly by the ATG5-ATG12 complex, then stabilized by ATG16L1, and further processed by microtubule-associated protein light chain (LC3/ATG8) under the strict control of ubiquitin-like conjugation systems (ATG10, ATG7, ATG3). The engulfment of random or selective cargo, closure of the autophagosome, and fusion with the lysosomal compartment is orchestrated by LC3, and the Beclin1-UV-irradiation resistance-associated gene (UVRAG) complex[13,14]. Defects in basal autophagy may yield accumulation of cytotoxic materials, damaged DNA, and thus, genomic instability, while alterations of induced autophagy especially lead to reduced cell survival[10,12]. By compromising cellular fitness defective autophagy has been ultimately related to several chronic inflammatory disease conditions, such as inflammatory bowel disease, like CD and cancer, neurodegeneration, and infectious disorders[10,11,15]. Gener-

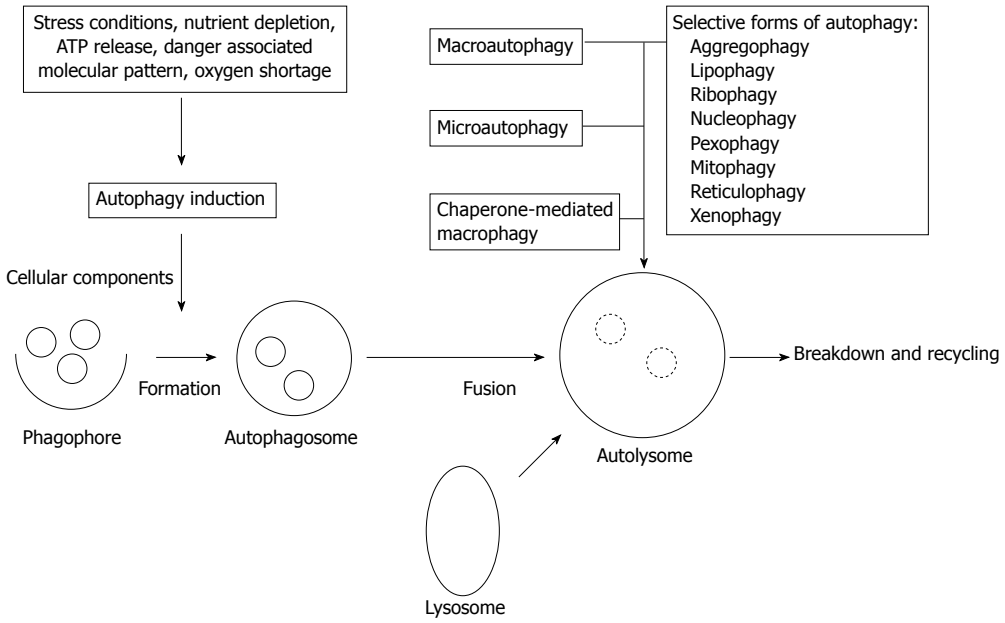

Figure 1 The autophagic process and types of autophagy.

ally autophagy deficiency is closely related to accelerated tumorigenesis. In autophagy-incompetent cells upon induced oxidative stress cell-autonomous mechanisms are exhibited in forms of accumulated DNA damage and chromatin instability[16]. However, inflammatory events as a non-cell-autonomous mechanism along with defective apoptosis could independently contribute to malignant transformation and cancer progression, partly by favoring cell necrosis[17]. Similar situation has been found in human IBD with high risk of malignancy, and in experimental cases of *Atg5*-/- or *Atg7*-/- mice displaying abnormalities resembling human IBD[18]. Autophagy and stress-responsive cellular degradation pathways of intrinsic and extrinsic apoptosis can fundamentally alter, activate or inhibit each other *via* an extensive molecular crosstalk, and in fact, cell destiny is determined by their actual functional status and interplay[19]. Their crosstalk is primarily regulated by the current status of the ATG6/Beclin-1 complex, a Bcl-2/Bcl-xL interacting element, since Bcl2 is a potent autophagy inhibitor. Dissociation of this complex can be achieved by toll-like receptor (TLR) adaptors (MyD88, TRIF), or activation of mitogen activated phosphokinase (MAPK)-JNK cascade, as well as by translocation of the damage-associated molecular pattern (DAMP) protein high-mobility-group B (HMGB)-1[13,19]. There is also diverse interaction between autophagy and the nuclear factor-κB (NF-κB) signaling pathways through positive and negative feedback regulatory loops[14]. The tumor suppressor *p53* gene exerts a typical dual role in autophagy regulation, depending primarily on its subcellular, nuclear or cytoplasmic distribution[13,14].

NOD-LIKE RECEPTORS AND CROHN'S DISEASE

NOD-like receptors (NLRs) are pattern recognition receptors (PRRs) and belong to the family of innate immune receptors sensing pathogen-associated molecular patterns (PAMPs). Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) is constitutively expressed intracellularly in macrophages and dendritic cells, and to lesser extent in intestinal epithelial cells and T cells. The centrally located motifs of NLRs are referred to NOD domains that are interacted with the caspase activation and recruitment domain ones. NOD2 recognizes *N*-acetyl--muramyl-peptide (MDP), a bacterial peptidoglycan component, and upon activation the induced receptorial conformation changes result a multiprotein, the inflammasome (NLRP3). Ligation of NOD2 triggers recruiment of the adaptor protein receptor interacting protein 2 (RIP2) causing a TRAF6-mediated ubiquitination of inhibitor of κB-kinase gamma (IKKγ; NEMO), and hence results in activation of downstream signaling pathways implicating NF-κB, MAPKs and proinflammatory caspases[20,21]. The Crohn's disease-associated *NOD2* genetic variants are located in the leucine-rich repeat (LRR) region of NOD2, *i.e.*, in the ligand-binding domain of this intracellular PRR[22]. The altered amino acid sequence is related either to insertion resulting in a frame-shift mutation, or to non-synonymous SNPs resulting in amino acid exchanges. The more commonly observed genetic variants (of missense or nonsense mutations) in CD are the SNP8 (R702W), SNP12 (G908R), and SNP13 (L1007fsC), respectively, however a number of rare *NOD2* variants have also been discovered, being localized again almost exclusively to the LRR region[22,23]. Upon MDP ligation the Crohn's disease-associated "loss-of-function" NOD2 variants abrogate RIP2 binding, and so fail to activate NF-κB[24,25]. Further, NOD2 is involved in the modulation of TLR signaling, as well. Thus, in case of Crohn's disease-related gene polymorphisms the TLR2-induced NF-κB activation is also decreased[26,27].

However, yet it is difficult to correctly interpret the real functional consequences of given mutations since they may activate additional, compensatory mechanisms resulting in a definite inflammatory phenotype. On the other hand NOD2 has a pivotal role in direct antibacterial defense by the induced release of defensins. NOD2-/- mice and patients with the CD NOD2 variants display diminished expression of antimicrobial α-defensins in Paneth cells, that contributes to impaired antibacterial capacity and decreased epithelial barrier function[28,29]. In contrast to hypomorphic functions the frame-shift gene mutation variant encodes a "gain-of-function" by actively suppressing interleukin-10 (IL-10) transcription[30].

AUTOPHAGY AND CROHN'S DISEASE

The autophagy machinery in IBD represents a recently developed pathway fundamentally contributing to the pathogenesis[10]. Functional polymorphisms of the autophagy genes *ATG16L1* (T300A) and immunity-related GTPase family M protein *(IRGM*; C313T) have been found as definite risk factors for CD[31-34]. The ATG16L1 protein is widely expressed in intestinal epithelial cells, and also in macrophages and lymphocytes. The ubiquitous *ATG16L1* seems to be fundamental in selective autophagy, *i.e.,* in xenophagy, nonetheless its defect has only been described within the gut[35].

In CD patients homozygous for the risk *ATG16L1* allele the "loss-of-function" deficiency due to failures of autophagosome formation results in impaired engulfment and degradation of cytoplasmic content (microbes), defective presentation of bacterial antigens to $CD4^+$ T cells, and further, in alterations of Paneth cell granule formation causing a disrupted granule exocytosis[18,36-38]. Additionally, ATG16L1-deficient Paneth cells in CD display a "gain-of-function" defect by increasing expression of inflammatory cytokines[18,38]. Moreover, upon stimulation with NOD2 ligands or with lipopolysaccharides (LPS) through TLR4, macrophages and myeloid cells with the ATG16L1 risk variant generate high levels of reactive oxygen species (ROS), and respond with inflammasome overactivation leading to enhanced IL-1β and IL-18 production *via* Myd88 and TRIF-dependent activation of caspase-1[36-38]. Generally, aberrant activation of PRR signaling pathways may result critically severe inflammation. *IRGM* is the only human gene representative for innate immunity-related GTPases, necessary for γ-interferon-mediated resistance to intracellular pathogens[39]. During initiation of autophagy IRGM expression is essentially required for the proper clearance of bacteria. The risk polymorphism of *IRGM* due to the impaired protein expression can lead to functional abnormalities in xenophagy[32,33]. Since IRGM is possibly regulated in a cell specific manner the CD risk allele may cause cell specific phenotypes.

NOD2 and autophagy

Functionally NOD2 is closely associated with autophagy, and yet interacts mechanically (*i.e.,* immunoprecipitated) with ATG16L1, therefore autophagy seems to be a key factor in CD[37,40,41]. Autophagy is mainly activated due to sensors of the innate immunity, *i.e.,* by PRR signaling upon recognition of PAMPs (MDP, LPS, ss/ds RNA, methylated DNA/CpG), but it could also be induced by DAMPs (like ATP, ROS, and misfolded proteins), pathogen receptors (like CD46), IKK, JNK and HMGB proteins[10,12,19,38]. Sensory PRR-molecules include TLRs, NLRs and RIG-I-like receptors (RLRs). Induction of NOD2 in dendritic and epithelial cells by bacterial ligands and leaving bacteria results in ATG16L1-dependent formation of autophagic vacuoles. However, the NOD2 variants of CD lack this activity, and further MDP-induced autophagy is also absent in cells with the ATG16L1 risk variant, suggesting that both NOD2 and ATG16L1 co-localized on plasma membrane are required for an optimal innate immune signaling[40-42]. In addition, a NOD2-dependent failure in autophagy-induction and consequently a diminished bacterial killing was found for *Salmonella typhimurium*, *Shigella flexneri*, and enteroadherent invasive *Escherichia coli* (*E. coli*)(AIEC)[40,41,43]. The normal NOD2, but not the CD-associated variants recruits ATG16L1 to the plasma membrane preferentially at the bacterial entry side, so physiologically NOD2 is critical for engulfing invading pathogens by autophagosomes[41,42]. Furthermore, in dendritic cells NOD2-dependent autophagy is also essential for the appropriate antigen processing and presentation and a subsequent induction of $CD4^+$ T-cells[41]. Dendritic cells from CD patients with either NOD2 or ATG16L1 variants display a failure to translocate bacteria to lysosomes and relocate MHC II to cell surface, as well[40]. When the disease-associated *ATG16L1* and *NOD2* alleles are present in combination, a synergistic genetic epistasis, *i.e.,* an increase in CD susceptibility was observed, underscoring the importance of a signaling crosstalk regarding the inflammasome and autophagy[44].

Endoplasmic reticulum stress and autophagy

The unfolded protein response (UPR) induced by endoplasmatic reticulum (ER) stress represents another pathway in IBD pathophysiology[45]. Genetically ER stress is associated with both forms of IBD and occurs upon excessive accumulation of misfolded or unfolded proteins in the ER, leading to UPR especially in cells with high secretory capacity, like goblet cells and Paneth cells[35,46]. UPR is regulated by different pathways (and related transcription factors) with the preference of the inositol-requiring enzyme 1/X-box binding protein 1 (IRE1/XBP1) axis[47]. *Via* this axis there is a conserved link between innate immunity (TLR and NOD signaling) and the UPR[47]. GWAS-based candidate gene studies revealed the role of *XBP1* SNPs in IBD-related ER-stress[48,49]. Decreased or absent XBP1 function in the intestinal epithelial cell (IEC) compartment through IRE1 hyperactivation results in uncontrolled ER-stress, *i.e.,* a proinflammatory overactivation, and further in dysfunction and premature apoptotic depletion of Paneth cells, with

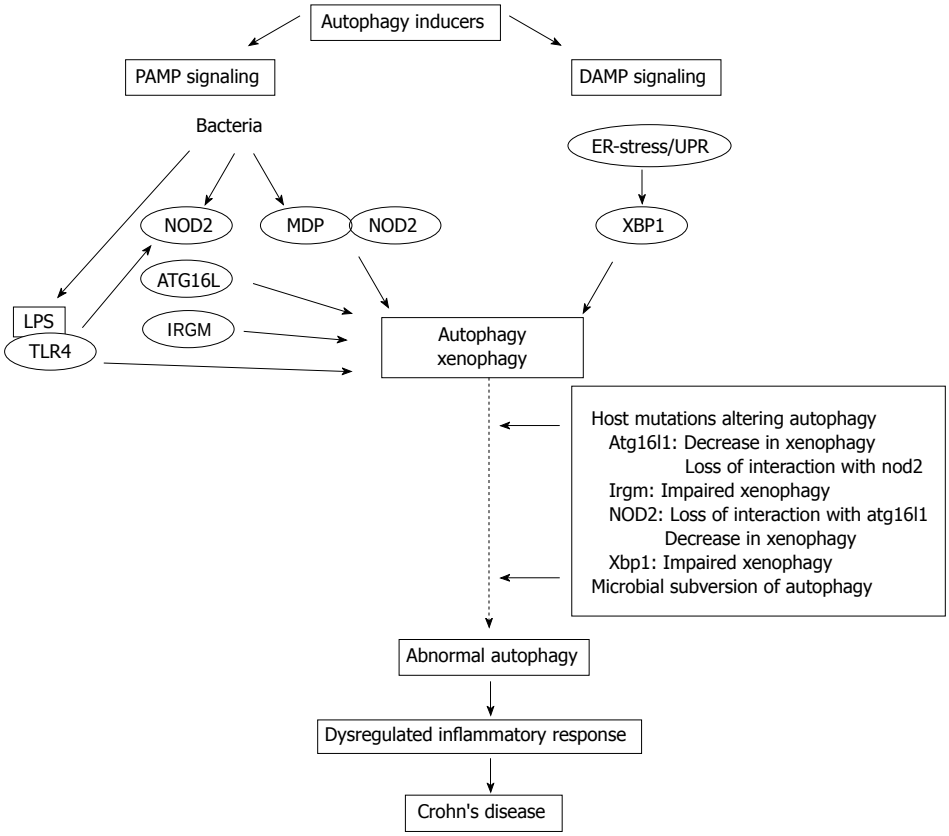

Figure 2 Schematic illustration of the crosstalk between autophagy and innate immunity in Crohn's disease. PAMP: Pathogen-associated molecular patterns; DAMP: Damage-associated molecular pattern; ER: Endoplasmic reticulum; UPR: Unfolded protein response; NOD: Nucleotide-binding oligomerization domain-containing protein; MDP: *N*-acetyl-muramyl-peptide; LPS: Lipopolysaccharide; TLR: Toll-like receptor; XBP1: X-box binding protein 1; IRGM: Immunity-related GTPase family M protein.

the consequent impaired handling of the microbiota[49]. Under ER stress autophagy is induced *via* JNK (downstream of IRE1), which is overactivated by the hypomorphic XBP1[50,51]. However, even defective autophagy *per se* is able to provoke ER stress, especially when the ATG7 protein involved in regulation of autophagosome formation is also depressed[52]. Regarding PI3K there is an antagonistic action, since in UPR it is responsible for the activation of XBP1, but in the contrary autophagy is suppressed by the canonical AKT-TOR pathway[53,54]. In IBD, IECs presumably are affected both by impaired UPR signaling and aberrant autophagy, but their exact interplay needs to be further clarified.

GUT MICROBIOTA AND CROHN'S DISEASE

The intestinal microbiota, which normally colonize mucosal surfaces in symbiotic mutualism with the host is unique and quite stable over time[55]. The basic challenge for the intestinal immune recognition is the requirement of a simultaneous delicate balance between tolerance and responsiveness towards microbes[56]. Several data suggest the existence of immune tolerance to antigens of the individual own bacterial flora, whereas its breakdown definitely contributes to IBD pathogenesis[37,57]. In CD there is a profound and complex host defect in sensing and responding intestinal (lumenal and mucosal) microbiome. Accordingly, reprogramming in the microbial composition, *i.e.,* a significant decreased load of commensal, protective resident bacteria (like *Bifidobacteria, Lactobacilli* and *Firmicutes*) along with the impaired immunity against the putative pathogenic (harmful) ones (such as *Bacteriodetes*, and *Proteobacteria*, including *E. coli*) provoke a deleterious inflammatory condition, corresponding to CD[58]. The exact nature of the distinct mucosal flora (dysbiosis), however has not yet been fully elicited. Specific strains of *E. coli* (termed AIEC) in CD affect especially the epithelial layer with the ability to adhere, invade and replicate in IECs, and further, a subpopulation even resides and survives within macrophages, and thereby induces increased production of tumor necrosis factor-α[43,59]. ATG16L1 and IRGM-deficient autophagosomes promote the AIEC survival as well[43]. Moreover, in the presence of CD-associated NOD2 variants or hypomorphic XBP1 dendritic cells exhibit diminished intracellular bacterial killing[41]. It is hypothesized, that AIEC possesses the capacity to circumvent innate immune responses leading to activation of NF-κB[60]. Thus, regarding the host interactions with microbes genetic risk factors of CD functionally render pathways of the innate immunity to converge to a deeply impaired autophagic process.

CONCLUSION

Overall, there is no doubt that autophagy can be considered as an apparently difficult regulatory network, being in close connection with several signal transduction pathways and cellular programs. Principle elements of immunological autophagy include the direct cell-autonomous pathogen elimination, the regulation of PRRs, and inflammasome activation, and the cytoplasmic antigen processing for MHC presentation to T cells. Recently significant advances have been achieved in understanding the importance of autophagy in CD, which previously had not been implicated in IBD pathology. In CD functional consequences of the underlying autophagy-related gene defects (*ATG16L1, IRGM, NOD2, XBP1*), in particular the inappropriate stimulation of antimicrobial and inflammasome pathways eventually result in uncontrolled inflammation (Figure 2). Therefore, autophagy in CD is predicted as a key regulator mechanism with the capacity to integrate several aspects of disease pathogenesis. Theoretically the complex autophagy signaling in CD offers a promising novel therapeutic target, since due to its induction potentially not only the load of cytoinvasive bacteria, and the perturbed immune responses, but the resulting inflammatory process, as well may simultaneously be reduced. Thus, autophagy boosting would represent an efficient biologic manipulation, and could provide an alternative therapeutic option. Several candidate pathways, *e.g.*, inhibition of mTOR, decrease of ER-stress, lowering of inositol triphosphate (IT3), *etc.*, could be considered. On the other hand, however, much cautiousness is required regarding its pleiotropic physiological repertoire, since pharmacologic autophagy modulation can initiate additional biologic effects not expected in CD. Further detailed functional analyses of the Crohn's disease-associated genetic polymorphisms are needed to explore and define more precisely the subcellular and molecular basis of the crosstalk between autophagy and the innate immune axis, hopefully allowing the introduction of selective new therapeutic approaches into daily practice.

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REVIEW

Autoimmune hepatitis in childhood: The role of genetic and immune factors

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Abstract

Autoimmune hepatitis (AIH) is a rare chronic inflammatory disease of the liver, which affects a group of patients who lost their immunological tolerance to antigens of the liver. It is clinically characterized by hypergammaglobulinemia, elevated liver enzymes, presence of autoantibodies and histological changes. Although being rare in children, it represents a serious cause of chronic hepatic disease that can lead to cirrhosis and hepatic failure. Clinical findings, exclusion of more common liver disorders and the detection of antibodies antinuclear antibodies, smooth muscle antibodies and anti-LKM1 are usually enough for diagnosis on clinical practice. The pathogenic mechanisms that lead to AIH remain obscure, but some research findings suggest the participation of immunologic and genetic factors. It is not yet knew the triggering factor or factors that stimulate inflammatory response. Several mechanisms proposed partially explain the immunologic findings of AIH. The knowledge of immune factors evolved might result in better markers of prognosis and response to treatment. In this review, we aim to evaluate the findings of research about genetic and immune markers and their perspectives of application in clinical practice especially in pediatric population.

Key words: Autoimmune hepatitis; Genetics; Clinical practice; Immunophenotype

Core tip: In this review article, we reported recent data on autoimmune hepatitis in pediatric patients highlighting the importance of genetic and immune markers. We also discuss the perspectives of the application of these new biomarkers in clinical practice.

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INTRODUCTION

Autoimmune hepatitis (AIH) is a chronic inflammatory disease of the liver that is rarely found in children and adolescents. AIH affects a group of patients who have lost their immunological tolerance to antigens of

the liver[1-4]. It is more frequent in female patients and is characterized by hypergammaglobulinemia, elevated liver enzymes, the presence of autoantibodies and histological changes[4-7]. The age of onset usually ranges from months to 75 years old, but it is very rare before the age of two years old, and the highest incidence occurs between 10 and 30 years old[2]. In addition to being considered rare in children, AIH represents a serious cause of chronic hepatic disease, which can result in cirrhosis and its complications. Immunosuppressive treatment results in a good response, but a delay in or absence of treatment can result in cirrhosis and liver failure[2,6]. This condition can also be complicated by association with autoimmune cholangitis, in which bile duct disease is present together with hepatitis, particularly in children[2,7,8].

Clinical and laboratory diagnosis

Because histological activity index (HAI) is a rare disorder, one crucial point for diagnosis is the exclusion of more common pathologies. The diagnosis is confirmed by clinical findings, laboratory and histopathology tests and the exclusion of other causes of chronic liver disease[4,6,7,9]. The clinical spectrum is broad, ranging from asymptomatic laboratory abnormalities to clinical symptoms similar to fulminant acute viral hepatitis. The classical presentation is jaundice, dark urine, fever, asthenia, anorexia and increased abdominal volume in an acute or insidious presentation[6,10]. Hepatomegaly, splenomegaly and signs of chronic liver disease, such as spider veins, collateral circulation and abdominal ascites, might be present. Approximately 20% of cases are associated with other autoimmune disorders[8].

According to the presence of autoantibodies, AIH can be classified into two forms: type 1 autoimmune hepatitis, in which antinuclear antibodies (ANAs) and/or anti-smooth muscle antibodies (SMAs) are detected; and autoimmune hepatitis type 2, in which anti-liver-kidney (anti-LKM1) autoantibodies are detected[9,11-13]. In adult patients, the presence of anti-soluble liver-kidney antigen and anti-liver-pancreas might be understood as a third form of AIH (AIH type 3), despite clinical features similar to type 1[14]. Type 1 is the most common type of AIH in any age group, while type 2 usually occurs in younger patients, with courses having a greater likelihood of acute liver failure[2,3].

During treatment, ANA and SMA levels can decrease, but neither level seems to have a correlation with prognosis[15-17]. Therefore, 10%-15% patients are negative for ANAs, SMAs and LKM-1 at clinical presentation but later show detectable levels of these autoantibodies, with only five percent remaining negative over time[15,18]. Other autoantibodies could facilitate in diagnosis and/or act as prognostic markers, and their possible clinical applications are listed in Table 1[19-38].

Most services do not perform routine assessment of the autoantibodies shown in Table 1, which remain reserved for research situations. The antibodies ANA, SMA and anti-LKM1 are usually sufficient for diagnosis in clinical practice. More research is needed to establish the clinical use of these autoantibodies and to investigate the presence of these autoantibodies in pediatric patients, thereby elucidating their role in this group of patients.

Diagnostic criteria

The International Autoimmune Hepatitis Group diagnostic criteria for AIH, published in 1993 and revised in 1999, guide diagnosis and facilitate early treatment[39-41]. A simplified scoring system, created in 2008, considers transaminases levels, autoantibodies, immunoglobulin G levels, liver biopsy, exclusion of Wilson disease and of viral hepatitis and cholangiogram[41,42]. The use of these criteria could also be helpful in children, but limitations must be recognized[43]. In children, it is difficult to differentiate AIH from primary sclerosing cholangitis or to identify autoimmune cholangitis overlap syndrome. The diagnosis of fulminant hepatitis cases has not been well determined because the use of 1/40 as a titer for autoantibodies is high to use in children (1/20 for ANA and SMA and 1/10 for anti-LKM1 are considered positive in this age group)[3,43]. For these reasons, histology is often included in the diagnostic criteria for HAI in children[3,44]. On histological examination, characteristic findings include the presence of piecemeal necrosis (interface hepatitis), lymphoplasmocytic infiltrates with numerous plasmocytes, and rosette formation[44,45]. Histology is a powerful tool for diagnosis, with high specificity (81%-99%) and predictability (62%-91%) but low sensitivity (36%-57%)[45]. Some cases also demonstrate biliary duct alterations, such as inflammatory infiltration of duct cells, cholestasis and ductopenia, which might represent an overlapping syndrome[46].

Genetic and immunologic markers

Some studies have unveiled the association of AIH with genetic markers, and the impact of immunophenotyping on clinical practice has been described.

Although the pathogenesis of AIH is not fully understood, susceptibility is partly determined by the presence of genes related to major histocompatibility complex Ⅱ (MHC Ⅱ) and most directly to human leukocyte antigen (HLA)[7,47]. The main associations are with HLA-DR3 and HLA-DR4 (DRB1*03 and DRB1*04) in Europeans and North Americans[48]. In children, HLA-DRB1*1301 is related to susceptibility to HAI, determining the prognosis and response to treatment[47,49]. The findings of the immunophenotyping in HAI are shown in Table 2.

Some conclusions can be drawn from these studies, in addition to some controversial findings[48-57]. Fortes Mdel *et al*[50] showed that patients presenting the HLA-DRB1*1301 allele were associated with a higher likelihood of developing cirrhosis. Czaja *et al*[56] concluded that patients with -DRB1*03 were younger at disease onset than patients with -DRB1*04, and they also had worse responses to corticotherapy. Patients expressing HLA

Table 1 Autoantibodies studies and their findings

Type of AIH	Autoantibodies	Antigen	Meaning
AIH type 1	Anti-actin	Actin	Poor response to treatment with corticosteroids[19-21]
AIH types 1 and 2 (80%-90% of cases)	Anti-asialoglycoprotein receptor	Asialoglycoprotein receptor	Liver specific antigen and indicative of prognosis[22,23]
AIH types 1 and 2 (8%-20% of cases)	Antimitochondrial antibody-M2	Mitochondria	Favorable response to corticosteroids[24,25]
AIH type 1 (39% of cases)	Anti-chromatin	Chromatin	High titers of immunoglobulin G and shows disease activity[26,27]
AIH type 2 (32% of cases)	Anti-liver-cytosol type 1	Enzyme formiminotransferase cyclodeaminase	Diagnostic tool and marker of liver inflamation[28-30]
AIH type 1	Antibody to histone and dsDNA	dsDNA	High titers of immunoglobulin G and poor-immediate response to corticosteroids[26]
AIH type 1 (47.5% of cases)	Anti-soluble liver antigen	t-RNAs	Presence of severe forms, associated with fatal outcome[31-35]
AIH type 2 (5%-19% of cases)	LKM-3	Uridinediphosphateglucuronyl tranferase	Allows diagnosis, being sometimes the only marker identified[36]
AIH type 1	Perinuclear antinuclear neutrophil cytoplasmic antibodies	Peripheral nuclear and perinuclear antigen	Presence of severe forms; Most frequent in primary sclerosing cholagitis and primary biliary cirrhosis[36-38]

AIH: Autoimmune hepatitis; dsDNA: Double-stranded DNA.

DRB1*04 are more often women, with a greater risk of comorbidity with other immune diseases and with good responses to corticosteroids[56,58].

In contrast, MHC Ⅱ antigens have shown significant heterogeneity among different ethnicities. Patients with HLA-DRB1*13 and -DRB1*03 have an earlier onset of disease compared to other patients, possibly because their ethnic groups that have a tendency toward AIH onset at younger ages. Moreover, certain ethnic groups have low prevalences of these immunophenotypes, such as the populations of Mexico and Japan, where HLA-DRB1*04 is more common, and these low rates seem to establish increased susceptibility to the disease in older people[50-52]. Few studies have demonstrated the role of immunophenotypes in HAI in children; to apply these markers as indicators of response to treatment and prognosis, more studies are needed.

The known physiopathological mechanism in AIH consists of an inflammatory response with T-lymphocyte cells, principally helpers, and B lymphocytes, macrophages and natural killer cells. The triggering factor or factors that stimulate this inflammatory response are not yet known. Several mechanisms have been proposed that would partially explain the immunologic findings of AIH[7,59].

Studies in adults and children have identified some potential pathways for the damage observed in AIH, such as the deregulation of immunoregulatory mechanisms. Some of the studies have shown that AIH patients have reductions in the number and function of T lymphocytes $CD4^+CD25^+$, which is one of the regulatory cells (T-regs) that normally represent 5%-10% of CD4 T cells in healthy humans[7,59-66]. These cells suppress the proliferation and cytokine responses of effectors CD4 and CD8 T cells, and they down-regulate the functions of macrophages, dendritic cells, natural killer cells, and B lymphocytes[62].

All immune findings are more pronounced in the initial presentation than after remission with treatment[61,62,66,67]. T-reg immunosuppressive functioning causes the production of anti-inflammatory cytokines, such as interleukin-4 (IL-4), interleukin-10 (IL-10) and transforming growth factor (TGF)-beta[68,69]. The surface markers involved in anti-inflammatory mechanisms are glucocorticoid-induced tumor necrosis factor receptor (CD62L), cytotoxic T lymphocyte-associated protein-4 (CTLA-4) and fork head/winged helix transcription factor (FOXP3)[62,70]. If the mechanisms of failure become known, new treatments, based on recuperation of the function of T-regulation, could be used in AIH[70-72].

Natural killer T cells ($CD3^+$ and $CD56^+$) are found in reduced numbers, producing lower levels IL-4 and IL-2 in AIH patients. These lower levels result in reduction of the surface expression of CTLA-4 in $CD4^+$T cells, playing a pivotal role in liver autoaggression, especially during the active phase of the disease[61,72]. Kurokohchi *et al*[73] also found that the levels of CTLA-4 were reduced in inflammatory cells from the peripheral blood of AIH patients, compared with controls, while levels of $CD80^+$ and $CD86^+$ were increased in liver-infiltrating cells. Other research has shown that the CCR5 cytokine receptor was preferentially expressed on Th1 cells. This cytokine plays a pivotal role in the recruitment of interferon-gama (IFN-γ) (a pro-inflammatory cytokine), producing $CD4^+$ T cells at inflammatory sites, such as hepatic tissue, and promoting hepatocyte damage in AIH[73,74]. Another possibility involves the presence of CD4 and/or CD8 self-reactive T cells, which could damage liver cells. These cells are found in healthy people, but in AIH patients, they are 10-fold higher in number[68,75].

Studies have also suggested that mutations in these genes act as precursors of the surface markers of immune cells and might also have significance in autoimmune diseases because changes in HLA (MHC) are absent in some patients. Mutations of several lympho-

Table 2 Major histocompatibility complex class Ⅱ human leukocyte antigen and its association with autoimmune hepatitis patients

Ref.	Total No. of patients/ controls (No. of children)	What was evaluated	Conclusions
Donaldson *et al*[48]	96/100 (no)	HLA-DR	HLA-DR3 and DR4 genes independently confer susceptibility to autoimmune hepatitis
Fortes Mdel *et al*[50]	41/111 (13)	HLA-A, -B, -C, -DR and DQ	Regarding HLA-A and -C there were no significant differences between groups; For HLA class Ⅰ, an increase in the frequency of B*08, B*18, B*45 and B*50 was observed. HLA B*40 was more frequent in healthy controls; For HLA class Ⅱ, an increase in the frequency of HLA-DQB1*02, -DQB1*04, HLA-DRB1*03, DRB1*13 and DRB3 was observed. HLA-DRB1*1301 and -DRB1*0301 were more frequent in children
Ota *et al*[51]	51/no (no)	HLA-DR and -DQ	Increased frequency of all HLA-DRB1*04 alleles, principally -DRB1*0405. Secondary association with -DRB1*15 and DRB1*16
Vázquez-García *et al*[52]	30/175 (not cited)	HLA-A, -B, -C, -DR and -DQ	A significant association with HLA-DRB1*0404 was found. It was present in patients with average age onset. DQB1*0301 had a low frequency in patients and may represent a protective factor; No association was found with any class Ⅰ antigen
Fainboim *et al*[53]	52/197 (all)	HLA-A, -B, -C, -DR and -DQ	No significant associations with HLA class Ⅰ antigens were found; HLA-DR6 group (HLA-DRB1) showed increased frequency, principally HLA-DRB1*1301; The analyses of HLA-DQ group showed an associations of HLA-DQB1*0603
Pando *et al*[54]	206/208 (122)	HLA-DR and -DQ	The frequencies of HLA-DRB1*1301, -DRB1*0301, -DQA1*0103, -DQB1*0603 were significantly increased on AIH patients; HLA-DRB1*1301 was associated with younger age at disease onset, being the allele associated with AIH in children and HLA-DRB1*1302 worked as a protective factor
Bittencourt *et al*[55]	139/129 (74)	HLA-DRB and -DQB1	In AIH type 1, there was significant increase in the HLA-DRB1*13, -DRB1*03, -DRB3 and -DQB1*06 alleles in patients. HLA-DRB1*13 was more frequent in children than adults. The low frequency of HLA-DQB1*0301 may indicate a protective role of this allele; In AIH type 2, a significant increase in DRB1*07, DRB1*03, DRB4 and DQB1*02 was observed
Czaja *et al*[56]	86/102 (not cited)	HLA-A, -B, -C, -DR and -DQ	DRB4*0103 is associated with immune diseases, DRB1*0301 with a poor treatment response, and DRB1*0401 with a lower frequency of hepatic death or transplantation
Czaja *et al*[57]	210/396 controls with other chronic liver disease/102 healthy controls (no)	HLA-DR B1*03, -DRB1*04 and -DRB1*13	The frequency of HLA DRB1*13 was higher in patients without -DRB1*03 and -DRB1*04; Primary sclerosing cholangite patients showed a similar frequency of HLA-DRB1*13 when compared with AIH patients

HLA: Human leukocyte antigen; MHC: Major histocompatibility complex; AIH: Autoimmune hepatitis.

cyte surface markers studied could represent molecular markers of autoimmunity in AIH. Among them is the CTLA-4 (CD152) gene mutation, which has appeared in controversial reports of the phenotypes that represent susceptibility to AIH[76-81]. For instance, in the Brazilian study by Bittencourt *et al*[77] no association was established between exon 1 *CTLA-4* gene polymorphisms at position 49 and AIH susceptibility, contradicting findings in a North American population[78].

CTLA-4, which is expressed on the surface of T cells, induces peripheral tolerance by biding CD80 and CD86 on antigen-presenting cells. In doing so, CTLA-4 competes with the co-stimulatory molecule CD28, reducing the immune response[47]. CTLA-4 is considered a critical coordinator in immune regulation. Based on this finding, some researchers have attempted to find a drug that simulates its mechanism and that could be used in the treatment of autoimmune conditions; one such drug is an immunoglobulin G-CTLA-4 (Abatacept), which was recently approved by the FDA for use in rheumatoid arthritis[82,83].

Furthermore, some studies have aimed to evaluate whether a *Fas* gene polymorphism or its increased expression on lymphocyte surfaces could be key mechanisms for autoimmunity in AIH. Fas (CD95) is part of the tumor necrosis factor family, and it induces receptor-mediated programmed cell death (apoptosis) through engagement with its ligand (FasL/CD95L). It indirectly controls the number of antigen-activated lymphocytes[84]. Ogawa *et al*[85] showed that AIH patients show an increase in CD95 (Fas)-positive $CD4^+$ and $CD8^+$ T cell numbers. These individuals show disease courses with high levels of conversion of naive $CD45RO^-$ to primed $CD45RO^+$ $CD4^+$ T cells. This course could indicate that constant activation of T lymphocytes and/or the persistent presence of activated lymphocytes requires continuous work from regulation cells, such as $CD95^+$ T $CD4^+$[85]. Tsikrikoni *et al*[86] also found a greater number of Fas^+ and $FasL^+$ cells in the mononuclear cells of AIH patients and increased TNF-α and IFN-γ production in cultured cells, suggesting that these cytokines could be involved in accelerating apoptosis. They also showed an increase in $CD14^+$ monocyte cell numbers, in accordance with the increased

expression of apoptotic markers, such as $CD14^+$ cells, responding to the clearance of apoptotic cells[87]. Concomitantly, the results of genetic studies have shown that some mutations can affect the function of Fas receptors, but more research is needed to determine these receptors' relationship with AIH[88-91].

A lack of consistent evidence has persisted for studies evolving genes of cytokines, such as tumor necrosis factor; TGF-beta1, and TBX21, (a regulator of T lymphocyte lineage development and a controller of the expression of IFN-γ)[91-98].

TREATMENT

An important feature of AIH is response to treatment with corticosteroids and immunosuppressants[2,6,99]. Prednisone alone or in combination with azathioprine is the main form of treatment[99]. This treatment has the goal of reducing hepatic inflammation, the induction of clinical remission, relief of symptoms and improvement of survival. The treatment response characterizes clinical improvement and a reduction of aminotransferases to normal or to no more them two times of the maximum of the reference value, while remission lies in clinical improvement, normalization of aminotransferases and gamma globulin, autoantibody reduction or extremely low titers of autoantibodies and histological resolution of inflammation with a reduction in fibrosis[3,44]. Moreover, relapse is characterized by increased transaminases after remission has been achieved, as shown by Ferreira *et al*[44,100]. Relapse is common during treatment and occurs in up to 40% of patients, requiring a temporary increase in the dose of corticosteroid[3,99]. Noncompliance play a prominent role in a percentage of relapses[44,100]. Some medications offer alternative treatment, such as cyclosporine, tacrolimus and mycophenolate mofetil. These drugs are reserved for patients who fail to respond to the first treatment choice[2,6]. In cases of autoimmune sclerosing cholangitis and autoimmune cholangitis, the use of ursodeoxycholic acid can be necessary to control bile duct disease[2].

Liver transplantation is the last-line treatment indicated for patients who have not responded to medication. The need for transplantation is present in 8.5% of children with HAI[8]. The total duration of immunosuppressive therapy has not been established, but in the face of the possible side effects with medication, discontinuation of treatment should be considered when the remission criteria are met in patients with type 1 AIH[3]. To meet this goal, the patient must present histological resolution of inflammation after at least two years of clinical and laboratory remission (normal liver enzymes, liver function and gamma globulin and autoantibodies in low or undetectable titers)[3]. Approximately 20% of patients with type 1 AIH can remain in remission after discontinuation of treatment, but relapses are frequent after the suspension of treatment[6,8,100]. In type 2 AIH, treatment discontinuation is not recommended because relapses are more frequent, and failure of remission upon suspension is almost certain in this condition[8].

The prognosis of patients who respond to immunosuppressive treatment is good, even when there is cirrhosis at baseline; there is a good quality of life and, in general, use of low doses of medication[2-4]. Except for the changed autoantibodies that were initially detected, no markers are currently used in clinical practice to choose and follow treatment.

CONCLUSION

In conclusion, recent studies have shown new possibilities for the diagnosis and prognostic evaluation of AIH, except for in the pediatric age group, which remains unrepresented in these assessments. Susceptibility to autoimmune diseases is multifactorial, but genetic and immunological factors play pivotal roles. MHC Ⅱ antigens could represent a susceptibility marker for AIH, considering the differences between ethnic groups, or they might predict treatment response and prognosis. Finally, in pediatric populations, the prevalence and titers of autoantibodies can be different from in adults, such as for the MHC Ⅱ HLA-DRB1*1301, which can be a marker of susceptibility in the pediatric population.

Perhaps in the future, knowledge of autoimmune mechanisms will reveal better markers for the diagnosis, monitoring and treatment of AIH and other autoimmune diseases, but there are still only few available studies with good suggestions for markers.

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ORIGINAL ARTICLE

Antioxidant properties of glutamine and its role in VEGF-Akt pathways in portal hypertension gastropathy

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Abstract

AIM: To investigate the effects of glutamine on oxidative/nitrosative stress and the vascular endothelial growth factor (VEGF)-Akt-endothelial nitric oxide synthase (eNOS) signaling pathway in an experimental model of portal hypertension induced by partial portal vein ligation (PPVL).

METHODS: Portal hypertension was induced by PPVL. The PPVL model consists of a partial obstruction of the portal vein, performed using a 20 G blunt needle as a guide, which is gently removed after the procedure. PPVL model was performed for 14 d beginning treatment with glutamine on the seventh day. On the fifteenth day, the mesenteric vein pressure was checked and the stomach was removed to test immunoreactivity and oxidative stress markers. We evaluated the expression and the immunoreactivity of proteins involved in the VEGF-Akt-eNOS pathway by Western blotting and immunohistochemical analysis. Oxidative stress was measured by quantification of the cytosolic concentration of thiobarbituric acid reactive substances (TBARS) as well as the levels of total glutathione (GSH), superoxide dismutase (SOD) activity, nitric oxide (NO) production and nitrotyrosine immunoreactivity.

RESULTS: All data are presented as the mean ± SE. The production of TBARS and NO was significantly increased in PPVL animals. A reduction of SOD activity was detected in PPVL + G group. In the immunohistochemical analyses of nitrotyrosine, Akt and eNOS, the PPVL group exhibited significant increases, whereas decreases were observed in the PPVL + G group, but no difference in VEGF was detected between these groups. Western blotting analysis detected increased expression of phosphatidylinositol-3-kinase (PI3K), P-Akt and eNOS in the PPVL group compared with the PPVL + G group, which was not observed for the expression of VEGF when comparing these groups. Glutamine administration markedly alleviated oxidative/nitrosative stress, normalized SOD activity, increased levels of total GSH and blocked NO overproduction as well as the formation of peroxynitrite.

CONCLUSION: Glutamine treatment demonstrated to reduce oxidative damage but does not reduce angiogenesis induced by PH in gastric tissue, demonstrating a beneficial role for the PI3K-Akt-eNOS pathway.

Key words: Partial portal vein ligation; Oxidative stress; Glutamine; Portal hypertension; Rats

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INTRODUCTION

Portal hypertension (PH) is a clinical syndrome that is usually secondary to obstruction of the intra- or extra-hepatic portal flow. It is considered the main complication of liver disease, being responsible for the development of other liver diseases, such as portal hypertensive gastropathy, ascites, spontaneous bacterial peritonitis, hepatorenal syndrome, hepatopulmonary syndrome, portopulmonary hypertension, hyperkinetic syndrome and hepatic encephalopathy[1].

PH is characterized by an increase in pressure above 5 mmHg in the portal venous system. When the pressure reaches 8 to 10 mmHg, in the esophagus and stomach, gastroesophageal varices arise, which develop from a network of collateral circulation through the vessels that form the splanchnic circulation. Bleeding from gastroesophageal varices can occur when the portal pressure gradient reaches values above 12 mmHg[2-4].

Numerous veins dilate, including the hemorrhoidal plexus, abdominal wall and esophagogastric junction. The umbilical vein communicates with and dilates the superficial veins of the abdominal wall, and the presence of abdominal collateral circulation is an important clinical sign of portal hypertension, which is characterized by dilated and tortuous veins radiating from the navel to the upper abdomen and lower chest[5,6]. Collateral circulation of the left gastric vein to the azygos vein is responsible for esophagogastric varicose veins and increased circulation in the gastric mucosa, which characterize the complications of portal hypertension referred to as portal hypertension gastropathy (PHG)[5].

The vascular endothelium releases vasodilators, including nitric oxide (NO) and prostacyclins, and vasoconstrictors, including endothelin, angiotensin and thromboxane. The function of vascular tone is maintained by balancing these agents. The increased peripheral resistance is maintained by elevation of vasoconstrictors or vasodilators or by reducing the levels of both. The blood exerts a force against endothelial cells, which are the main agonist in the release of NO[7].

Increases in NO synthesis have also been reported in the liver of rats with PH. Moreover, NO production has been implicated in the pathogenesis of PHG, with increases in NO serum levels being detected in patients with PHG[5]. When present in high concentrations, NO acts as a free radical, forming two molecules of dinitrogen trioxide (N_2O_3) or peroxynitrite (ONOO), which are responsible for cytotoxic effects such as inflammation and septic shock. Recently, NO has been presented as an important signal of the maintenance of homeostasis, as well as a cytotoxic agent involved in numerous diseases[8-10].

Increased formation of blood vessels in the splanchnic region occurs through the process of angiogenesis, which is involved in the maintenance of hyperdynamic circulation in portal hypertension. This hypothesis is based on recent studies that demonstrate the presence of increased splanchnic angiogenesis and neovascularization, which are responsible for the formation of portosystemic collaterals in experimental models of PH[11]. The stimulus for the proliferation of new blood vessels occurs through a complex cascade of angiogenic events, and it is the main modulator of this mechanism, vascular endothelial growth factor, vascular endothelial growth factor (VEGF), that stimulates the proliferation and migration of endothelial cells. Overproduction of NO is stimulated by endothelial nitric oxide synthase (eNOS), which can be stimulated by both VEGF and phosphatidylinositol-3-kinase (PI3K). In turn, PI3K-Akt also receives stimulation through the VEGF pathway, and the shear stress that occurs in PH can be a factor in stimulation *via* PI3K-Akt as well[12-14]. The Akt protein directly stimulates eNOS by increasing the capacity of eNOS to generate NO. There are several ways to stimulate the Akt pathway, including through growth factors, cytokines and the mechanical force of shear stress in a blood vessel, which activates the NO release mechanism in a PI3K-dependent manner[15,16]. Overproduction of vascular NO plays a central role in both systemic and splanchnic vasodilations, which are characteristics of portal hypertension that cause it to be recognized as a major complication of liver cirrhosis. The increased expression and activity of eNOS are well-established events in chronic models of portal hypertension[17,18].

Glutamine, a nonessential amino acid, has received increasing attention because it becomes essential during stress and catabolic conditions[19]. Glutamine administration can result in an enhanced antioxidant capacity in various situations, such as critical illness or sepsis[20]. In the stomach, glutamine is able to protect against peptic ulceration and improves the healing of ulcers[21]. The present study was designed to investigate the potential beneficial effects of glutamine administration on gastric oxidative stress and to evaluate the role of the VEGF-PI3K-Akt-eNOS pathway in NO overproduction in an experimental model of PHG.

MATERIALS AND METHODS

Ethics

All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23, revised 1985).

Animals and experimental groups

Male Wistar rats with a mean weight of 250 g were used. The animals were obtained from the Center for Breeding

of Laboratory Animals of the Federal University of Rio Grande do Sul. The rats were held in cages at 20-24 ℃ with a 12 h light/dark cycle and given free access to food and water. They were randomly divided into the following four groups of fourteen animals each: (1) [sham-operated (SO) rats receiving only NaCl as a vehicle]; (2) SO + G (SO rats receiving glutamine); (3) PPVL (PPVL rats receiving vehicle); and (4) PPVL + G (PPVL rats receiving glutamine).

Partial portal vein ligation and sham operation

During the procedure, rats were anesthetized with a ketamine (Ketalar, Parke Davis, 100 mg/kg) and xylazine 2% (Rompun, Bayer, 50 mg/kg) cocktail *ip*. PH was induced by partial portal vein ligation (PPVL) as described by Moreira *et al*[22]. Briefly, the portal vein was isolated, and a 3-0 silk ligature was tied around both the portal vein and an adjacent 20 gauge blunt-tipped needle. The needle was subsequently removed, and the vein was allowed to re-expand. The abdomen was then closed, and the animal was allowed to recover. Control rats underwent a similar operation but without ligature of the portal vein. Sham-operated animals received only vehicle (NaCl, 1 mL/kg, *ig*). Glutamine was administrated daily (14 mg/kg, daily, *ig*) for 7 d beginning on the eighth day after the surgical protocol. All rats were anesthetized and sacrificed on the fifteenth day of the protocol. Their stomachs were immediately removed and divided into four subsamples that were stored in a freezer at -80 ℃ for analysis of oxidative stress, total glutathione, immunohistochemistry and Western blotting.

Oxidative stress determinations

Gastric oxidative stress was determined by measuring the concentration of aldehydic products (TBARS). Briefly, the frozen tissue was homogenized in a solution containing 140 mmol KCl and 20 mmol phosphate buffer (pH 7.4) and centrifuged at 14000 *g* for 10 min. For TBARS analysis, the amount of aldehydic products generated by lipid peroxidation was measured *via* the thiobarbituric acid reaction using 3 mg of protein per sample. The samples were incubated at 90 ℃ for 30 min following the addition of 500 mL of 0.37% thiobarbituric acid in 15% trichloroacetic acid and then centrifuged at 2000 *g* for 15 min. The spectrophotometric absorbance of the supernatant was determined at 535 nm[23].

Nitric oxide quantification

Nitric oxide production in the gastric tissue was measured indirectly using a quantitative colorimetric assay based on the Griess reaction. This method is sensitive for both nitrite and nitrate ions[24]. Briefly, the samples were deproteinized and subsequently centrifuged for 20 min at 12000 *g*. After incubation of the supernatants with *E. coli* nitrate reductase (37 ℃, for 30 min) to convert nitrates to nitrites, 1 mL of Griess reagent (0.5% naphthylethylenediamine dihydrochloride, 5% sulfonylamide, 25% phosphoric acid) was added. The reaction was allowed to proceed at room temperature for 20 min, after which the absorbance at 546 nm was measured using a sodium nitrate solution as a standard.

Antioxidant enzyme activities

Cytosolic superoxide dismutase (SOD; EC 1.15.1.1) was assayed spectrophotometrically based on the rate of epinephrine autooxidation, which is progressively inhibited by increasing amounts of SOD in the homogenate; the amount of enzyme that results in 50% of the maximum inhibition of autooxidation is defined as 1 unit of SOD activity[25]. For analysis of glutathione (GSH) and GSSG, the livers were homogenized with 5% (w/v) metaphosphoric acid. After centrifugation (16000 *g* for 2 min), the tissue homogenate was assessed spectrophotometrically (415 nm) in a microplate reader employing a modified version of the 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, Sigma)/GSSG reductase (Sigma) recycling method using the 43 *N*-ethylmaleimide (NEM, Fluka) conjugating sample preparation technique for GSSG. Samples (10 L) for both GSH and GSSG determination were assayed in 96-well polystyrene plates (Corning) at 37 ℃ in the presence of 10 mmol DTNB, 0.17 mm β-NADPH (Sigma, dissolved in 0.5% (w/v) $NaHCO_3$ as a Stabilizing agent) and 0.5 U/mL GSSG reductase[26].

Western blotting analysis

The technique used for this measurement was protein expression by Western blotting analysis employing the system described by Laemmli[27] for electrophoresis and the blotting technique described by Towbin *et al*[28]. Proteins (80 g) were separated in a 10%-15% polyacrylamide gel and transferred electrically to polvinylidene difluoro membranes (Millipore, Bedford, MA, United States). Subsequently, the membranes were placed in Tris/saline-tamponade/Tween-20 blocking solution (TBST-5% milk powder in Tris-buffered saline containing 0.05% Tween 20) for 60 min at 37 ℃. The membrane was incubated overnight at 4 ℃ with polyclonal eNOS and VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, United States), P-Akt and PI3K (Cell Signaling Technology, Danvers, MA, United States). Thereafter, the membranes were washed with TBST and incubated for one hour at room temperature with an anti-rabbit immunoglobulin antibody coupled to HRP (SIGMA, Glostrup, Denmark). The proteins were detected by chemiluminescence using a commercial ECL kit (Amersham Pharmacia Biotech, Uppsala, Sweden), and the density of specific bands was quantified using a densitometer image (Image J, United States).

Immunohistochemistry

Tissues sections (4 m) soaked in a formalin fixative and embedded in paraffin were subjected to immunohistochemical analysis[29]. This technique consisted of the following steps: deparaffinization, rehydration, antigen retrieval, inactivation of endogenous peroxidase and blocking of nonspecific reactions. The samples were incubated with the primary antibody for 12 h at 4 ℃ us-

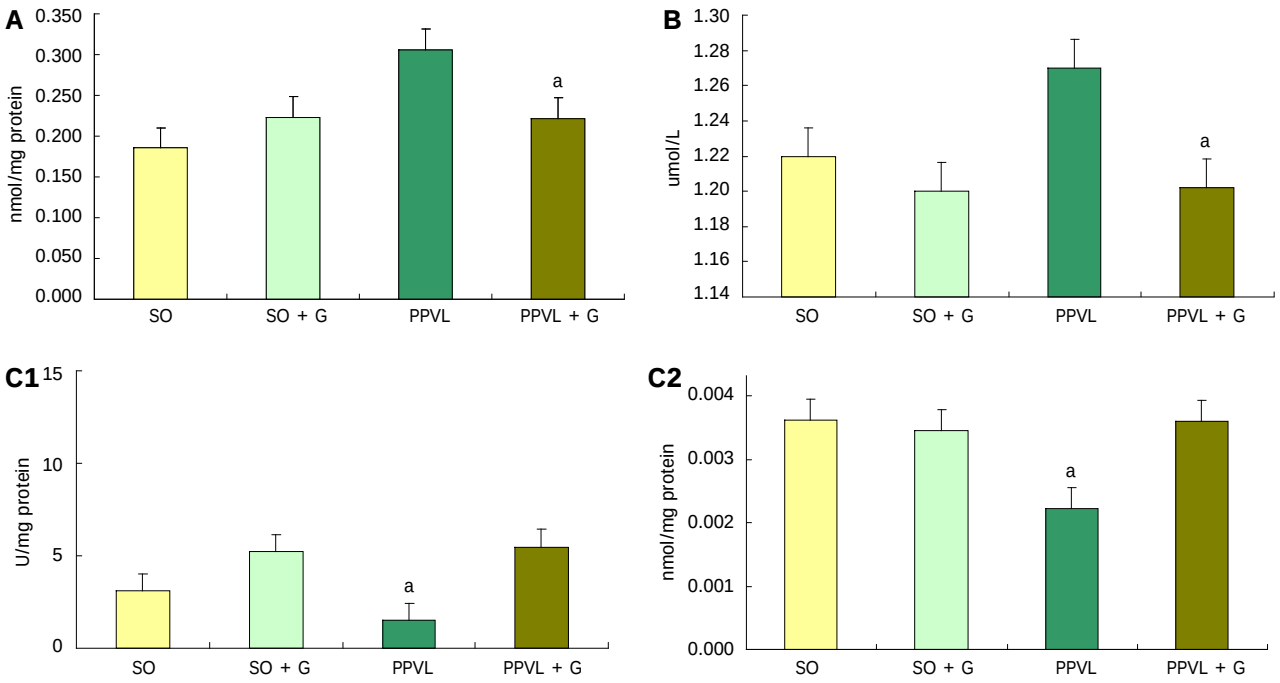


Figure 1 Effect of partial portal vein ligation and glutamine administration on gastric oxidative stress, nitric oxide production, antioxidant enzyme activities. A: Effect of partial portal vein ligation and glutamine administration on gastric oxidative stress; B: Effect of partial portal vein ligation and glutamine administration on gastric nitric oxide production; C: Effect of partial portal vein ligation and glutamine administration on gastric antioxidant enzyme activities: (1) SOD activity; and (2) GSH activity. TBARS concentration. Values are the mean ± SE for 14 rats. $^{a}P < 0.05$ *vs* the sham-operated group. PPVL: Partial portal vein ligation; G: Glutamine; SOD: Superoxide dismutase; GSH: Glutathione; TBARS: Thiobarbituric acid reactive substances; SO: Sham-operated.

ing the specific dilution of each antibody indicated in the instructions. After we applied the streptavidin-biotin complex (LSAB, DAKO) using a diaminobenzidine revelation tetrahydrochloride Kit (DAB, DAKO) and the samples were counterstained with hematoxylin. The antibodies used in the gastric mucosa samples were eNOS and VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, United States), AKT (Cell Signaling Technology, Danvers, MA, United States) and NTT (Sigma, United States).

Statistical analysis

The data were calculated and analyzed using analysis of variance. A *post hoc* multiple comparisons test was performed using the Student Newman-Keuls test. Values were considered significant when $P < 0.05$. All calculations were performed using the statistical program Graphpad Prism, version 14.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Markers of oxidative stress

The cytosolic concentration of TBARS increased in PPVL group, compared to SO. And decrease significantly on PPVL + G group compared to PPVL. Glutamine administration was effective in diminishing TBARS production (Figure 1A).

Nitric oxide levels

The concentrations of nitrites in the gastric tissue (Figure 1B), the values were significantly higher in PPVL group compared to SO. Moreover, nitrite concentrations were similar to PPVL + G and SO groups. This parameter was restored to baseline levels in animals PPVL receiving glutamine.

Antioxidant enzyme activities

Analysis of antioxidant enzyme activities showed that portal vein ligation induced a considerable reduction of gastric SOD in PPVL group compared to SO, SO + G and PPVL + G. Glutamine treatment increased SOD activity and GSH activity (Figure 1C) on PPVL + G group.

Western blotting analysis

Analyses of the protein expression of PI3K (Figure 2B), P-Akt (Figure 2A) and eNOS (Figure 3A) showed that there was reduced expression in the PPVL + G group compared with the PPVL group. This effect was not observed for the expression of VEGF (Figure 3B), for which a significant increase was observed in the PPVL group compared with the SO group, although no reduction was observed when compared with the PPVL + G group.

Immunohistochemistry

Analyses of the reactivity of the eNOS (Figure 4A), Akt (Figure 4C) and NTT (Figure 4D) proteins in the gastric mucosa of rats showed that there was reduced expression in the PPVL + G group compared with the PPVL group. This effect was not observed for the expression of VEGF (Figure 4B), for which a significant increase

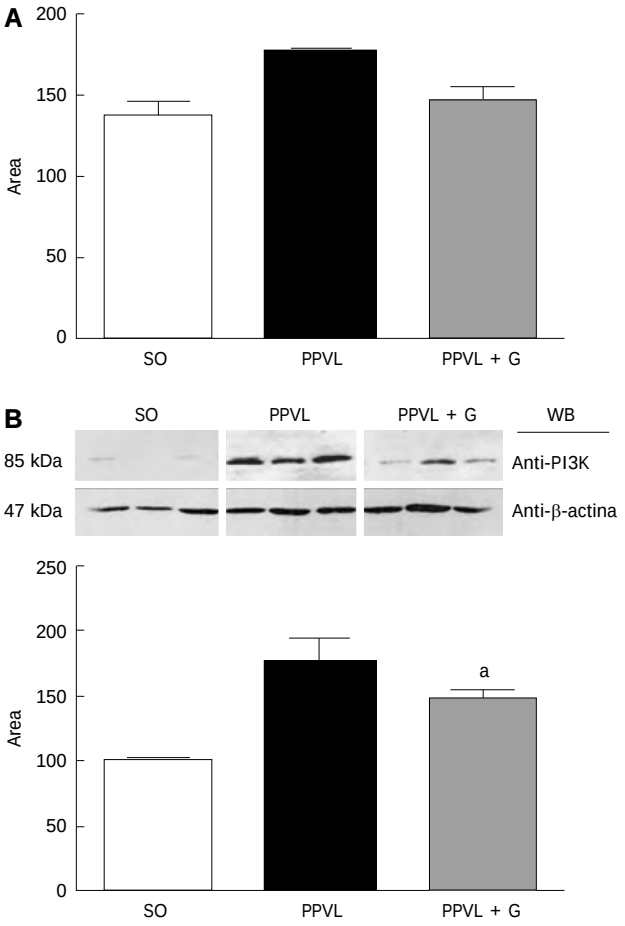


Figure 2 Expression of P-Akt and phosphatidylinositol-3-kinase determined by Western blotting in gastric tissue from rats in the sham-operated group, the partial portal vein ligation group and rats subjected to portal vein ligation and glutamine treatment. A: An increase in P-Akt protein expression was detected in the PPVL group *vs* the SO group; B: An increase in PI3K protein expression was found in the PPVL group *vs* the SO group. The PPVL + G group showed reduced expression of this enzyme *vs* the PPVL group. SO: Sham-operated; PPVL: Partial portal vein ligation; G: Glutamine; PI3K: Phosphatidylinositol-3-kinase; WB: Western blotting. [a]*P* < 0.05 *vs* the SO group.

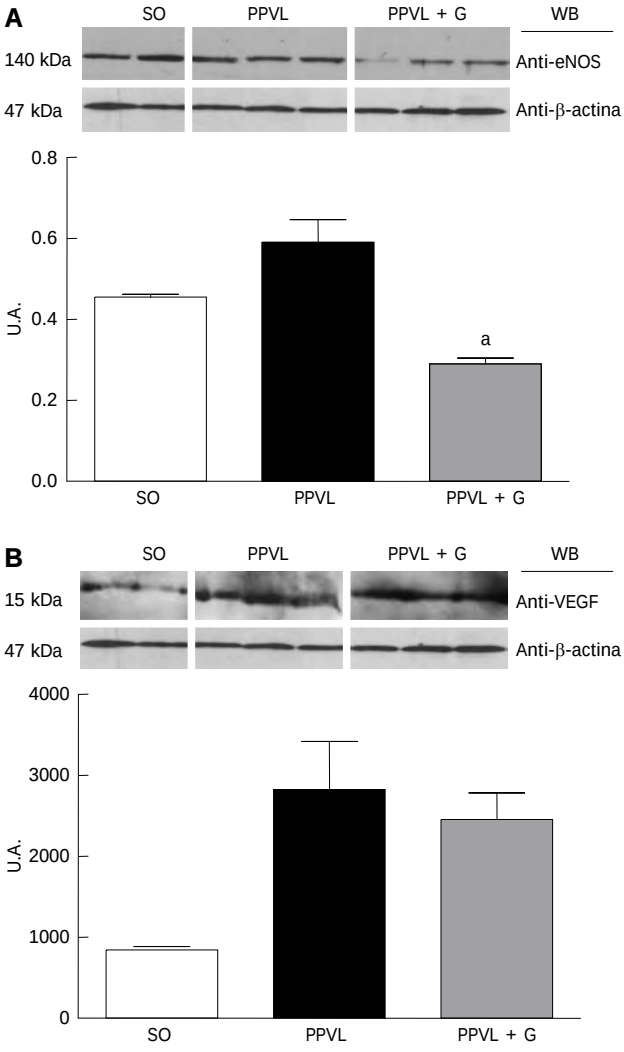


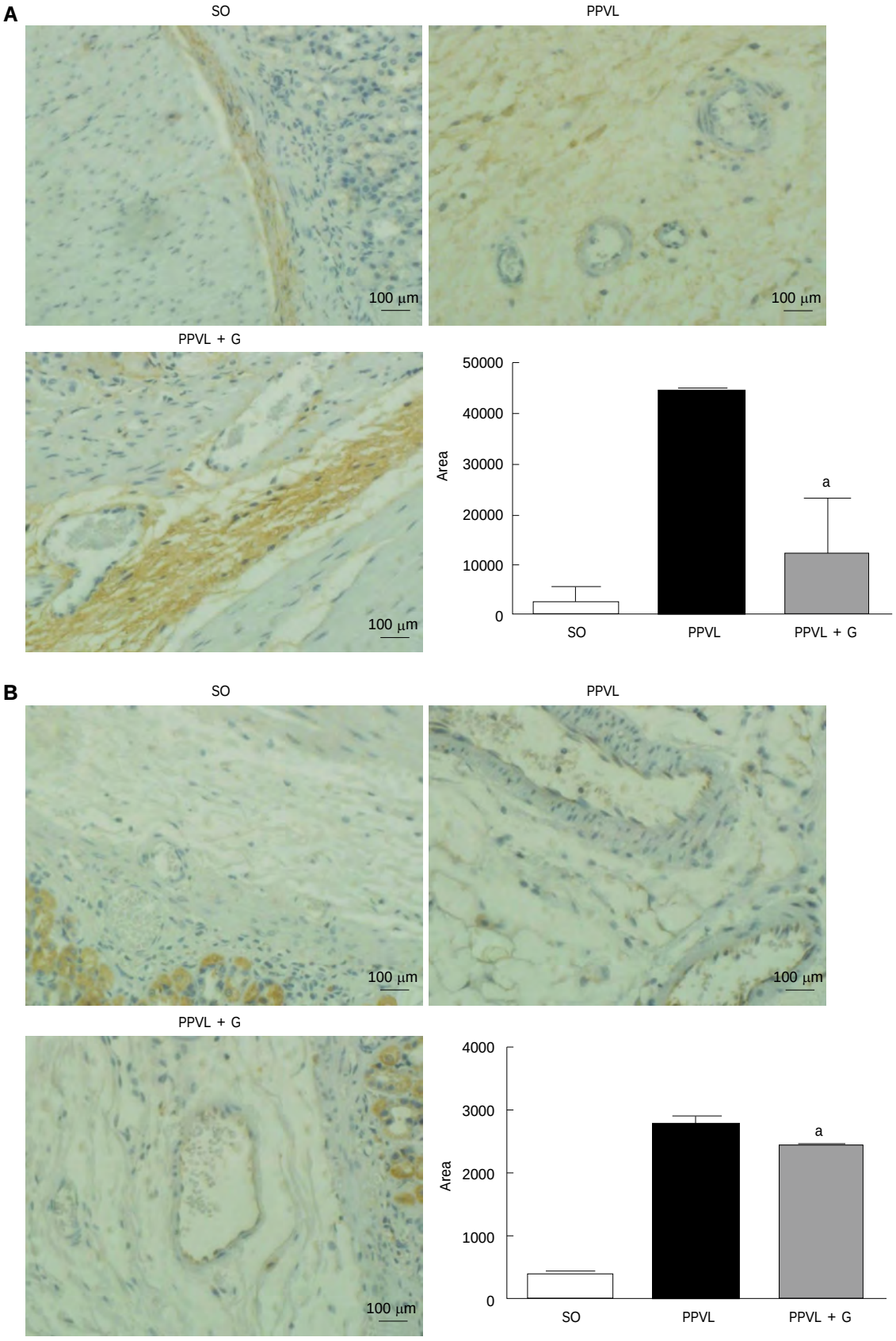
Figure 3 Expression of endothelial nitric oxide synthase and vascular endothelial growth factor determined by Western blotting in gastric tissue from rats in the sham-operated group, the partial portal vein ligation group and rats subjected to partial portal vein ligation and glutamine treatment. A: An increase in eNOS expression was observed in the PPVL group *vs* the SO group. The PPVL + G group showed reduced expression of this enzyme *vs* the PPVL group; B: An increase in VEGF expression was detected in the PPVL group *vs* the SO group. The PPVL + G group did not show a reduction in the expression of VEGF *vs* the PPVL group. SO: Sham-operated; PPVL: Partial portal vein ligation; G: Glutamine; VEGF: Vascular endothelial growth factor; eNOS: Endothelial nitric oxide synthase; WB: Western blotting. [a]*P* < 0.05 *vs* the SO group.

was observed in the PPVL group compared with the SO group, although no reduction was observed when compared with the PPVL + G group.

DISCUSSION

PHG is recognized as a clinical condition in PHI, but the exact pathogenesis of PHG is still unclear. The PPVL model has been extensively studied and found to be a useful tool for understanding the pathophysiology of PHI and PHG. This model has been developed in different animal species, such as rats, mice and rabbits, and it is presently accepted to be suitable for investigating the pathogenesis of PHI and PHG because is highly reproducible and easy to perform, and portal hypertension develops very rapidly. The model used in our study is characterized by prehepatic portal hypertension with maintenance of hepatic structure, hyperdynamic circulation and portal-systemic shunting. Treatment of portal hypertension to prevent complications, particularly gastrointestinal bleeding, is of fundamental importance and occurs under three clinical scenarios: prevention of first bleeding (primary prophylaxis), treatment after an episode of bleeding and prevention of secondary bleeding (secondary prophylaxis). These treatments can be performed using vasoconstrictors, which reduce portal venous flow; vasodilators, which reduce intrahepatic resistance; or a combination of both treatments[30].

Treatment using chemicals or natural products that would prevent complications of PH would represent a significant advance in therapy and could be a way to decrease mortality. Development of experimental models exhibiting pathogenic characteristics similar to those of human disease may help in understanding the mecha-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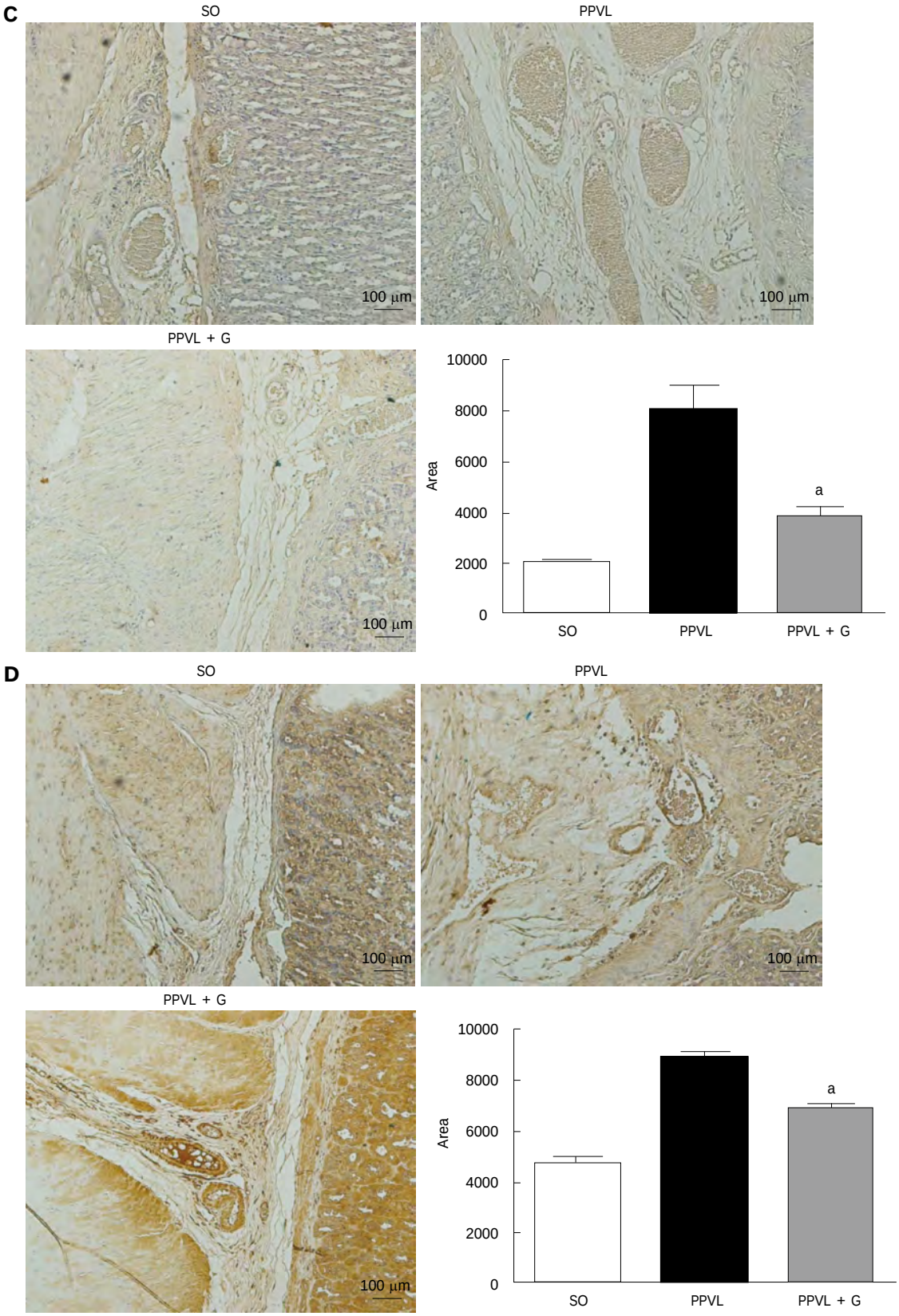

Figure 4 Representative expression of endothelial nitric oxide synthase, vascular endothelial growth factor, Akt and nitrotyrosine determined by immunohistochemical analysis of gastric tissue from rats in the sham-operated group, the partial portal vein ligation group and rats subjected to partial portal vein ligation and glutamine treatment. A: An increase in endothelial nitric oxide synthase (eNOS) expression was observed in the partial portal vein ligation (PPVL) group compared with the sham-operated (SO) group. The PPVL + glutamine (G) group showed reduced expression of this enzyme *vs* the PPVL group; B: An increase in vascular endothelial growth factor (VEGF) expression was observed in the PPVL group *vs* the SO group. The PPVL + G group did not show a reduction in the expression of VEGF *vs* the PPVL group; C: An increase in Akt protein expression was found in the PPVL group *vs* the SO group. The PPVL + G group showed a reduction in the expression of this enzyme *vs* the PPVL group; D: An increase in nitrotyrosine expression was observed in the PPVL group *vs* the SO group. The PPVL + G group showed reduced expression of this enzyme *vs* the PPVL group. Analysis of the field was carried out precisely in the submucosal region, where there is evidence of edema and vasodilatation. eNOS staining; original magnification × 10. $^{a}P < 0.05$ *vs* the SO group.

nisms of portal hypertension and allow testing of new therapeutic modalities. Thus, an experimental model of PH was employed in this study and has contributed to improving our understanding of the pathophysiological conditions presented in humans. PH may be triggered by various agents, leading to cirrhosis.

A study assessing the damage caused by oxidative stress in the gastric mucosa showed that there was an increase of LPO and decreases in the levels of the enzymes SOD, CAT and GPx in injured animals, suggesting that oxidative stress is involved in gastric tissue damage[31]. When we assessed lipid peroxidation in the stomachs of rats, an increase in the level of substances reacting with thiobarbituric acid (TBA-RS) was detected in the PPVL group compared with the SO group, which is suggested to be related to increased oxidative stress. This conclusion was supported by the amounts of TBA-RS observed in the PPVL + G group compared with the PPVL group. Similar results have been shown in other studies that used the flavonoid quercetin and *N*-acetylcysteine, suggesting that the amino acid glutamine exhibits antioxidant potential[32]. In the present study, glutamine reduced submucosal edema and vasodilation as well as reducing lipid peroxidation in homogenized stomachs from animals in the PPVL + G group. Glutamine, as a potential antioxidant, appears to protect the gastric mucosa and decrease oxidative damage to the gastrointestinal tract, which was observed both in this study and in previous studies of colitis employing glutamine[33].

The enzyme SOD, which is responsible for the dismutation of superoxide anion radicals into hydrogen peroxide, is the first line of defense against cellular oxidative stress. The SOD activity determined in the gastric mucosa in a study of patients with liver disease showed that this enzyme is found at reduced levels in these patients compared with control subjects with cirrhosis. However, individuals with chronic liver disease showed no significant changes in the levels of SOD. In liver diseases such as hepatitis, cirrhosis and hepatocellular carcinoma, there are high levels of LPO in the stomach, suggesting involvement of oxidative stress in gastric mucosal lesions in these liver diseases[34]. Studies relate the decrease in SOD enzyme activity to increased oxidative stress[35,36]. In the present study, we observed a decrease in SOD activity in the PPVL group compared with other groups, which could be related to the inactivation of superoxide anions, as this enzyme was acting in the dismutation of EAOS and the formation of H_2O_2. In contrast, animals in the PPVL + G group maintained values of SOD enzyme activity similar to controls. This increased activity of SOD in the PPVL group was due to the increased formation of EAOS, causing oxidative stress. Total GSH has a special physiological importance because glutamine serves as a substrate for glutathione formation. Studies have demonstrated the direct involvement of GSH in colitis[37]. A relationship was shown with glutathione colitis, in which rats with experimental colitis caused by TNBS showed a significant decrease in the level of glutathione compared with the control group[38].

In the evaluation of NO metabolites in stomach homogenates, we observed a reduction in the production of these metabolites in the PPVL + G group compared to the PPVL group. This increase in NO production in the PPVL group can be explained by the process of angiogenesis, which occurs in this model for the purpose of shunting of blood from the obstructed area to the systemic circulation. Moreover, these increased levels of nitric oxide could be reacting with superoxide anions to form peroxynitrite radicals, which are extremely harmful. This NO release becomes more pronounced due to the need for the formation of new blood vessels so that blood can reach the systemic circulation, triggering an increase in RL, thus stimulating lipid peroxidation and oxidative stress[39].

Oxidative stress plays an important role in the pathogenesis of PH because in association with the overproduction of superoxide anions and nitric oxide observed in the model of partial portal vein ligation, peroxynitrite formation occurs. Researchers have noted specific actions of ONOO as a modulator of intracellular signaling pathways regulating inflammatory responses, including induction of angiogenesis and VEGF.

The significant reduction of lipid peroxidation observed in animals in the PPVL + G group demonstrates effective action of the glutamine in the process of lipid peroxidation. This result is consistent with the expected capacity of glutamine in the formation of inactivating EAOS, especially in reducing the formation of peroxynitrite. Peroxynitrite reacts with free tyrosine and tyrosine residues in protein molecules to produce nitrotyrosine. Alternatively, EAOS can activate tyrosine to form tyrosyl, a radical that in turn oxidizes NO to produce nitrotyrosine (NTT)[40,41]. The generation of peroxynitrite was assessed based on the level of expression and immunoreactivity of NTT, with significant reductions being observed for both parameters in animals in the PPVL + G group, demonstrating the effectiveness of glutamine with respect to the oxidative/nitrosative damage that occurs in the gastric mucosa.

Upregulation of eNOS initiates the post-translational level mediated by Akt, which increases its activity at any concentration of cytosolic Ca^{2+}[42]. During early cirrhosis, this pathway is stimulated by different types of stimuli, such as VEGF, inflammatory cytokines and mechanical shear forces[42]. In advanced stages of portal hypertension, bacterial translocation also activates eNOS *via* tumor necrosis factor, increasing tetrahydrobiopterin, which is an essential element that acts as a cofactor of the enzyme. According to several studies, other mechanisms, such as changes in the subcellular localization of eNOS[43], *S*-nitrosylation[44] or degradation of asymmetrical dimethylarginine, may be involved in regulating the activity of eNOS[45]. We observed in this study that glutamine reduced eNOS protein expression and the immunoreactivity of the enzyme in the gastric mucosa of animals in the PPVL + G group. One explanation for

this finding is that glutamine, due to its involvement in NO synthesis, is involved in reduction of the cytosolic levels of Ca^{2+}, which is related to the levels of Akt; in turn, Akt levels are stimulated by mechanical stress shear. Throughout this process, this hemodynamic mechanism would be inhibited due to reduced levels of NO triggered by the action of glutamine.

Angiogenesis is characterized by the formation of new vascular structures and a pathophysiological phenomenon that has been further investigated in recent years due to the critical role it plays in the pathogenesis of disease and its potential as a therapeutic target. Additionally, angiogenesis contributes to a number of physiological processes, such as wound healing and the reproductive cycle[46]. NO released by dilation and increased vascular permeability as well as the migration, proliferation and survival of endothelial cells plays a crucial role in angiogenesis. Many molecules have been implicated as modulators of the angiogenic process, such as tumor necrosis factor, interleukins, angiopoeitins and growth factors, including VEGF. Indeed, VEGF stimulates NO production by NOS, increasing vascular permeability and the proliferation and survival of endothelial cells[47].

Furthermore, overproduction of ONOO, which is formed by the reaction of superoxide anions and nitric oxide, is observed under inflammatory conditions[48], whereas cytotoxic actions of ONOO have been reported *via* the modulation of intracellular signaling pathways that regulate inflammatory responses, including induction of angiogenesis and increased levels of VEGF. Studies in the retina of rats with diabetes induced by streptozotocin demonstrated the formation of nitrotyrosine and increased effects regarding stimulation of VEGF. These conditions have been studied because they induce the generation of reactive oxygen species (ROS). EAOS are involved in triggering the overexpression of VEGF in various cell types[49,50], but the molecular mechanisms underlying this effect remain to be elucidated. Studies in patients, in animals and *in vitro* indicate that formation of nitrotyrosine, a marker of ONOO, is associated with increased VEGF expression during diabetic microvascular disease, atherosclerosis and tumor angiogenesis[51,52]. In addition, studies using a cultured cell line showed that formation of ONOO stimulates an increase in VEGF[53]. In the present study, we observed increased expression and immunoreactivity of VEGF in animals in the PPVL group, but there was no significant reduction of these parameters in animals in the PPVL + G group. We suggest that these results demonstrate direct action of glutamine on NO synthesis to reduce its levels, rather than only affect the action of growth factors and the associated cascade of events.

VEGF also acts by stimulating PI3K, which activates eNOS in an Akt-dependent manner, resulting in activation of eNOS and increased NO production. PI3K phosphorylates Akt, which can rapidly activate eNOS[54]. The Akt pathway has emerged as a signaling pathway in all cells of higher eukaryotes, and Akt has been found to be one of the most important and versatile kinases for understanding human physiology and disease. Recent studies have been performed to elucidate the molecular details of the regulation of Akt and its role in human disease. The NO released due to eNOS stimulation is involved in vasodilation, vascular remodeling and angiogenesis[55]. The Akt signaling pathway also leads to increased production of transcription factors induced by hypoxia (HIF1α and HIF2α), partly through the activation of an mTOR-dependent mechanism[56]. The activation of eNOS through Akt/B kinase leads to increased NO production, vasodilatation and increased splanchnic circulation[57]. Akt activation occurs due to an increase in shear stress-induced endothelial disruption, although other mechanisms may be involved[58].

In this study, we observed a significant increase in the expression and immunoreactivity of Akt in the PPVL group and a reduction in these parameters in the PPVL + G group. These results can be explained by the role that glutamine plays in the production of NO, which in turn, triggers a chain reaction, is overproduced and dilates the splanchnic vessels, gradually increasing shear stress, which is the mechanical stimulus for the activation of Akt. In addition, it was observed that VEGF levels are unchanged in the PPVL + G group compared to the PPVL group, and the VEGF RTK pathway acts as a means for the release of PIK3, eNOS and P-Akt, leading to the production of more NO, which is a feature present in the hyperdynamic circulation associated with PH. Therefore, glutamine acts to mitigate this situation not by reducing the levels of NO *via* VEGF, but by reducing the shear stress triggered by NO synthesis through inhibition of *L*-arginine, thereby reducing the levels of Akt, which is stimulated by mechanical stress in blood vessels. In conclusion, we describe the beneficial effects of glutamine treatment on oxidative stress, including reduced portal pressure, normalization of SOD and reduction of NO production by eNOS, mediated by the PI3K-Akt-eNOS pathway. VEGF levels tended to decrease, although this trend was not found to be statistically significant.

COMMENTS

Background

Portal hypertension (PH) is the main complication of cirrhosis and is through its development is arising other liver diseases. Among them we mention the gastropathy of portal hypertension, which is characterized by the development of hyperdynamic splanchnic circulation. The main modulator of this process is nitric oxide release and their pathways in the pathophysiology of PH is still being studied. As there is no effective treatment for PH, it was decided to study the role of glutamine, an amino acid that has been used in the treatment of colitis, the clinic and evaluate its involvement both in oxidative stress, as in the intracellular stimulus for release nitric oxide.

Research frontiers

Studies are relevant in clinical hypertension, especially in hepatology. In addition, PH may be associated with other diseases such as schistosomiasis and budd-chiari syndrome. Therefore, the study of PH has a great clinical relevance for gastroenterology, since there is still no effective therapeutic treatment.

Innovations and breakthroughs

There are numerous studies using the experimental model of partial portal vein ligation,with which it mimics the complications of PH. Have been evaluated for

treatment of other molecules such as PH, quercetin and *N*-acetylcysteine. The results were similar with glutamine to these molecules, but there is when the comparative results on the role of intracellular pathways.

Applications

These results suggest that the amino acid glutamine is a molecule with therapeutic potential and can be used in the treatment of portal hypertension, reducing damage to the gastric mucosa caused by hypertension portal gastropathy.

Terminology

PH is characterized by increased vascular resistance and/or blood splanchnic flow; Glutamine: Essential amino acid involved in several cellular functions, among them acting as a substrate for the synthesis of antioxidants such as glutathione; Gastropathy of portal hypertension: Clinical syndrome secondary to cirrhosis, which is characterized by the formation of edema and dilation of gastric submucosal tissue.

Peer review

This is a quantitative study, in which the authors analyze the antioxidant effect and the possible involvement of glutamine in the gastric mucosa in an animal model of PH. The results are interesting and show that this molecule may have therapeutic potential in treatment of gastric mucosal lesions triggered by PH.

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ORIGINAL ARTICLE

Resistin mediates the hepatic stellate cell phenotype

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Abstract

AIM: To describe the role of resistin in liver fibrosis.

METHODS: For the *in vivo* animal study, Sprague Dawley rats were subjected to bile duct ligation (BDL) for 4 wk. Rat liver, adipose tissue (epididymal fat) and serum were analyzed for resistin expression. For the *in vitro* experiment, rat primary hepatic stellate cells (HSCs) and Kupffer cells (KCs) were used. HSCs were exposed to recombinant resistin, and collagen Ⅰ, transforming growth factor β1, α smooth muscle actin, tissue inhibitor of metalloproteinase 1 and connective tissue growth factor expression were analyzed. Resistin gene and protein expression was quantified as was the expression of pro-inflammatory cytokines including tumor necrosis factor α (TNFα), interleukin (IL)-1, IL-6, IL-8 and monocyte chemotactic protein-1 (MCP-1). The effects of resistin on HSC proliferation, migration and apoptosis were determined. The effects of resistin on KCs were also investigated.

RESULTS: Following BDL, rat epididymal fat and serum rather than liver showed higher resistin expression compared to control rats. In liver, resistin was expressed in quiescent HSCs and KCs. Resistin treatment resulted in enhancement of *TNFα*, *IL-6*, *IL-8* and *MCP-1* gene expression and increased IL-6 and MCP-1 protein in HSCs. Resistin activated HSC phospho-MAPK/p38, and p38 inhibition diminished IL-6 and MCP-1 expression. Furthermore, resistin facilitated HSC proliferation and migration, but decreased apoptosis which was *via* an IL-6 and MCP-1 mechanism. Finally, resistin-induced transforming growth factor β1 from KCs enhanced HSC collagen Ⅰ expression.

CONCLUSION: Resistin directly and indirectly modulates HSC behavior towards a more pro-fibrogenic phenotype.

Key words: Resistin; Hepatic stellate cell; Kupffer cell; Liver fibrosis; Monocyte chemotactic protein-1

Core tip: Resistin activated hepatic stellate cells (HSCs) phospho-MAPK/p38, and p38 inhibition diminished interleukin 6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) expression. Furthermore, resistin facilitated HSC proliferation and migration, but decreased apoptosis which was *via* an IL-6 and MCP-1 mechanism. Finally, resistin-induced transforming growth factor β1 from Kupffer cells enhanced HSC collagen Ⅰ expression. Resistin directly and indirectly modulates HSC behavior towards a more pro-fibrogenic phenotype.

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INTRODUCTION

Metabolic alterations such as glucose intolerance, increased energy expenditure, and negative nitrogen balance with depletion of fat and skeletal muscle mass are frequently encountered in patients with cirrhosis[1,2]. In particular, glucose intolerance and insulin resistance are almost universal[3,4] and, in part, mediate the progression of fibrosis[5]. However, the mechanisms whereby metabolic alterations mediate disease progression are unclear. Adipose tissue secreted proteins (adipokines) such as leptin and adiponectin modulate metabolic homeostasis and have direct effects on the hepatic fibrogenic cascade. For example, leptin promotes liver fibrosis, while adiponectin is anti-inflammatory and anti-fibrotic[6-9]. Resistin, another adipokine, has been reported to be associated with impaired insulin sensitivity and glucose intolerance[10-13], but its role in hepatic fibrosis has not been adequately delineated[14-17].

Resistin is almost exclusively expressed in the white adipose tissue of rodents, but is expressed in humans predominantly by monocytes/macrophages[18]. Several reports indicate that the serum levels of resistin are elevated in cirrhosis[14-16], increasing progressively with worsening liver function as determined by the Child-Pugh class[17]. Furthermore, in patients with liver disease, resistin levels are correlated with the extent of insulin resistance and with clinical complications and prognosis[16]. In a recent animal study[19], hyperinsulinemia and increased tumor necrosis factor α (TNFα) secretion following bile duct ligation (BDL) were shown to up-regulate adipose tissue resistin gene expression which could subsequently contribute to liver fibrosis. A recent human study noted that resistin expression was low in normal liver, but was increased in severe fibrosis, suggesting that intra-hepatic resistin derived from monocytes/macrophages might contribute to fibrosis[15,20,21].

In the present study, we undertook *in vivo* and *in vitro* studies to elucidate the role of resistin in liver fibrosis. We show that resistin has increased expression in the epididymal fat and serum of cirrhotic rats. Resistin has a pro-inflammatory role in mediating the release of TNFα, interleukin (IL)-6, IL-8 and monocyte chemotactic protein-1 (MCP-1) in hepatic stellate cells (HSCs). Importantly, we demonstrate that resistin directly and indirectly mediates HSC activated phenotype in IL-6/MCP-1 and transforming growth factor (TGF) β1 dependent mechanisms, respectively, indicating that resistin contributes to the pro-inflammatory and pro-fibrotic phenotype of activated HSCs.

MATERIALS AND METHODS

Materials

Recombinant mouse resistin protein, recombinant IL-6, and MCP-1 ELISA kits, IL-6, MCP-1 and TGFβ1 antibodies were purchased from RD Systems (Minneapolis, MN, United States). Nycodenz, α smooth muscle actin (αSMA) mouse antibody was purchased from Sigma-Aldrich (St. Louis, MO, United States). Pronase E, DNase I and collagenase B were purchased from Roche Applied Sciences (Indianapolis, IN, United States). Resistin rabbit polyclonal antibody was purchased from Abbiotec TM (San Diego, CA, United States). p-p38, pERK1/2, pJNK, nuclear factor κB (NF-κB), p-p65 and p-p50 mouse monoclonal antibodies, p-p38 inhibitor (SB203580) and pJNK inhibitor (SP600125) were purchased from Cell Signaling Technology, Inc (Beverly, MA, United States). The BrdU ELISA kit was purchased from Roche Diagnostics (Castle Hills, NSW, Australia). Anti-mouse IgG conjugated to horseradish peroxidase was purchased from GE Healthcare Life Sciences (Piscataway, NJ, United States). DMEM medium was obtained from Invitrogen (Carlsbad, CA, United States).

Animals

Male Sprague Dawley (SD) rats were obtained from the Animal Resources Centre (Perth, Australia). All animals were maintained under 12-h light/dark cycles with food and water *ad libitum*. For the *in vivo* experiment, BDL or a sham surgical procedure was performed on rats. After 4 wk, rat liver, epididymal fat and serum were collected for resistin quantification. All experimental protocols were approved by the Sydney West Area Health Service Animal Research Ethics Committee.

Isolation and culture of rat hepatic stellate cells and Kupffer cells

Rat HSCs were isolated by a two-step (collagenase B and pronase E) perfusion method under ketamine and xylazine anesthesia as reported previously[6]. Briefly, rat liver was perfused through the portal vein using Ca^{2+}- and Mg^{2+}-free Gey's Balanced Salt Solution (GBSS, Sigma, United States) and then sequentially with pronase E followed by collagenase B (Roche Applied Science, Castle Hills, NSW, Australia). The liver was excised, gently dispersed in GBSS containing 0.01% DNase I and the cell suspension filtered through a sterile nylon mesh and subjected to low-speed centrifugation. The resultant cell pellet was mixed with 30% Nycodenz to obtain an 11% final Nycodenz/cell suspension. After centrifugation at 1400 *g* for 20 min, HSCs were collected, resuspended in culture medium, and plated on 6 well plates with 10% FCS/DMEM at a density of 0.8×10^6 cells/well. Cell viability was assessed by trypan blue exclusion and was routinely more than 95%. Purity was 95% as determined by morphology, vitamin A autofluorescence and desmin positivity. HSCs were maintained in 95% air and 5% CO_2 in DMEM (Gibco, United States) with 10% FCS and 1% penicillin/streptomycin. KCs were further obtained and purified by elutriation[6]. KCs were identified by their ability to phagocytose latex beads; viability was > 96% and purity > 98%. KCs were cultured in 10% FCS/DMEM/1% penicillin-streptomycin.

Treatments: For recombinant mouse resistin (RD Systems, Minneapolis, MN, United States), we undertook a dose ranging study based on previous reports[20-23] using 10, 50, 250 and 500 ng/mL. We found that 500 ng/mL

was the optimal dose which was used in all subsequent experiments. Primary rat HSCs and KCs were cultured for the time periods indicated and serum starved (0.2%) for 4 h prior to treatment. Subsequently, control (vehicle) and resistin (500 ng/mL) were added to the culture wells. After 24 h or extended culture as indicated, total RNA and protein were extracted. For the KC-HSC co-culture experiment, control (vehicle) and resistin (500 ng/mL) were added to cultured KCs at day 2 for 24 h, then KCs were washed three times with PBS and fresh medium was added and cultured for another 24 h. Afterwards, KC conditioned medium (KM) was transferred to HSCs at day 4 for 24 h co-culture. In one experiment, lipopolysaccharide (LPS, 50 ng/mL) was used to further activate cultured KCs.

Real-time reverse transcription polymerase chain reaction

Total cellular RNA was prepared from HSCs using TRI@ REAGENT (Molecular Research Center, INC., Cincinnati, OH, United States). Complementary DNA (cDNA) was synthesized from 1 μg RNA using Super-Script Ⅲ reverse transcriptase and 0.5 nmol of random primers (Invitrogen, Carlsbad, CA, United States). Real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed using SYBR Green Platinum SYBR Green SuperMix (Invitrogen, United States). The synthesized cDNA was amplified using the following sequence specific primers: resistin 5'-CAAGACTTCAGCTCCCTACTGC-3' (forward) and 5'-GACGGTTGTGCCTTCTGG-3' (reverse); collagenα1 (Ⅰ) 5'-TTCACCTACAGCACGCTTGTG-3' (forward) and 5'-TCTTGGTGGTTTTGTATTC-GATGA-3' (reverse); TGFβ1 5'-TCGACATGGAGCT-GG TGAAA-3' (forward) and 5'-GAGCCT-TAGTTTGGACAGGATCTG-3' (reverse); αSMA5'-CGATAGAACACGGCATCATC-3' (forward) and 5'-CATCAGGCAGTTCGTAGCTC-3' (reverse); tissue inhibitor of metalloproteinase 1 (TIMP1) 5'-AAGGGC-TACCAGAGCGATCA-3' (forward) and 5'-GGTATT-GCCAGGTGCACAAAT-3' (reverse); connective tissue growth factor (CTGF) 5'-CGCCAACCGCAAGATTG-3' (forward) and 5'-ACACGGACCCACCGAAGAC-3' (reverse); IL-6 5'- CCCTTCAGGAACAGCTATGAA-3' (forward) and 5'-ACAACATCAGTCCCAAGAAGG-3' (reverse); IL-1 α 5'-ACATCCGTGGAGCTCTCTT-TACA-3' (forward) and 5'-TTAAATGAAC-GAAGTGAACAGTACAGATT-3' (reverse); IL-1β 5'-TACCTATGTC TTGCCCGTGGAG-3' (forward) and 5'-ATCATCCCACGAGTCACAGAGG-3' (reverse); TNFα 5'-GCCCAGACCCTCACACTC-3' (forward) and 5'-CCACTCCAGCTGCTCCTCT -3'(reverse); IL-8 5'-TCTGCAGCTCTGTGTGAAGG-3' (forward) and 5'-AATTTCTGGTT TGGCGCAGT-3' (reverse); MCP-1 5'-AGCATCCACGTGCTGTCTC-3' (forward) and 5'-GATCATCTTGCCAGTGAATGAG-3' (reverse). The relative amount of mRNA was calculated by reference to a calibration curve. The final result for each sample was normalized to the respective β actin value.

Immunoblotting: Cell culture media were removed and the cells washed with PBS and lysed on ice in a buffer containing 20 mmol/L Tris, 0.5 mmol/L $MgCl_2$, 1 mmol/L Dithiothreitol (DTT), 3 mmol/L NaN_3, and a mixture of protease and phosphatase-inhibitors. Cell lysates were disrupted using a sonicator on ice. After centrifugation at 13000 *g* for 15 min, the supernatant was collected as cytoplasmic protein. Nuclear protein was extracted as described previously[6]. The protein concentration was determined using the Bradford Protein Assay (Bio-Rad, Sydney, Australia). Immunoblotting was performed as previously described with some modifications[6,24]. Total protein (20 μg per lane) was resolved by electrophoresis on 12% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) under reducing conditions. The electrophoresed proteins were electrotransferred onto Polyvinylidene difluoride membranes (Immobilin-P, Millipore, Bedford, MA, United States). The membranes were blocked with 5% skim milk (Resistin, TGFβ1, p-p38, pERK1/2, pJNK, NF-κB p-p65, NF-κB p-p50 and αSMA) for 60 min and then incubated overnight with primary antibody (Resistin 1:200, p-p38 1:1000, pERK1/2 1:1000, pJNK 1:1000, p-p65 1:1000, p-p50 1:1000, TGFβ1 1:500, αSMA 1:2000) at 4 ℃. After 3 washes with 0.05% Tween-20/TBS, anti-mouse IgG (peroxidase conjugate) secondary antibody was applied. Blots were visualized by enhanced chemiluminescence (Pierce Perbio, Rockford, IL, United States). All images were quantified by densitometry.

Sirius red staining and quantification of collagen in HSCs

Sirius red staining and collagen quantification were performed according to a previously published protocol[6,25]. Briefly, Sirius red F3BA solution (0.1% in saturated picric acid) was added to cell layers fixed in Bouin's solution. After 1 h the cell layers were washed in tap water and again in 0.01 mol/L HCl to remove unbound dye. For collagen quantification, the dye was dissolved in 0.1 mol/L NaOH and absorbance determined at 450 nm. The amount of collagen was normalized to the protein concentration using the Bradford Reagent. Assays were performed in duplicate.

Enzyme-linked immunosorbent assay

The media were collected from cultured HSCs following 24 h stimulation with resistin (500 ng/mL). Levels of IL-6 and MCP-1 in the media were determined according to the manufacturer's instructions (RD Systems). Rat serum was collected and resistin concentration detected using a resistin rat ELISA kit (Boivendor) according to manufacturer's instructions.

HSC proliferation: Cell proliferation was analyzed using a BrdU-based enzyme-linked immunosorbent assay (Roche Diagnostics) according to the manufacturer's

instructions. HSCs at day 4 were treated with resistin (500 ng/mL) or other agents as indicated for 24 h. The cells were subsequently labeled with BrdU for 2 h at 37 ℃. Cells were then fixed and incubated with a peroxidase-conjugated anti-BrdU antibody for 90 min at room temperature. After adding the peroxidase substrate, 3,3',5,5'-tetramethylbenzidine, BrdU incorporation was determined by measuring optical densities at 450 nm (background 620 nm).

HSC migration: HSC migration was assessed both with the wound scratch assay and a modified Boyden chamber. For the wound scratch assay, using a sterile 200 μL pipette tip, three separate wounds were generated through the cell monolayer. HSCs (90% confluence) at day 6 cultured in 12-well plates were treated with resistin (500 ng/mL) or other agents as indicated. The scratch area was photographed immediately and 6 h after scratching and cell migration into the scratch area calculated as the area covered by cells in the percentage of the initial scratch area. For the second method, a cell culture insert (12 well, BD) was used and the porous membrane (pore size 8 μm) of the filter was coated with 30 μg/mL collagen I at 37 ℃ for 30-60 min. HSCs at day 6 were trypsinized and placed into the upper chamber (10^5 cells/mL). The lower wells were filled with resistin (500 ng/mL) or other agents as indicated. After 6 h of incubation at 37 ℃, cells adhering to the upper side of the filter were removed with a cotton swab. The filters were then fixed with 100% methanol and stained with HEMA-3. The numbers of HSCs on the lower side of the filter were counted in five randomly chosen microscopic fields at a magnification of × 400 by changing the focus.

HSC apoptosis: Annexin-V/PI labeling was used to detect HSC apoptosis. Briefly, trypsinized HSCs were washed twice in PBS, stained with annexin-V (10 μL) and PI (5 μL) for 10 min, and the apoptotic rate quantified by FACS Calibur flow cytometry (Becton Dickinson Inc.) at 488 nm. More than 1×10^4 cells were detected, and the results were analyzed with FlowJo software (Treestar, United States). The population of apoptotic cells was identified as annexin V+/PI-. The percentage of apoptotic cells was calculated according to total annexin V+/PI- divided by total cells.

Statistical analysis

The results are expressed as mean ± SD. Comparisons between 2 groups were analyzed using the Student *t* test. For the comparison of more than two groups, we used two-way ANOVA. *P* values < 0.05 were considered statistically significant. All calculations were performed using Statistical Program for Social Sciences (SPSS) software 13.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Resistin expression is up-regulated in cirrhotic rats

Resistin expression in liver, epididymal fat and serum in BDL and sham rats was examined. We noted that resistin expression in epididymal fat was considerably higher than that in liver in the BDL or sham rats (Figure 1A and B, all $P < 0.01$). BDL rat epididymal fat mRNA and protein level were further up-regulated compared to sham rats (Figure 1A and B, both $P < 0.05$). Similarly, BDL rat serum resistin level was also elevated (Figure 1C, $P < 0.05$). However, liver resistin mRNA and protein were unchanged in the BDL and sham groups (Figure 1A and B). These findings suggest that increased adipose resistin rather than liver resistin may play a vital role in resistin-mediated liver injury in rodents. Therefore, we undertook detailed *in vitro* experiments in order to explore the impact of exogenous resistin on HSC activated phenotype.

Resistin is expressed in quiescent HSCs and KCs

Resistin mRNA was detected in quiescent rat HSCs (Figure 1D) at day 1 and was reduced by 90% ($P < 0.01$) following activation for 8 d on plastic. Resistin mRNA was expressed in quiescent KCs (day 1) and activated KCs (3 d), without significant changes over time. LPS (50 ng/mL for 24 h) stimulation of KCs at day 3 did not enhance resistin expression (Figure 1D). Consistent with the mRNA data, resistin protein expression declined 6-fold in HSCs at day 8 with no change in KCs at day 3 and after LPS stimulation (Figure 1E). These data indicated that autocrine HSC resistin and paracrine KC resistin are unlikely to be of major importance in mediating any effects on activated HSCs.

Resistin promotes a pro-inflammatory phenotype in HSCs

Pro-inflammatory cytokines and chemokines play a permissive role in liver fibrosis[26-29] and previous reports suggest that resistin increases MCP-1 secretion. We evaluated the expression of TNFα, IL-1α, IL-1β, IL-6, IL-8 and MCP-1 in rat HSCs after stimulation with resistin. As demonstrated, resistin (500 ng/mL) stimulation for 24 h markedly up-regulated the expression of TNFα, IL-6, IL-8 and MCP-1 mRNA (Figure 2A, all $P < 0.05$), but not that of IL-1α and IL-1β. To rule out any potential effects of inadvertent endotoxin contamination, we repeated these studies in the presence of Polymyxin B and noted no difference in the gene expression profile (data not shown). Finally, using trypan blue staining and LDH assays at 24, 48 and 72 h, we excluded the possibility of direct cellular toxicity due to the resistin dose used (data not shown). Since IL-6 and MCP-1 are well documented to play a role in mediating hepatic fibrosis, their protein concentrations were estimated in conditioned medium. As shown in Figure 2B, resistin administration increased IL-6 and MCP-1 concentrations 1.7 and 1.8 fold after 24-h of treatment (both $P < 0.05$).

Resistin enhances HSC proliferation and migration but diminishes HSC apoptosis via an IL-6 and MCP-1 pathway

During the process of chronic liver injury, activated

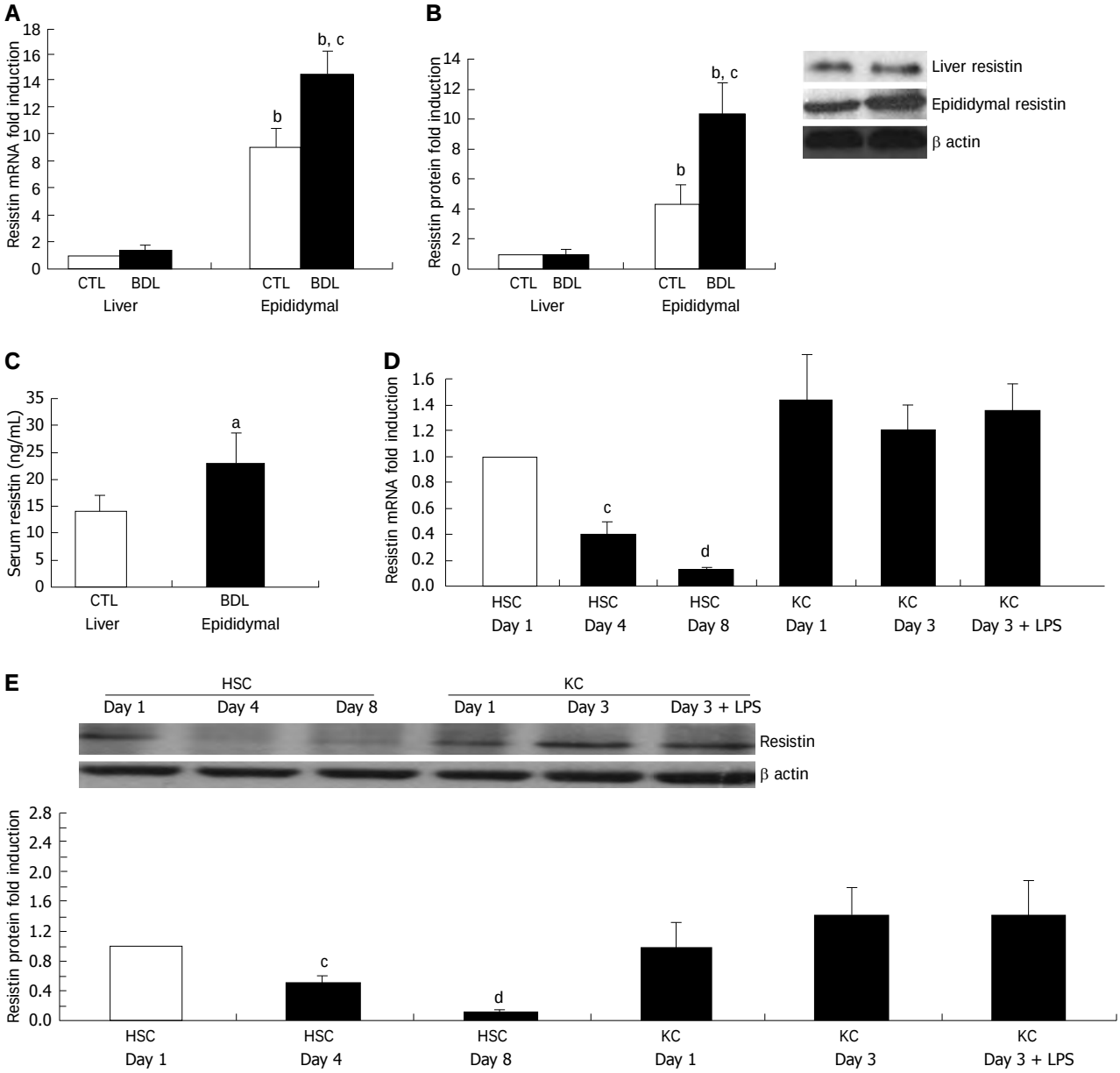

Figure 1 Rat extrahepatic but not intrahepatic resistin is up-regulated in cirrhosis. For the *in vivo* animal study, Sprague Dawley rats were subjected to bile duct ligation (BDL) for 4 wk. Liver, adipose tissue (epididymal fat) and serum were collected to determine resistin expression by quantitative polymerase chain reaction (qPCR), immunoblot and enzyme-linked immunosorbent assay. For the cell culture study, rat hepatic stellate cells (HSCs) and Kupffer cells (KCs) were isolated and cultured on plastic. HSC and KC total RNA/protein were extracted at different culture times (day 1, 4 and 8 for HSCs; day 1 and 3 for KCs). One group of KCs at day 2 were treated with Lipopolysaccharide (LPS) (50 ng/mL) for 24 h. qPCR and Immunoblot were performed for quantification of resistin mRNA and protein. β actin was used as an internal control. A: mRNA expression of resistin in liver and epididymal fat; B: Protein expression of resistin in liver and epididymal fat; C: Serum resistin concentrations; D: mRNA expression of resistin in HSCs and KCs on different culture days; E: Protein expression of resistin in HSCs and KCs on different culture days. Results are mean ± SD of at least three independent experiments performed in triplicate. $^{a}P < 0.05$ and $^{b}P < 0.01$ increased *vs* rat liver, HSC control at day 1 or sham rat serum; $^{c}P < 0.05$ and $^{d}P < 0.01$ decreased *vs* liver of control sham rat or HSC control at day 1.

HSCs proliferate and migrate to sites of inflammation and have reduced apoptosis. This phenotype is part of the expected adaptive wound healing response to injury. Hence, we sought to determine the role of resistin in mediating activated HSC behavior. As demonstrated in Figure 3A, resistin enhanced HSC proliferation by approximately 90% compared to the control ($P < 0.01$). Using the wound scratch assay and a modified Boyden chamber, compared to the control, resistin treatment resulted in an approximately 80% and approximately 220% increase in HSC migration, respectively ($P < 0.05$, Figure 3A and B). We next examined the role of resistin on HSC apoptosis. In contrast, resistin significantly reduced HSC apoptosis (56%, $P < 0.05$, Figure 3A), as shown by annexin V/IP flow cytometry. Finally, we determined whether up-regulation of IL-6 and MCP-1 was responsible for the changed HSC phenotype by resistin. As expected, resistin-mediated HSC proliferation, migration and apoptosis were partially, but significantly reversed (all $P < 0.05$, Figure 3A and B) by IL-6 (5 μg/mL) and

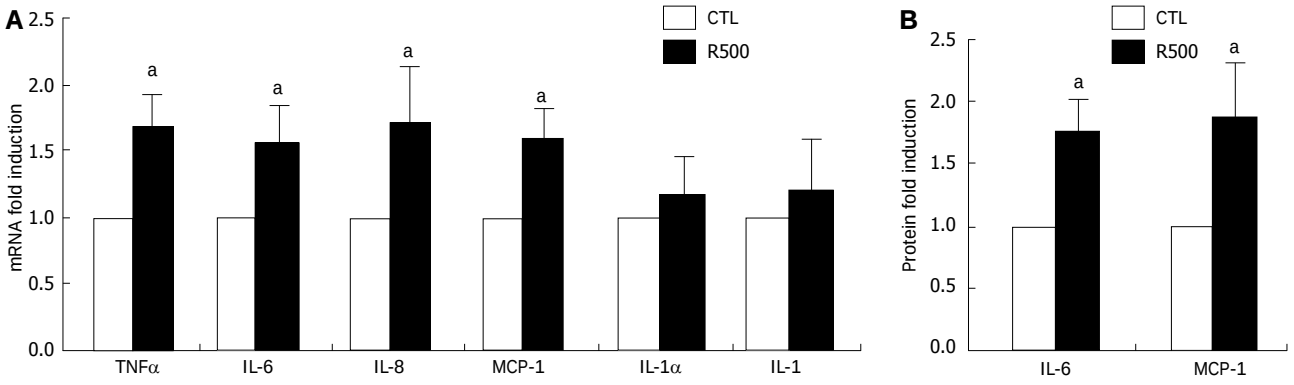

Figure 2 Resistin enhances the expression of tumor necrosis factor α, interleukin 6, interleukin 8 and monocyte chemotactic protein-1 in hepatic stellate cells. Rat hepatic stellate cells (HSCs) at day 4 were cultured with resistin (500 ng/mL) (R500) for 24 h. Total RNA was extracted and quantitative polymerase chain reaction was performed to quantify mRNA expression. Media were collected and enzyme-linked immunosorbent assay conducted to determine interleukin 6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) protein concentrations. A: mRNA expression of tumor necrosis factor α (TNFα), IL-6, IL-8, MCP-1, IL-1α and IL-1; B: IL-6 and MCP-1 protein levels. Data are expressed as mean ± SD. At least three independent experiments were conducted in triplicate for data analysis. [a]*P* < 0.05 *vs* controls (untreated).

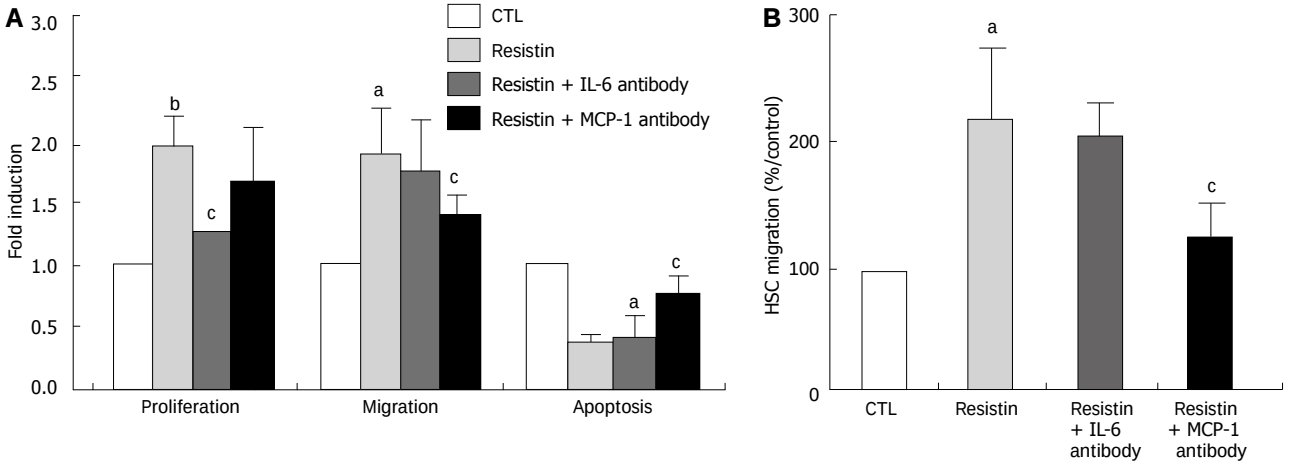

Figure 3 Resistin promotes hepatic stellate cells proliferation and migration but decreases hepatic stellate cells apoptosis in an interleukin 6 and monocyte chemotactic protein-1 dependent mechanism. BrdU, enzyme-linked immunosorbent assay, Wound Scratch Assay (or Boyden chamber) and annexin V/IP flow cytometry were performed to determine hepatic stellate cells (HSCs) proliferation, migration and apoptosis, respectively. For the interleukin 6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) inhibition experiments, IL-6 and MCP-1 neutralizing antibodies (5 μg/mL and 10 μg/mL, respectively) were added to the culture 1 h before resistin (500 ng/mL) administration. Resistin (500 ng/mL) was added to rat HSCs at day 4 for 24 h. Absorbance was measured and apoptosis assessed. For the migration assay, rat HSCs at day 6 were used. After a scratch wound was made, resistin (500 ng/mL) was added and the cells were cultured for 6 h and photographed. For the Boyden chamber assay, the detailed procedure is described in the Materials and Methods section. A: Resistin promoted HSC proliferation and migration, but inhibited HSC apoptosis, while IL-6 and MCP-1 antibodies reversed the resistin-induced HSC phenotype; B: The Boyden chamber assay confirmed that resistin enhanced HSC migration and MCP-1 neutralization reversed this effect. Results are mean ± SD of at least three independent experiments performed in triplicate. [a]*P* < 0.05 and [b]*P* < 0.01 *vs* control (untreated); [c]*P* < 0.05 *vs* resistin treatment alone.

MCP-1 (10 μg/mL) neutralization, respectively. These data suggest that resistin triggered HSC IL-6 and MCP-1 production, thereby modulating HSC phenotype.

Resistin activates HSC MAPK/p38 and nuclear NF-κB p65

Mitogen-activated protein kinases (MAPK) and NF-κB play critical roles in the induction of pro-inflammatory cytokines and chemokines, and regulate cell biological behaviors. Therefore, we determined whether resistin activates HSC MAPK (p38, ERK1/2 and JNK) and NF-κB. Phoshor-p38, ERK1/2 and JNK in the cytoplasm as well as NF-κB p65 and p50 in cytosolic and nuclear extracts were analyzed by immunoblotting. The results showed that cytoplasmic p-p38 and nuclear p-p65 were up-regulated (both $P < 0.05$, Figure 4A and C). Changes in cytosolic pERK1/2, pJNK (data not shown), p65 and cytosolic and nuclear p-p50 (data not shown) were not observed. In the p-p38 inhibition experiment using SB203580, we found that p-p38 activation was responsible for IL-6 and MCP-1 induction in HSCs ($P < 0.05$, Figure 4B). Furthermore, resistin (500 ng/mL) enhanced NF-κB DNA binding ability (luciferase mRNA. $P < 0.05$, Figure 4D). As expected, NF-κB inhibition by pyrrolidine dithiocarbamate (PDTC) (100 μmol/L) attenuated the resistin-induced increase in nuclear p-p65 and NF-κB DNA binding ability ($P < 0.05$, Figure 4C and D). Similarly, PDTC reversed resistin-induced up-regulation of IL-6 and MCP-1 (Figure 4E).

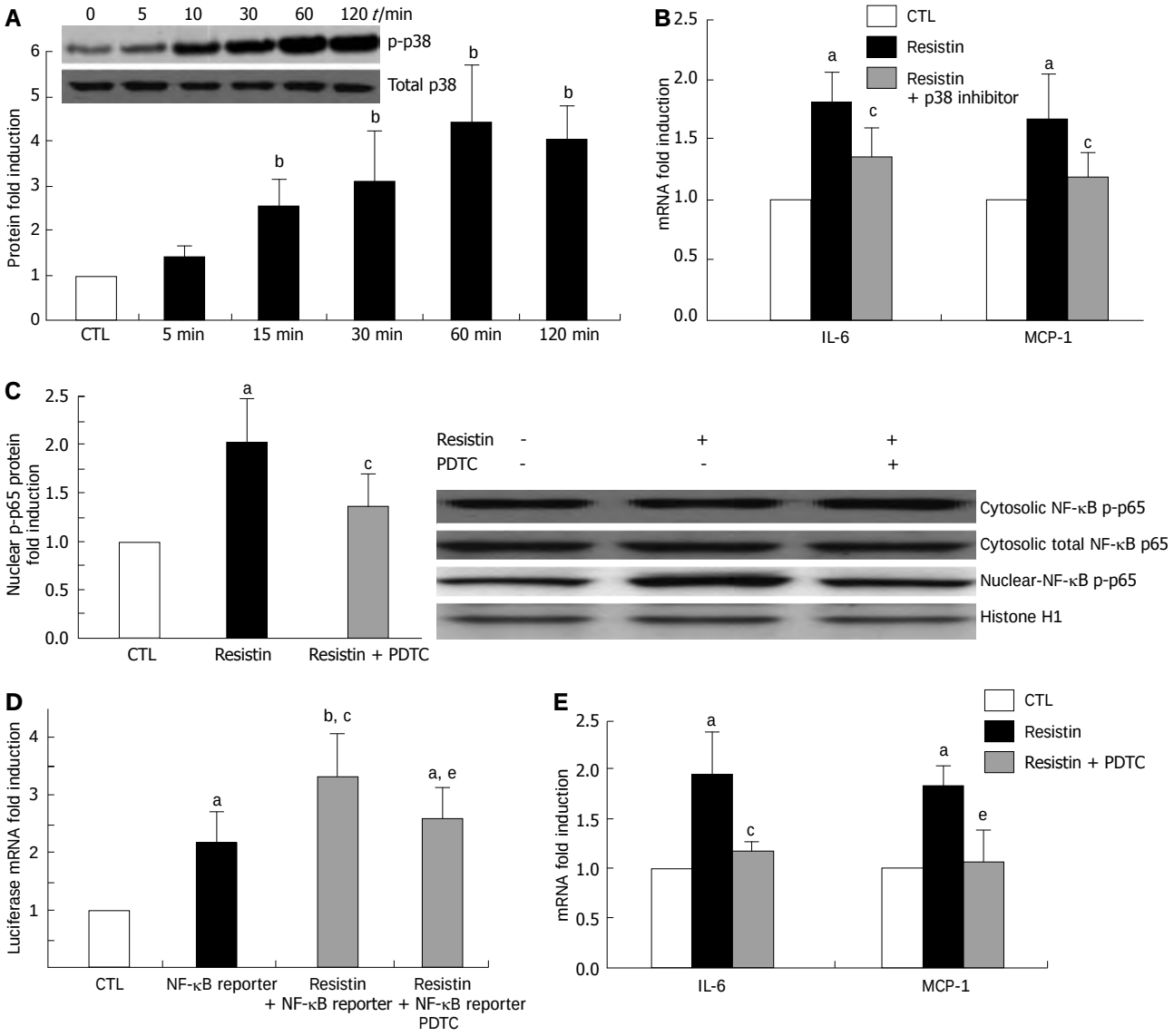

Figure 4 Resistin activates hepatic stellate cells MAPK/p38 and nuclear factor κB p65. Rat hepatic stellate cells (HSCs) at day 4 were cultured with resistin (500 ng/mL) for 120 min. Cytosolic and nuclear proteins were extracted and Immunoblot performed to quantify p-p38 and nuclear factor κB (NF-κB) p-p65. For NF-κB DNA binding capacity, 3 × NF-κB /Luc reporter was added to the culture for 24 h and Luciferase mRNA quantified by quantitative polymerase chain reaction. For the p-p38 and NF-κB inhibition experiments, SB203580 (20 μmol/L, p-p38 inhibitor) or pyrrolidine dithiocarbamate (PDTC) (100 μmol/L, NF-κB inhibitor) was added 1 h before resistin treatment. Resistin (500 ng/mL) was added to rat HSCs for 24 h. A: p-p38 was enhanced by resistin; B: p-p38 inhibition (24 h) diminished resistin-induced interleukin 6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) increase by HSCs; C: Nuclear p-p65 was increased by resistin exposure and decreased by PDTC (120 min); D: Luciferase mRNA was augmented by resistin and diminished by PDTC (24 h); E: PDTC reversed resistin-induced enhancement of IL-1 and MCP-1 (24 h). Data are expressed as mean ± SD. At least three independent experiments were conducted in triplicate for data analysis. [a]$P < 0.05$ and [b]$P < 0.01$ *vs* controls (untreated); [c]$P < 0.05$ *vs* resistin treatment alone or NF-κB reporter treatment alone; [e]$P < 0.05$ *vs* combination of resistin and NF-κB reporter.

Resistin indirectly promotes HSC collagen I expression through the actions of KCs

To determine whether resistin affects KCs and whether KCs participate in the process of resistin-mediated HSC phenotype, the appropriate experiments were undertaken. As shown in Figure 5A and B, resistin up-regulated TGFβ1 and CTGF mRNA in KCs and enhanced TGFβ1 protein in KC medium (both $P < 0.05$). Co-culture of HSCs and KC conditioned medium resulted in a significant increase in HSC collagen I and CTGF expression (Figure 5C and D, all $P < 0.05$), however, TGFβ1 (10 μg/mL) neutralization diminished this increase (Figure 5C and D, all $P < 0.05$). Downstream signaling responsible for increased KC TGFβ1 and CTGF expression by resistin were further analyzed. We found that pJNK and p-p38 were activated following exposure to resistin. Furthermore, pJNK and p-p38 inhibition partially, but significantly reversed resistin-induced TGFβ1 and CTGF enhancement (Figure 5E and F, $P < 0.05$ and 0.01). These data suggest that resistin affected HSC activated phenotype by increased TGFβ1 from KCs.

DISCUSSION

Resistin is suggested to play a pathogenic role in insulin resistance and altered glucose metabolism in rodents[3,4].

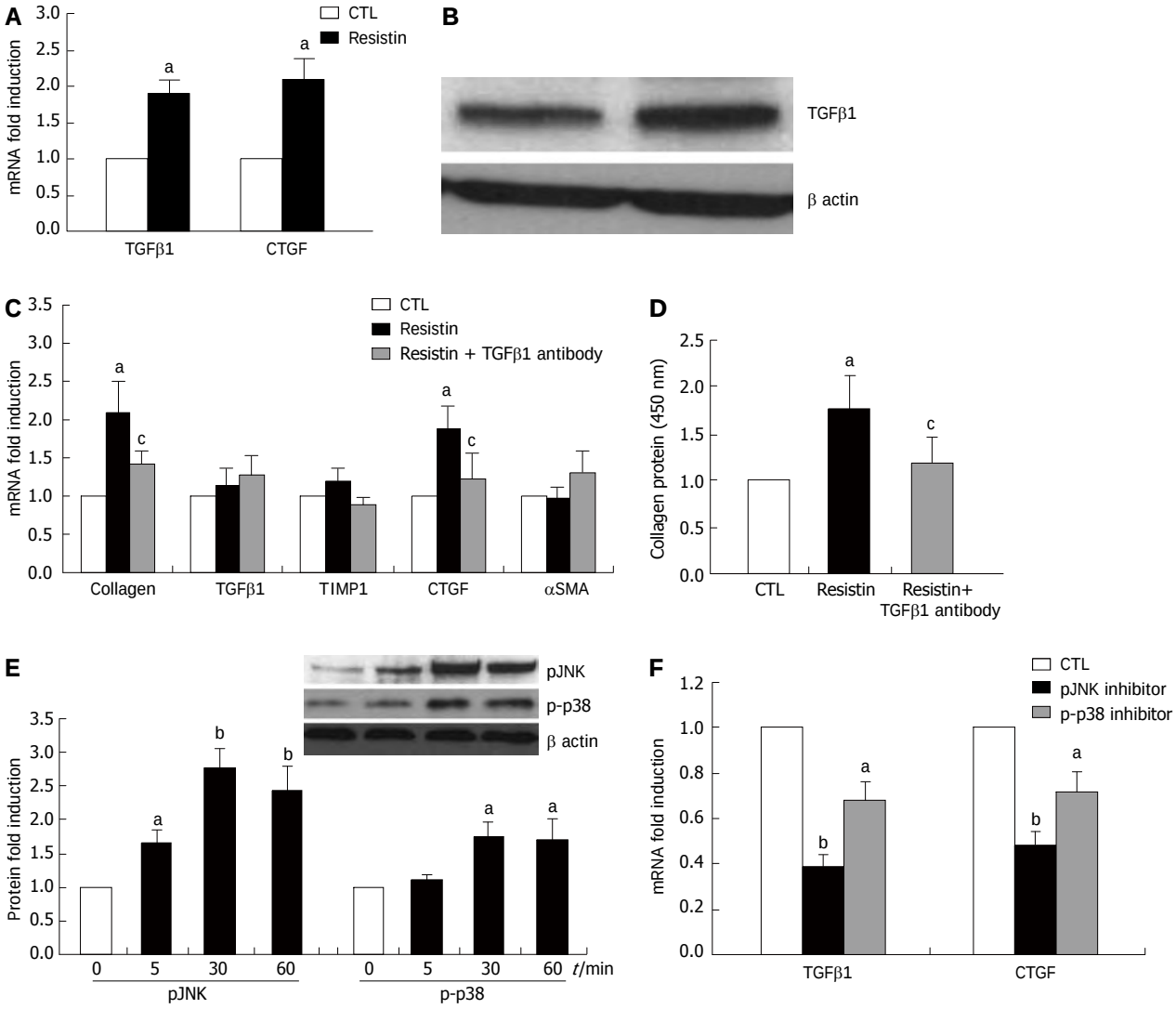

Figure 5 Resistin indirectly enhances hepatic stellate cells collagen I expression through a transforming growth factor β1 dependent mechanism *via* the action of Kupffer cells. Rat Kupffer cells (KCs) at day 2 were cultured with resistin (500 ng/mL) for 24 h. Total RNA was extracted and quantitative polymerase chain reaction was performed. Transforming growth factor β1 (TGFβ1) protein expression in KC conditioned medium (KM) was quantified by immunoblotting. Sirius red was used for collagen I protein quantification in the medium. For the KC-hepatic stellate cell (HSC) co-culture experiment, KCs at day 2 were incubated with resistin for 24 h and then washed three times with phosphate-buffered saline, fresh medium was subsequently added to the culture for another 24 h. KM was then transferred to HSCs at day 4 for 24 h. HSC collagen I , TGFβ1, tissue inhibitor of metalloproteinase 1 (TIMP1), connective tissue growth factor (CTGF) and α smooth muscle actin (αSMA) expression were determined. For inhibition experiments, TGFβ1 monoclonal antibody (10 μg/mL), SP600125 (50 μmol/L) and SB203580 (20 μmol/L) were added to KCs for 24 h. A: Resistin promoted KC T*GFβ1* and *CTGF* gene expression; B: Resistin augmented TGFβ1 expression in KM medium; C: HSC collagen I and CTGF mRNA were augmented by resistin conditioned KM reversed by TGFβ1 neutralization (10 μg/mL); D: HSC collagen protein was increased by resistin conditioned KM but reversed by TGFβ1 neutralization (10 μg/mL); E: Resistin increased KC JNK and p38 phosphor-protein; F: pJNK inhibitor (50 μmol/L, SP600125) and p-p38 inhibitor (20 μmol/L, SB203580) partially, but significantly reversed resistin-induced TGFβ1 and CTGF expression by KCs. Data are expressed as mean ± SD. At least three independent experiments were conducted in triplicate for data analysis. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* controls (untreated); $^{c}P < 0.05$ *vs* resistin treatment alone.

For example, lowering plasma resistin in insulin-resistant mice decreases blood glucose levels and improves insulin sensitivity[10,30,31], while treatment of normal mice with resistin impairs glucose tolerance and insulin actions[10,30]. Resistin may also play a pivotal role in inflammation since it up-regulates IL-6 and TNFα expression in human peripheral blood mononuclear cells *via* NF-κB activation[22]. Furthermore, the addition of resistin protein from both mice and humans to macrophages results in enhanced secretion of pro-inflammatory cytokines including TNFα and IL-12[32]. In human cirrhosis, resistin levels in the liver and plasma are elevated and increase further with the severity of liver disease[14-17,33]. This suggests that the pro-inflammatory activities of resistin may modulate liver inflammation and drive disease progression in cirrhosis.

This study provides evidence that in cirrhotic rats, adipose tissue (epididymal fat) and blood resistin are up-regulated, and adipose tissue may be the main source of resistin secretion. Rat liver, HSCs and KCs express resistin, but are unlikely to be important sources of resistin secretion in cirrhosis. Resistin exerts pro-inflammatory activities on HSCs with enhanced secretion of pro-

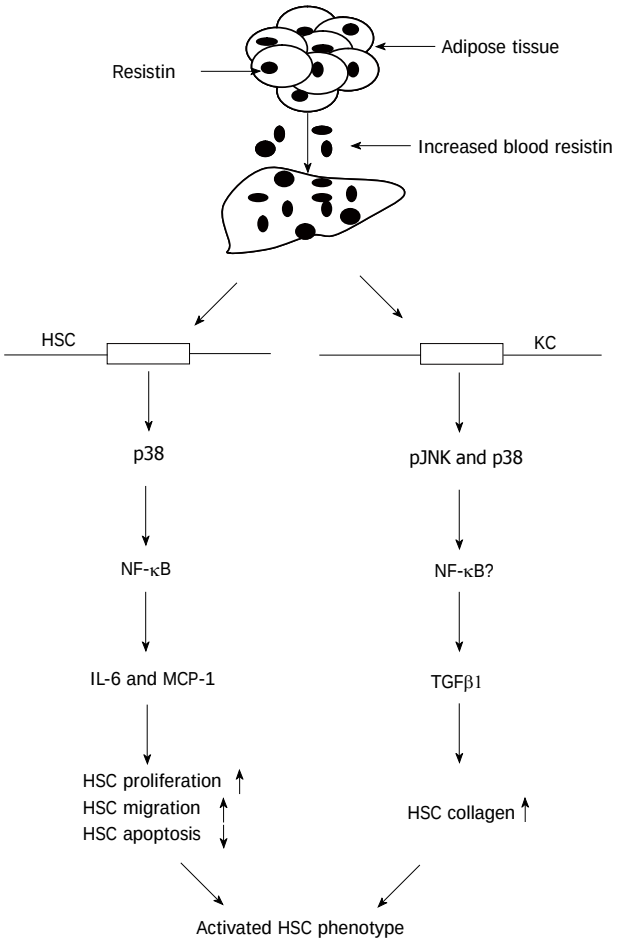

Figure 6 Schematic diagram illustrating a possible mechanism by which resistin potentiates hepatic stellate cells profibrogenic phenotype. Adipose tissue is a predominant source of resistin expression and secretion in rodents. Increased adipose resistin released into the bloodstream and liver stimulates HSCs to produce increased amounts of pro-inflammatory cytokines and chemokines (IL-6 and MCP-1), leading to enhanced HSC proliferation and migration, but attenuation of HSC apoptosis. In addition, resistin promotes KC TGFβ1 which subsequently activates HSC by up-regulation of collagen I. Therefore, resistin is considered one of the pro-fibrogenic adipocytokines. TGFβ1: Transforming growth factor; NF-κB: Nuclear factor κB; IL-6: Interleukin 6; MCP-1: Monocyte chemotactic protein-1; HSCs: Hepatic stellate cells.

inflammatory cytokines (TNFα, IL-6, IL-8 and MCP-1). Most importantly, resistin promotes HSC proliferation and migration, while inhibiting their apoptosis *via* an IL-6 and MCP-1 mechanism. KCs participate in this process by up-regulating HSC collagen I through increased TGFβ1. Taken together, our data suggest that resistin promotes the progression of liver injury.

Resistin is almost exclusively expressed by white adipose tissue in rodents, but is expressed by monocytes and macrophages in humans[18,34,35]. Liver infiltrating CD43 cells and KCs have been suggested as key sources of resistin in the liver of cirrhotic patients[20,21], thus resistin was more abundant in adipose tissue than in human liver[20,21]. In this study, although rat quiescent HSCs expressed resistin, it declined markedly on activation. The relevant mechanism is unclear, however, adipogenic transcriptional regulation may be required for maintenance of the quiescent HSC phenotype[26]. KCs also expressed resistin but no change was found on activation or LPS stimulation. Therefore, it is unlikely that resistin derived from HSCs and KCs contributed to the increase in serum resistin in BDL rats, thus HSCs and KCs are non-critical sources of resistin. However, other liver cell types may not represent a likely source of resistin production as hepatocytes and endothelial cells do not express resistin[15]. Thus, adipose tissue, including epididymal fat, could be the predominant source of resistin in liver injured rodents. It has been demonstrated in *in vivo* and *ex vivo* studies, that increased TNFα and insulin in BDL cirrhotic rats stimulate adipose resistin expression[14,15,19].

Why LPS was unable to trigger resistin secretion by KCs is unknown. KCs belongs to the macrophage family, and many studies have shown that LPS exposure induced resistin production in human and rodent macrophages[36,37]. The mechanisms involved require further clarification.

As expected, HSC expression of TNFα, IL-6, IL-8 and MCP-1 mRNAs was increased on resistin exposure, as was IL-6 and MCP-1 protein. Bertolani *et al*[20] reported similar findings in human HSCs and noted that resistin up-regulated human HSC MCP-1 that was dependent on a Ca^{2+}/NF-κB-dependent pathway[20]. We further demonstrated that resistin directly augmented HSC proliferation and migration, but reduced HSC apoptosis *via* an IL-6 and MCP-1 mechanism. These novel data imply that IL-6 and MCP-1 inhibition may prevent resistin-induced liver fibrogenesis. The pro-fibrogenic effects of IL-6 and MCP-1 are well documented in the literature[38-40]. Moreover, we found that resistin was able to promote KC activation as it stimulated enhancement of KC TGFβ1 expression. Thus, increased TGFβ1 led to up-regulation of HSC collagen I and HSC activation. This is an important finding, as TGFβ1 is a potent profibrogenic cytokine. Interestingly, this phenomenon is similar to our previous report[6]. We observed that the profibrogenic role of leptin could be achieved at least through TGFβ1 from KCs[6]. Collectively, these data indicate that resistin is able to modulate HSC behaviors towards a more pro-fibrogenic phenotype.

Although many functions of resistin in inflammation and inflammation-related diseases have been described, the relevant intracellular signaling pathway of resistin is not yet completely understood. We further demonstrated that resistin mediated HSC IL-6 and MCP-1 *via* p38 and KC TGFβ1 *via* pJNK and p-p38 (Figure 6). These results may provide evidence to prevent resistin-mediated liver injury/fibrosis using relevant signaling inhibitors.

In summary, this study demonstrates that in rodents, resistin production in the context of liver injury is principally non-hepatic in origin. Extrahepatic resistin could contribute to liver fibrosis by its direct and indirect pro-fibrogenic effects on HSCs. Further studies on resistin knockout and transgenic animals are needed.

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COMMENTS

Background

Metabolic abnormalities usually cause the progression of liver fibrosis. To date, the mechanism whereby metabolic alterations mediate disease progression are unclear. Resistin, an adipokine, has been reported to be associated with metabolic alterations, however, its role in hepatic fibrosis has not been clearly investigated.

Research frontiers

Although many functions of resistin in inflammation and inflammation-related diseases have been described, the relevant intracellular signaling pathways of resistin in liver fibrosis are not yet completely understood.

Innovations and breakthroughs

To date, there have been a limited number of studies regarding the impact of resistin on the phenotype of hepatic stellate cells and how it functions in liver fibrosis. In this study, the authors employed a direct analysis to identify the significant correlation between resistin and hepatic stellate cells (HSCs). The authors confirmed that resistin mediated-HSCs move towards a more pro-fibrotic phenotype which is dependent on interleukin 6/monocyte chemotactic protein and/or transforming growth factor β1.

Applications

By understanding the mechanism whereby resistin mediates HSC activation, this study may provide evidence to prevent resistin-mediated liver injury/fibrosis using relevant signaling inhibitors.

Terminology

The serum levels of resistin are elevated in cirrhosis, and the changed phenotype of HSCs play an important role in the pathogenesis of liver fibrosis. Resistin is involved in this process.

Peer review

The paper reported the effects of the adipokine resistin on the biology of hepatic stellate cells and Kupffer cells. It is well presented.

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ORIGINAL ARTICLE

Influence of up-regulation of Notch ligand DLL4 on biological behaviors of human gastric cancer cells

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Abstract

AIM: To investigate the potential roles of Delta-like ligand 4 (DLL4) on the biological behavior of gastric cancer cells and its molecular mechanisms.

METHODS: A recombinant eukaryotic expression vector containing human *DLL4* gene was constructed and transfected into the human gastric cancer cell line SGC7901. Clones with up-regulated DLL4 were selected and amplified. The effect of DLL4 up-regulation on gastric cancer cell growth was assessed using cell growth assay. The migration and invasion were assessed using a transwell migration assay and matrigel invasion assay. Matrix metalloproteinases were detected using the zymogram technique. Cells were implanted subcutaneously into male BALB/c nu/nu mice. Tumor volumes were then calculated and compared. DLL4 staining in the implanted tumor was performed using immunohistochemistry technique.

RESULTS: Growth curves over a six-day time course showed significantly promoted cell proliferation of SGC7901 cells with up-regulated DLL4. DLL4 up-regulation in SGC7901 cells promoted the migration (205.4 ± 15.2 *vs* 22.3 ± 12.1, $P < 0.05$) and invasion (68.8 ± 5.3 *vs* 18.2 ± 6.0, $P < 0.05$) *in vitro* and tumorigenicity *in vivo* (2640.5 ± 923.6 mm^3 *vs* 1115.1 ± 223.8 mm^3, $P < 0.05$). Furthermore, significantly increased mRNA level and increased secretion of matrix metalloproteinase-2 (MMP-2) proenzyme were observed in SGC7901 cells with up-regulated DLL4. However, increased MMP-9 mRNA level but decreased extracellular MMP-9 proenzyme level was observed.

CONCLUSION: Our observations indicated a mechanism by which activation of DLL4-mediated Notch signaling promotes the expression and secretion of MMP-2 proenzyme and influences the progress of gastric cancer.

Key words: Gastric cancer; Delta-like ligand 4/Notch; Matrix metalloproteinase; Migration; Invasion

Core tip: Delta-like ligand 4 (DLL4), one of the five notch signaling ligands in mammals, has been researched mainly with regard to vasculogenesis and tumor angiogenesis. To the best of our knowledge, there is rare study to investigate its role and mechanism in human gastric cancers. We found that DLL4

promotes cellular proliferation, migration, invasion and tumorigenicity in gastric cancer cells. Furthermore, increased mRNA level and increased secretion of matrix metalloproteinase-2 proenzyme, while increased matrix metalloproteinase (MMP)-9 mRNA levels but decreased extracellular MMP-9 proenzyme levels were observed. These results indicated a mechanism by which activation of DLL4-mediated Notch signaling promotes the expression and secretion of MMP-2 proenzyme and influences the progress of gastric cancer.

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INTRODUCTION

Gastric cancer is one of the most common cancers and lethal malignancies worldwide[1,2]. Approximately 738000 patients with gastric cancer died in 2011[2]. Of these, 80% died within a short period after curative surgery due to locoregional recurrence (87%) and distant metastases (30%)[3,4]. The 5-year survival of gastric cancer is less than 40%, even with adjuvant chemo-radiotherapy[5]. Therefore, there is an urgent need for new therapeutic strategies to improve clinical outcomes of this disease.

Notch signaling, as an evolutionarily conserved signaling pathway, is involved in a variety of cellular processes including cell fate, differentiation, proliferation, survival rate, and apoptosis[1,6]. The Notch signaling in mammals consists of five ligands [Delta-like ligand 41/3/4 (DLL1/3/4) and Jagged 1/2] and four receptors (Notch 1-4). Notch proteins are synthesized as full-length unprocessed proteins and cleaved at the S1 site by furin-like convertase to generate the mature receptor. Notch-ligand binding induces the cleavages of a Notch receptor by metalloprotease and γ-secretase to release the Notch intracellular domain (NICD). The NICD subsequently translocates to the nucleus, where it forms a complex with the members of the CSL (C-promoter binding factor-1, suppressor of hairless in *Drosophila* and lag in *Caenorhabditis elegans*) transcription factor family and regulates the expression of downstream genes such as Hairy/Enhancer of Split (*HES1/5/6/7*) and the HES-related proteins (*HEY1/2/L*)[6-10].

Interestingly, Notch signaling may play distinct biological roles in different tumors. It acts as an oncogene in pancreatic cancer[11], colon cancer[12], breast cancer[13] and most other solid tumors[14,15], whereas it acts as an anti-oncogene in some types of skin cancer[16], lung cancer[17] and prostate cancer[18]. Compared with other solid tumors, less literature relates to the role of DLL4-mediated Notch signaling in gastric cancer.

Although most Notch-related genes are expressed in multiple tissue and cell types, DLL4 is largely restricted to the vascular endothelia and has been researched mainly with regard to vasculogenesis and tumor angiogenesis[7,19-21]. Blockade of DLL4 signaling has been shown to lead to inhibition of tumor angiogenesis by nonproductive angiogenesis in some types of murine tumor models[20,21]. However, the precise function and mechanism of DLL4 in gastric cancer remain unclear. In the present study, we up-regulated the expression of DLL4 in the gastric cancer cell line SGC7901, and assessed its biological function both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell lines and animals

Human gastric cancer cell line SGC7901 was purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Cells were propagated in RPMI-1640 medium (Gibco-Invitrogen, Carlsbad, CA, United States) supplemented with 10% fetal bovine serum (FBS) (Gibco-Invitrogen), penicillin (100 units/mL) and streptomycin (100 μg/mL) in a humidified atmosphere containing 5% CO_2 at 37 ℃.

Animal studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the protocol was approved by the Animal Research Committee of Zhejiang University, Hangzhou, China. Mouse protocols were conducted in accordance with stringent regulations laid out by Zhejiang University Laboratory Animal Center. Twenty-four 4-wk-old male BALB/c nu/nu mice (Shanghai SLAC Laboratory Animal Co, Shanghai, China) weighing 10-12 g used for subcutaneous tumor implantation were randomly divided into control, SGC7901-vector and SGC7901-DLL4 groups. Animals were housed in a sterile environment, and maintained on daily 12-h light/12-h dark cycle, which was controlled by qualified staff in the Zhejiang University Laboratory Animal Center.

Recombinant eukaryotic expression vector pEGFP-C1-DLL4 construction

A human full-length DLL4 cDNA fragment was amplified using a forward primer 5'-GGAATTCACCATGGCGGCAGCGTCC-3' and a reverse primer 5'-CGGGATCCTTATACCTCCGTGGCAATGACAC-3', verified by sequencing, and then *Eco*RI-*Bam*HI-digested. The 2-kb fragment was purified using a AxyPrep DNA Gel Extraction Kit (Axygen, Union City, CA, United States) and was ligated to a EcoRI-BamHI-digested pEGFP-C1 vector DNA (Clontech, Mountain View, CA, United States). Positive clones were further confirmed by *Eco*RI-*Bam*HI digestion and sequencing.

Transfection and clone selection

SGC7901 cells in logarithmic growth phase were collected and seeded into six-well plates at 4×10^5 cells/well to obtain approximately 80% confluence after overnight

Table 1 Primer sequences for real-time polymerase chain reaction

Genes	Forward sequences	Reverse sequences	Product size (bp)
DLL4	5′-CCCTGGCAATGTACTTGTGAT-3′	5′-TGGTGGGTGCAGTAGTTGAG-3′	73
Notch1	5′-GCCTCAACATCCCCTACAAGA-3′	5′-CCACGAAGAACAGAAGCACAAA-3′	120
HES1	5′-GTCAACACGACACCGGATAA-3′	5′-TTCAGCTGGCTCAGACTTTC-3′	113
HES5	5′-TGGAGAAGGCCGACATCCT-3′	5′-GGCGACGAAGGCTTTGC-3′	65
HEY1	5′-CGCGTTATCTGAGCATCATT-3′	5′-TGGGAAGCGTAGTTGTTGAG-3′	88
MMP-2	5′-GCTGACGGTAAGGACGGACTC-3′	5′-CGTTGCCATTGAACAAGAAGG-3′	158
MMP-9	5′-TTTGACAGCGACAAGAAGTGG-3′	5′-AGGGCGAGGACCATAGAGG-3′	189
E-cadherin	CGTTAGAGGTGGGTGACTACAAA	GAACAGCAAGAGCAGCAGAAT	220
GAPDH	5′-TGCCACTCCTCCACCTTTG-3′	5′-CGAACCACCCTGTTGCTGT-3′	104

DLL: Delta-like ligand 4; MMP: Matrix metalloproteinases; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

incubation. Cultured SGC7901 cells were divided into three groups: (1) transfected cells with recombinant pEGFP-C1-DLL4 vector (SGC7901-DLL4 group); (2) transfected cells with pEGFP-C1 vector (SGC7901-vector group); and (3) nontransfected cells (control group). Transfection was performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The cells were then cultured in RPMI-1640 medium containing 10% FBS and G418 (600 μg/mL) for 21 d. G418-resistant clones were selected and amplified in complete medium containing G418 (300 μg/mL). Up-regulation of DLL4 was verified by real-time polymerase chain reaction (PCR) and Western blotting assay.

RNA extraction and real-time PCR

Extraction of total RNA from cultured cells was performed using the TRIzol method (Invitrogen). Total RNA (1 μg) was reverse-transcribed using RevertAid First Strand cDNA Synthesis Kit (Fermentas, ThermoFisher Scientific, Waltham, MA, United States). Real-time PCR was performed in triplicate using SYBR Green PCR Master Mix (TaKaRa, Tokyo, Japan) in an ABI PRISM Stepone Plus Sequence Detection System (Applied Biosystems, Foster City, CA, United States) according to the manufacturer's instructions. Forty cycles were used to amplify DLL4/Notch-related genes (denaturation at 95 ℃ for 5 s, annealing and extension at 60 ℃ for 32 s). Relative quantitation of gene expression was detected using the method described by Pfaffl[22]. The primers for real-time PCR were described in Table 1.

Western blotting analysis

Total protein extracts were prepared and run on 10% polyacrylamide gels. Fractionated proteins were electrotransferred to polyvinylidenefluoride membranes. Antibodies against human DLL4 (1:1000, Abcam, Cambridge, United Kingdom) and β-actin (1:1000, Abcam) were used for detection at 4 ℃ overnight. Horseradish peroxidase-conjugated anti-rabbit antibody was applied as a secondary antibody at 25 ℃ for 1 h. Antigens were identified by luminescent visualization using an ECL Western blotting Detection System (Millipore, Billerica, MA, United States). Signal intensity was measured using a Bio-Rad XRS chemiluminescence detection system (Bio-Rad).

Cell growth assay and growth curve

3-(4,5-dimethyl-2-yl)-5-(3-arboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assays using the CellTiter 96 AQueous nonradioactive cell proliferation MTS agent (Promega, Madison, WI, United States) were performed to evaluate cell growth. Approximately 500 cells in 100 μL of medium with 0.5% FBS were plated in 96-well plates and allowed to attach for 24 h. Then 20 μL of the Promega MTS reagent was added to each well, and further incubation was conducted in a humidified incubator for 2 h. Absorbance of each well at 490 nm was determined using a Microplate Reader (Bio-Rad). The cell-growth curve was plotted using the optical densities obtained over 6 consecutive days.

Transwell migration assay

Transwell units with 8.0-μm pore-size polycarbonate filters (Corning Costar, Tewksbury, MS, United States) were used to investigate chemotactic cell migration. Cells were harvested and suspended at approximately 4×10^5 cells/mL in RPMI 1640 medium containing 0.5% FBS. A total volume of 100 μL of suspension was added into the upper compartment of the transwell unit. After 30 min of attachment, the units were transferred to wells containing 600 μL RPMI 1640 medium with 20% FBS as a chemoattractant, and further incubation was conducted for 20 h. After removing the cells on the upper surface of the membrane with a cotton bud and 15-min staining with 0.1% crystal violet, cell numbers on the underside were determined using light microscopy. Five randomly selected fields were counted per insert.

Matrigel invasion assay

Transwell units with 8.0-μm pore-size polycarbonate filters (Corning Costar) were precoated with 50 μL of 1:5 diluted matrigel (Becton Dickinson Biosciences, Franklin Lakes, NJ, United States) and used to investigate cell invasion. A total volume of 100 μL of suspension containing approximately 40000 cells was added to each upper compartment of precoated units. After 30 min of attachment, the units were transferred to wells containing 600 μL RPMI 1640 medium with 20% FBS as a chemoattractant, and incubation was conducted for 20 h. After removing the cells and Matrigel on the upper surface of

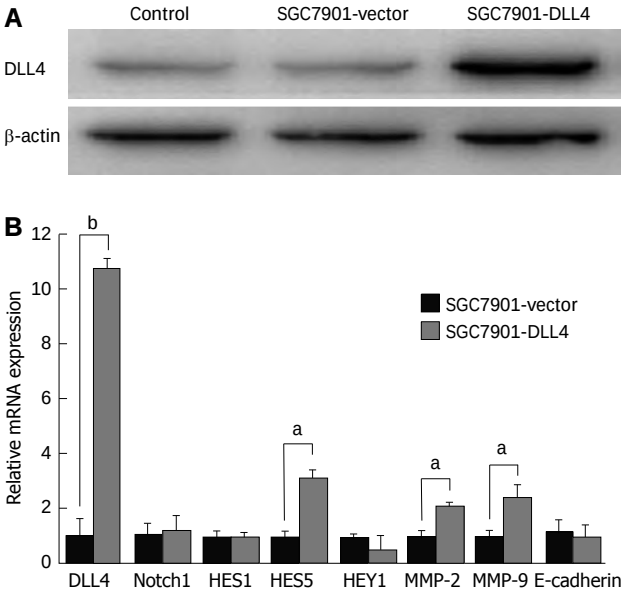

Figure 1 Up-regulation of Delta-like ligand 4 changed downstream gene expression in SGC7901 cells. A: Western blotting confirmed the up-regulation of Delta-like ligand 4 (DLL4) in the SGC7901-DLL4 group at the protein level; B: Real-time polymerase chain reaction was used to assess *Notch1*, *DLL4*, and downstream genes *HES1*, *HES5*, *HEY1*, as well as the matrix metalloproteinases-2 (*MMP-2*), *MMP-9*, and the adhesion protein E-cadherin at the mRNA level. The results showed increased expression of *DLL4*, *HES5*, *MMP-2*, and *MMP-9*, but no significant change of *HES1* and *HEY1* ($^aP < 0.05$, $^bP < 0.01$ *vs* the SGC7901-vector group). Data show mean ± SD of gene expression compared with the control group. Endogenous references was glyceraldehyde 3-phosphate dehydrogenase; SGC7901-vector: SGC7901 cells transfected with an empty vector; SGC7901-DLL4: SGC7901 cells transfected with a vector encoding human *DLL4* gene; Control: Non-transfected SGC7901 cells.

the membrane with a cotton bud and 15-min staining with 0.1% crystal violet, cell numbers on the underside were determined using light microscopy. Five randomly selected fields were counted per insert.

Gelatin zymography

Matrix metalloproteinases (MMPs) were detected using the zymogram technique according to the standard procedure[23]. Cells were allowed to grow in serum-free medium for 24 h. Then the medium was collected and diluted 1:1 with × 2 sample buffer and run on 10% polyacrylamide gels containing gelatin (1 mg/mL) until the bromophenol blue tracking dye reached the bottom of the gel. After electrophoresis, the gel was washed twice in Triton X-100 solution (2.5%) at room temperature for 15 min, transferred to 100 mL of development buffer containing 2 mL of Triton X-100 solution, and incubation was conducted at 37 ℃ for 72 h. Staining with Coomassie Blue R-250 solution proceeded overnight, then destaining was performed until the bands were clearly visible. Areas of protease activity appeared as clear bands against a dark blue background where the protease has digested the substrate.

Mouse tumor models

Twenty-four 4-wk-old male BALB/c nu/nu mice weighing 10-12 g were purchased and randomly divided into control, SGC7901-vector and SGC7901-DLL4 groups. Cells (1×10^6 cells/animal) in a total volume of 0.1 mL of PBS were implanted subcutaneously into the flank of each mice on day 0. Tumor size was measured on days 7, 14, 28, 35, 42. The tumor volume was calculated using the formula: volume = $D \times d^2 \times$ pi/6, where D and d represent the longer diameter and shorter diameter respectively.

Immunohistochemistry

For DLL4 staining, we used a rabbit monoclonal anti-human DLL4 antibody (1:200, Abcam). Paraffin-embedded tissue blocks were serially sectioned 4 μm in thickness, dewaxed, and rehydrated in serial alcohol washes. Endogenous peroxidase activity was blocked with 0.03% hydrogen peroxide in PBS for 20 min. Immunostaining for DLL4 was done by incubation for 1 h with primary antibody in blocking buffer and visualized using 3,3'-diaminobenzidine chromogen (Invitrogen) with hematoxylin (Invitrogen) counterstaining after treatment with HRP-conjugated Goat anti-rabbit immunoglobulin G (1:100 dilution).

Statistical analysis

Numerical results are shown as mean ± SD. Data were analyzed using SPSS ver. 13.0 statistical software (SPSS, Inc., Chicago, IL, United States). Differences among three groups were examined using one-way analysis of variance analysis. Means between two groups were compared using the Student's t test. Statistical significance was considered a P value of < 0.05. All experiments were performed at least three times.

RESULTS

Up-regulation of DLL4 changed downstream gene expression in SGC7901 cells

SGC7901 cells were transfected with vector encoding human *DLL4* (SGC7901-DLL4 group) or empty vector (SGC7901-vector group) and were then selected by G418 for at least 3 wk. Non-transfected SGC7901 cells were used as a control group. The up-regulating effect of the vector on DLL4 protein levels in the SGC7901-DLL4 group was confirmed by western blot assay (Figure 1A). Real-time PCR was further used to assess the expression of *Notch1*, *DLL4*, and downstream genes including *HES1*, *HES5*, *HEY1*, as well as the mRNA levels of MMP-2, MMP-9 and the adhesion protein E-cadherin. The results show that the mRNA level of DLL4 in the SGC7901-DLL4 group was approximately 10-fold higher than in the SGC7901-vector group. Accordingly, up-regulation of *DLL4* expression resulted in increased expression of *HES5*, *MMP-2* and *MMP-9* (Figure 1B).

Effects of DLL4 up-regulation on gastric cancer cell proliferation

MTS cell proliferation assays (Promega) were used to investigate the effect of DLL4 transfection on gastric

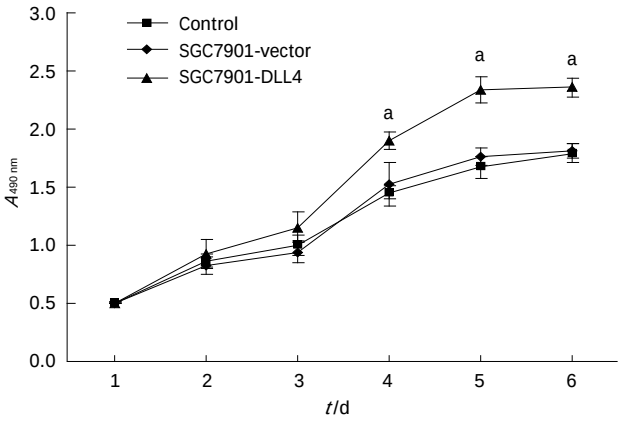

Figure 2 Up-regulation of Delta-like ligand 4 promoted cell proliferation in SGC7901 cells. Growth curve comparing SGC7901-Delta-like ligand 4 (DLL4), SGC7901-vector and SGC7901 cells over a 6-d time course. Up-regulation of DLL4 significantly promoted the proliferation of SGC7901 cells *in vitro*. Data are shown as the mean ± SD of three independent experiments ($^aP < 0.05$ *vs* the SGC7901-vector group); SGC7901-vector: SGC7901 cells transfected with an empty vector; SGC7901-DLL4: SGC7901 cells transfected with a vector encoding human *DLL4* gene; Control: Non-transfected SGC7901 cells.

cancer cells. A growth curve was plotted based on the optical densities obtained during the 6 d after attachment. The results showed that up-regulation of DLL4 resulted in significantly accelerated cell proliferation in the SGC7901-DLL4 group when compared to the SGC7901-vector group ($P < 0.05$, Figure 2).

Up-regulation of DLL4 accelerated migration of SGC7901 cells

The effects of DLL4 up-regulation on SGC7901 cell migration were investigated using 8.0-μm pore-size Corning Costar Transwell units. Approximately 4×10^4 cells from each of the groups were plated on the insert. The results show that the number of SGC7901 cells transfected with DLL4, which migrated across the insert, was 8.3 times higher than those transfected with empty vector (205.4 ± 15.2 *vs* 22.3 ± 12.1, $P < 0.05$) (Figure 3).

Up-regulation of DLL4 accelerated invasion of SGC7901 cells

Matrigel invasion assays were performed using 8.0-μm pore-size Corning Costar transwell units precoated with 50 μL Matrigel (1:5 diluted), which permitted cell migration across the filter. Approximately 4×10^4 cells from each group were plated in the insert. The results show that the number of SGC7901 cells transfected with DLL4, which migrated across both the Matrigel and the insert, was 2.83 times higher than those transfected with an empty vector (68.8 ± 5.3 *vs* 18.2 ± 6.0, $P < 0.05$) (Figure 3).

Effect of DLL4 up-regulation on the secretion and activation of extracellular MMPs

Gelatin zymography was used to analyze the effect of DLL4 up-regulation on the secretion and activation of MMPs, such as MMP-2 and MMP-9. From each group, 20 μL of culture supernatant were diluted 1:1 with × 2 sample buffer, incubated for 30 min at 25℃, and added to a zymogram comprising 10% polyacrylamide, 1 mg/mL gelatin, and electrophoresed at a constant voltage of 110 V until the bromophenol blue tracking dye reached the bottom of the gel. Coomassie Blue R-250 staining of the zymogram revealed significantly increased MMP-2 proenzyme level but decreased MMP-9 proenzyme level in the medium of the SGC7901-DLL4 group compared to the SGC7901-vector group (Figure 4).

Up-regulation of DLL4 accelerated tumor growth in vivo

To examine the effect of DLL4 up-regulation on gastric cancer growth *in vivo*, 24 four-wk-old male BALB/c nu/nu mice were divided randomly and averagely into three groups and implanted subcutaneously with control/SGC7901-vector/SGC7901-DLL4 cells. Six weeks after inoculation, the gastric cancer cells grew as subcutaneous implants in each nude mouse (100%). The size of subcutaneously formed tumor masses of the SGC7901-DLL4 group (2640.5 ± 923.6 mm^3) ($P < 0.05$ relative to the SGC7901-vector group) was significantly larger than the control (1011.1 ± 273.6 mm^3) and SGC7901-vector groups (1115.1 ± 223.8 mm^3) (Figure 5A). Immunohistochemistry staining of DLL4 confirmed the up-regulation of DLL4 expression in the SGC7901-DLL4 group (Figure 5B).

DISCUSSION

The finding that DLL4/Notch signaling participates in vascular development and homeostasis, as well as the evidence that DLL4 is predominantly expressed in the developing endothelium and in some tumor endothelia, suggest that DLL4-mediated Notch signaling activation is involved in tumor angiogenesis[24-26]. However, literature regarding the biological properties of DLL4-mediated Notch signaling in gastric cancer is very limited. The aim of our study is to investigate the potential roles of DLL4 on the biological behavior of gastric cancer cells and its molecular mechanisms.

In this study, we investigated the effect of DLL4 up-regulation on cell growth, migration and invasion *in vitro* and tumor growth *in vivo*. The results showed that up-regulation of DLL4 significantly promotes proliferation, migration, and invasion of SGC7901 gastric cancer cells *in vitro* and tumor growth *in vivo*. Our findings indicate the oncogenic function of DLL4/Notch signaling in gastric cancers, which is in accord with previous studies on other cancers. For example, small interfering RNA (siRNA)-induced knockdown of DLL4 resulted in decreased proliferation, increased apoptosis and retarded growth of tumor *in vitro* and *in vivo*[27-31]. Other Notch-targeting approaches such as chemical inhibitors of gamma-secretase significantly suppressed cell growth in colon cancer cell lines[32,33] and sensitized oxaliplatin- and 5-Fu-induced apoptosis and growth inhibition[34]. These findings indicate that DLL4/Notch signaling is an important molecular pathway involved in oncogenesis and chemoresis-

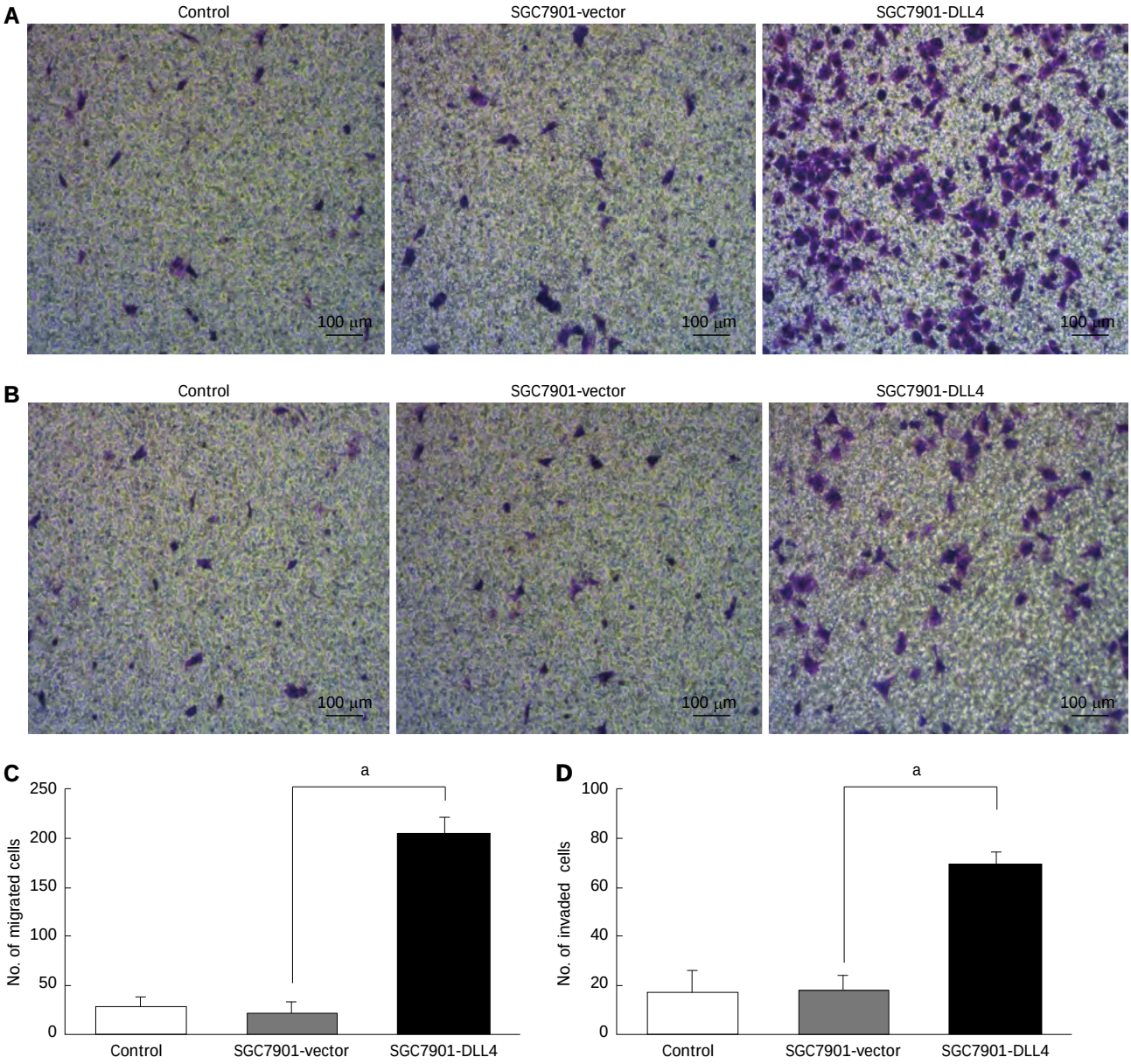

Figure 3 Up-regulation of Delta-like ligand 4 promoted cell migration and invasion in SGC7901 cells. A: Crystal violet staining revealed the migrated cells of each group; B: Crystal violet staining reveals invaded cells from each of the groups; C: The number of cells in the SGC7901-Delta-like ligand 4 (DLL4) group that had migrated was 8.31 times higher than those transfected with an empty vector (205.4 ± 15.2 *vs* 22.3 ± 12.1, $^{a}P < 0.05$); D: The number of cells in the SGC7901-DLL4 group that had migrated across both the Matrigel and the insert was 2.83 times higher than that in the SGC7901-vector group (68.8 ± 5.3 *vs* 18.2 ± 6.0, $^{a}P < 0.05$). SGC7901-vector: SGC7901 cells transfected with an empty vector; SGC7901-DLL4: SGC7901 cells transfected with vector encoding human *DLL4* gene; Control: Non-transfected SGC7901 cells.

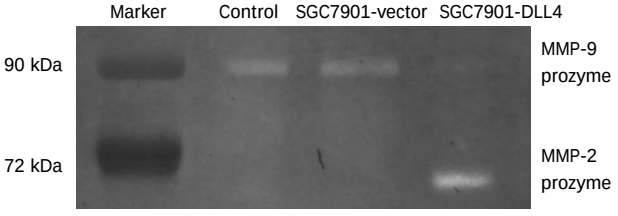

Figure 4 Effect of Delta-like ligand 4 up-regulation on the secretion and activation of extracellular matrix metalloproteinases. Twenty microlitres of culture supernatant from each group was used. Coomassie Blue R-250 staining of the zymogram revealed significantly increased matrix metalloproteinases (MMP)-2 proenzyme but decreased MMP-9 proenzyme in the SGC7901-Delta-like ligand 4 (DLL4) group *vs* the SGC7901-vector group. SGC7901-vector: SGC7901 cells transfected with an empty vector; SGC7901-DLL4: SGC7901 cells transfected with vector encoding human *DLL4* gene; Control: Non-transfected SGC7901 cells.

tance. Targeting DLL4/Notch signaling might constitute a novel molecular therapy for cancers.

Wang *et al*[31] reported that inactivation of Notch signaling in prostate cancer cells leads to decreased expression and activity of MMP-9, which contributes to the inhibition of cell migration and invasion. Our study shows that up-regulation of DLL4 leads to decreased activity of MMP-9 with increased MMP-9 expression at mRNA level. One explanation for this observation could be that Notch signaling targeting genes, as well as MMP-9, have complex post-transcriptional regulations. This explanation might also contribute to diverse roles of Notch signaling in different types of cancers. Another possible explanation is that the activation of MMP-9 is inhibited by

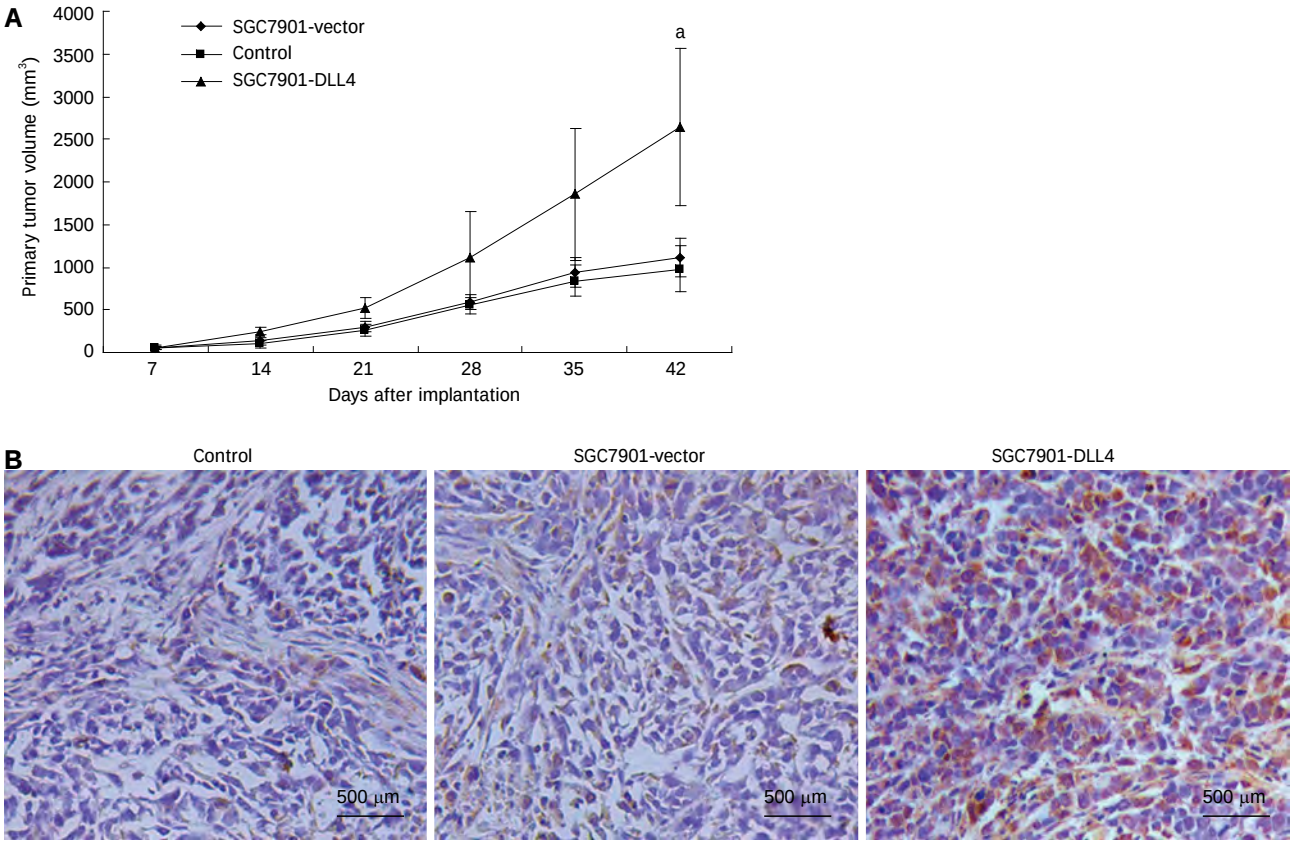

Figure 5 Up-regulation of Delta-like ligand 4 promoted tumorigenesis of SGC7901 cells *in vivo*. Twenty-four 4-wk-old male BALB/c nu/nu mice were divided randomly into three groups and implanted subcutaneously with control/SGC7901-vector/SGC7901-Delta-like ligand 4 (DLL4) cells. A: Six weeks after inoculation, the size of subcutaneous formed tumor masses of the SGC7901-DLL4 group (2640.5 ± 923.6 mm^3) was significantly larger than the control (1011.1 ± 273.6 mm^3) and SGC7901-vector groups (1115.1 ± 223.8 mm^3); B: Immunohistochemistry staining of DLL4 confirmed the up-regulation of DLL4 in the SGC7901-DLL4 group. $^aP < 0.05$ *vs* the SGC7901-vector group.

other signaling pathways or molecules induced by DLL4 up-regulation, such as tissue inhibitor of metalloproteinase-1 (TIMP-1), TIMP-2, or other TIMPs. Furthermore, significantly increased mRNA levels and secretion of MMP-2 proenzymes were observed in gastric cancer cells with up-regulated DLL4. These results suggest that MMP-9 signaling might not be sufficient to exert an effect alone in gastric cancer progression[35], while other molecules such as MMP-2 might play a major role.

In summary, to our knowledge, activation of DLL4-mediated Notch signaling effectively promotes proliferation, migration, and invasion of gastric cancer cells, and desensitizes the cells to chemotherapeutically induced cell senescence. Our data suggest that DLL4-mediated Notch signaling may play an important role in the progression of gastric cancer, and that DLL4-mediated Notch signaling could be a potential target for gastric cancer biotherapy. Meanwhile, we identified MMP-2 as a novel target of DLL4-mediated Notch signaling in gastric cancer cells. However, the precise molecular mechanism of DLL4-mediated Notch signaling in gastric cancer remains unclear. Further studies are required to elucidate possible downstream target genes or potential interacting molecules of DLL4-mediated Notch signaling.

In summary, up-regulation of DLL4 significantly promoted cellular proliferation, migration, and invasion *in vitro* and tumor growth *in vivo*. Significantly increased MMP-2 expression at both the mRNA and the protein level was observed in gastric cancer with up-regulated DLL4. Our observations indicated a mechanism by which activation of DLL4-mediated Notch signaling promotes the expression and secretion of MMP-2 proenzyme and influences the progress of gastric cancer.

COMMENTS

Background

Gastric cancer is one of the most common cancers and lethal malignancies worldwide. Discovering novel biomarkers that correlate with gastric cancer may present opportunities to reduce the severity of this disease. As one of the five Notch signaling ligands in mammals, Delta-like ligand 4 (DLL4) has been researched mainly with regard to vasculogenesis and tumor angiogenesis. However, the precise function and mechanism of DLL4 in gastric cancer remain unclear.

Research frontiers

Notch signaling, as an evolutionarily conserved signaling pathway, is involved in a variety of cellular processes, including cell fate, differentiation, proliferation and apoptosis. Previous data indicates its distinct biological roles in different tumors. It may work as an oncogene or anti-oncogene. Their observations indicated a mechanism by which activation of DLL4-mediated Notch signaling promotes the expression and secretion of matrix metalloproteinase (MMP)-2 proenzyme and influences the progress of gastric cancer.

Innovations and breakthroughs

Previous studies indicated that DLL4 is largely restricted to the vascular endothelia. Recent reports were focused on its roles on vasculogenesis and tumor

angiogenesis. The authors discovered that DLL4 up-regulation promotes cellular proliferation, migration, invasion and tumorigenicity in gastric cancer cells. Increased mRNA level and increased secretion of matrix metalloproteinase-2 proenzyme, while increased MMP-9 mRNA level but decreased extracellular MMP-9 proenzyme level were observed.

Applications

In understanding the role and mechanism of DLL4-mediated Notch signaling in gastric cancer, this study may represent a future strategy as a therapeutic target and/or a way to improve clinical treatment for gastric cancer.

Peer review

The authors found that ectopic expression of DLL4 significantly promoted cellular proliferation, migration, invasion *in vitro* and tumor growth *in vivo*. Significantly increased mRNA levels and secretion of MMP-2 proenzymes were observed, increased MMP-9 mRNA but decreased extracellular MMP-9 proenzyme were observed. The paper is well presented and the results are interesting.

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ORIGINAL ARTICLE

Basic transcription factor 3 is involved in gastric cancer development and progression

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Abstract

AIM: To further analyse cancer involvement of basic transcription factor 3 (BTF3) after detection of its up-regulation in gastric tumor samples.

METHODS: BTF3 transcription rates in human gastric tumor tissue samples (n = 20) and adjacent normal tissue (n = 18) specimens as well as in the gastric cancer cell lines AGS, SGC-7901, MKN-28, MKN-45 and MGC803 were analyzed *via* quantitative real-time polymerase chain reaction. The effect of stable BTF3 silencing *via* infection with a small interfering RNA (siRNA)-BTF3 expressing lentivirus on SGC-7901 cells was measured *via* Western blotting analysis, proliferation assays, cell cycle and apoptosis profiling by flow cytometry as well as colony forming assays with a Cellomic Assay System.

RESULTS: A significant higher expression of BTF3 mRNA was detected in tumors compared to normal gastric tissues (P < 0.01), especially in section tissues from female patients compared to male patients, and all tested gastric cancer cell lines expressed high levels of BTF3. From days 1 to 5, the relative proliferation rates of stable BTF3-siRNA transfected SGC7901 cells were 82%, 70%, 57%, 49% and 44% compared to the control, while the percentage of cells arrested in the G_1 phase was significantly decreased (P = 0.000) and the percentages of cells in the S (P = 0.031) and G_2/M (P = 0.027) phases were significantly increased. In addition, the colony forming tendency was significantly decreased (P = 0.014) and the apoptosis rate increased from 5.73% to 8.59% (P = 0.014) after BTF3 was silenced in SGC7901 cells.

CONCLUSION: BTF3 expression is associated with enhanced cell proliferation, reduced cell cycle regulation and apoptosis and its silencing decreased colony forming and proliferation of gastric cancer cells.

Key words: Basic transcription factor 3; Gastric cancer; Small interfering RNA; Proliferation; Apoptosis; Cell cycle

Core tip: After we found that basic transcription factor 3 (BTF3) transcription rates in human gastric tumor tissue samples were significantly higher than in adjacent normal tissues, we extended our study on gastric cancer cell lines. We silenced BTF3 in SGC7901 cells, which led to 82%, 70%, 57%, 49% and 44% proliferation rates of the control within the first 5 d after infection with a

small interfering RNA-BTF3 containing lentivirus. After BTF3 silencing, the percentage the G_1 phase arrested SGC7901 cells was decreased (P = 0.000), the percentages of cells in the S (P = 0.031) and G_2/M (P = 0.027) phases were increased and the apoptosis rate increased from 5.73% to 8.59% (P = 0.014).

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INTRODUCTION

Basic transcription factor (BTF3) is a 27 kD protein that in humans is encoded by the *BTF3* gene[1,2] and evolutionarily conserved in a variety of cells[3]. BTF3 was initially discovered as a member of the general transcription machinery and functions as a transcriptional initiation factor from proximal promoter elements by forming a stable complex with RNA polymerases[4,5]. There are two ubiquitously expressed isoforms of the *BTF3* gene that encode BTF3a and BTF3b proteins. The BTF3a is the transcriptional active form of BTF3, while the isoform BTF3b, which lacks the first 44 amino acids of the BTF3a N-terminus is transcriptionally inactive, although it is able to bind to the RNA polymerase Ⅱ[6]. However, previous studies indicated that BTF3 was not in fact essential for specific, *in vitro* initiation of transcription, but its biological importance was shown by the fact that mouse embryos, homozygous for a loss of function mutation in the *BTF3* gene, died at the early stage of development indicating its important role during development[7]. In addition, BTF3 was up-regulated strongly in mouse pregnancy, indicating an involvement in alveolar growth[8]. In cancer, it has been reported that the *BTF3* gene has been overexpressed in colorectal cancer, glioblastomas and hepatocellular carcinomas[9-12]. In the pancreatic ductal adenocarcinoma, the median level of the BFT3 and BFT3a mRNA was increased by 1.3 and 4.6 folds compared to the normal tissues, respectively. Down-regulation of the BTF3 expression using small interfering RNA (siRNA) resulted in reduced expression of several cancer-associated genes, including ephrin receptor B2, which is mainly expressed during development and involved in tumor cell survival[13,14] and heparanase 2 an extracellular matrix degrading enzyme involved in cell adhesion. In addition, ataxia-telangectasia mutated gene, which is implicated in cell cycle arrest[15], DNA repair[16] or apoptosis and the oncogene V-abl Abelson murine leukemia viral oncogene homolog 2, a nuclear protein tyrosine kinase for cell differentiation, cell division and cell adhesion were also had reduced expression[17]. By BFT3 silencing, up-regulated genes were *k-ras* oncogene-associated gene, related ras viral oncogene homolog 2, nuclear factor kappa-B, murine retrovirus integration site 1 and mucosal vascular addressing cell adhesion molecule 1, all known to be involved in tumor development[18]. In addition, BTF3 interacts with either 17β-estradiol or epidermal growth factor activated estrogen receptor α (ERα) *via* its AF1 domain in the breast cancer cell line MCF-7 and up-regulates transcriptional responses of ERα reporter genes[19,20]. In this study, we analyzed the expression of BTF3 mRNA and protein in gastric tumors and normal samples and compared BTF3 expressions in different gastric tumor cell lines. Finally we used siRNA-BTF3 to down-regulate BTF3 expressions in gastric tumor cells and measured the changes of proliferation and apoptosis to investigate the relationship between BTF3 and gastric cancer.

MATERIALS AND METHODS

Patients

Human tissue samples of gastric tumor (n = 20) and adjacent normal tissue (n = 18) specimens were obtained from patients with a median age of 63 years (range, 35-83 years) who received gastric resections from November 2011 to March 2012 in the Second Xiangya Hospital of Central South University, Changsha, China. All samples were confirmed histologically. Freshly removed tissues (within 5 min after surgical excision) were: (1) fixed in paraformaldehyde solution for 12-24 h and then paraffin embedded for histological analysis; (2) kept in RNAlater (Ambion Ltd., Huntingdon, Cambridgeshire, United Kingdom) for RNA analysis; or (3) snap-frozen in liquid nitrogen and maintained at -80 ℃ for protein analysis. All studies were approved by The Human Ethics Committee of the First Affiliated Hospital of Hunan Normal University in China, and written informed consent was obtained from all patients.

Cell culture

Gastric cancer cell lines (AGS, SGC-7901, MKN-28, MKN-45 and MGC803) were grown in complete DMEM medium (Gibco, New York, United States), supplemented with 10% fetal calf serum (Gibco, New York, United States) and 5 U/mL penicillin, and incubated at 37 ℃ in 5% CO_2 atmosphere.

Plasmid and vector construction

The selected and optimized siRNA (sense: GCCGAAGAAGCCTGGGAATCA, anti-sense: TGATTCCCAGGCTTCTTCGGC) against human BTF3 and negative control (sense: TTCTCCGAACG TGTCACGT, anti-sense: ACGTGACACGTTCGGAGAA) were used in generating the lentiviral vector. Basically, the BTF3 siRNA transcript template was synthesized, digested with Age I/EcoRI enzymes, and then ligated with pGCSIL-GFP vector. After checking by agarose electrophoresis, successful BTF3-siRNA-pGCIL-GFP recombinants were amplified, purified and sequenced. In order to evaluate the inhibitive effect of BTF3 sequences, the confirmed pGCSIL-GFP recombinants were transfected into 293T cells (data not shown). The expressed BTF3 protein was detected by Western blotting.

Table 1 Primer sequences

Primer	Sequence
GAPDH-F	TGACTTCAACAGCGACACCCA
GAPDH-R	CACCCTGTTGCTGTAGCCAAA
BTF3-F	GCGAACACTTTCACCATTACAG
BTF3-R	AACTTCATCATCATCATCCTCTCC

BTF3: Basic transcription factor 3; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Quantitative real-time polymerase chain reaction

Cells were split during the log-phase growth before plating into the 6-well plate with complete medium, and incubated at 37 ℃ with 5% CO_2. An appropriate amount of lentiviral vectors was added to cells when 30% confluence was reached. Total RNA was extracted 5 d after treatment from cells with GFP expression by Trizol (Invitrogen, San Diego, United States). RNA (2 μg) was reversely transcribed using M-MLV-RTase (Promega, Madison, United States) following manufacturer's instructions. Real-time polymerase chain reaction (PCR) was performed using the tested primer set (Table 1) and SYBR Master Mixture (*TAKARA, DRR041B*) following the manufacturer's instructions with a TAKARA's TP800 Real Time PCR Instrument. Following the normalization with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene, relative quantification of BTF3 expression was done by the comparative CT method ($2^{-\Delta\Delta C}$ method).

Total protein extraction, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting

Cells were harvested 36-48 h following the transfection. Pellets were washed twice with cold phosphate-buffered saline (PBS) (Gibco, New York, United States) and then cells were lysed on ice for 10-15 min using proper lysis buffer [1 mol/L Tris-HCl 100 mmol/L, 2% 2-mercaptoethanol, 20% glycerol, 4% sodium dodecyl sulfate (SDS) (Life Technologies, New York, United States)]. After sonication, total protein extraction was performed by centrifugation at 1200 *g* for 15 min at 4 ℃. Protein was separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), with 4% separating and 3%-15% gradient stacking gel. After SDS-PAGE, protein samples were wet transferred to polyvinylidenfluorid (PVDF) (Life Technologies, New York, United States) membranes for immunoblotting. To detect the target protein, the PVDF membrane was incubated with appropriate primary antibodies, either 2 h at the room temperature or overnight in the cold room (Mouse Anti-GFP Santa-Cruz SC-9996, 1:2000, Mouse anti-GAPDH Santa-Cruz SC-32233 1:5000), following 3 times of 10 min TBS-Tween 20 buffer (Life Technologies, New York, United States) washes, membranes were incubated with secondary antibodies for 2 h at room temperature (Goat Anti-Mouse IgG Santa-Cruz SC-2005 1:5000). After further 3 times washing, target proteins were detected using ECL™ Western blotting system (Amersham Cat. No. RPN2135).

Flow cytometric assays

Cells were harvested by Trypsin/EDTA (Life Technologies, New York, United States) solution from the 6-cm dishes at 80% confluence followed by setting up the designed assays. Pellets were then washed with PBS and collected into 5 mL centrifuge tubes in triplicate. After fixation using pre-cooled 4 ℃ 70% ethanol for 1 h, cells were washed again and then stained with propidium iodide (FACSCalibur, BD, New York, United States).

Colony forming assay

Five hundred cells were plated per well in 96-well plates in triplicate. After the colony number reached more than 5 in most of the wells, the colonies were scanned using Cellomics system following the manufacturer's protocol. The colony size and cell numbers within each colony were analyzed by Cellomics system (Thermo, Philadelphia, United States). The data were then statistically analyzed.

Statistical analysis

Results of all calculations were expressed as mean ± SD. Analysis of variance (ANOVA) with multiple comparisons using Bonferroni's test or one-way ANOVA was performed where appropriate. The difference between two groups was analyzed by unpaired *t* test GraphPad Prism (version 3.02-2000). The results of statistical tests were considered significant if the *P* value was < 0.05 and < 0.01.

RESULTS

Level of BTF3 expression in gastric cancer and tumor cell lines

A quantitative real-time PCR was performed to identify the transcription levels of the *BTF3* gene in 20 human gastric tumors and the relative tumor free margins ($n = 18$) as healthy tissue control. A significantly higher transcription of BTF3 mRNA was detected in tumors compared to normal gastric tissues (Figure 1A, $P < 0.01$), especially in female patients' section tissues compared to male patients (Figure 1B) and between the gastric body tumor and adjacent normal tissue, but no significant difference was detectable between gastric antrum or cardia tumor and adjacent normal tissues (Figure 1C). In order to further confirm that the *BTF3* gene was widely expressed in our gastric tumor cell lines, quantitative real-time PCR was performed in five different cell lines AGS, SGC-7901, MKN-28, MKN-45 and MGC803. SGC-7901, MKN-45 and MGC803 which are all poorly differentiated adenocarcinomas, while the MKN-28 and AGS are high and moderately differentiated cell lines. The expected products of BTF3 mRNA were detected in all the above five cell lines (Figure 1D).

Silencing of BTF3 by siRNA

Based on the significant BTF3 up-regulation from the clinical data, we sought that the regulation of BTF3 in gastric tissue might be related to cancer initiation promotion and progression. To investigate this, the gene was

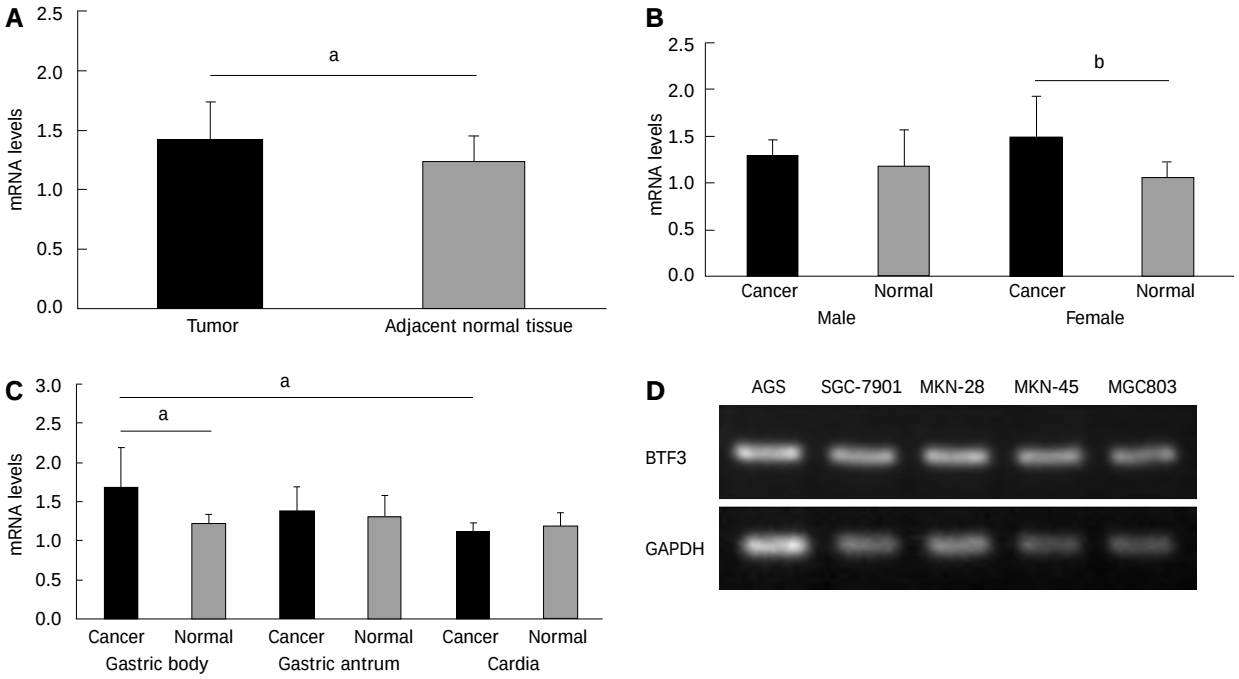


Figure 1 Level of basic transcription factor 3 expression in gastric cancer and tumor cell lines. A: Bar charts represent the mRNA levels of basic transcription factor 3 (BTF3) in gastric tumor and adjacent normal tissue measured by quantitative real-time polymerase chain reaction (PCR); B: BTF3 mRNA expression data from (A) further classified by gender; C: BTF3 expression pattern among gastric body, gastric antrum and cardia tumors; D: The expression of BTF3 mRNA was detected by quantitative real-time PCR among different cell lines. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene. Black bars represent tumors and gray bars normal tissues. The difference in BTF3 expression, $^{a}P < 0.05$, $^{b}P < 0.01$ between groups was assessed by unpaired *t* test and considered significantly different.

silenced using lentiviral vectors expressing the specifically designed synthetic siRNA-BTF3. In pilot experiments, the siRNA-BTF3 was successfully transfected into human SGC7901 and 293T cell lines for mRNA expression profiles and protein analyses *via* GFP expression to estimate the transfection efficiency (Figure 2A). In SGC7901 cells, a significant down-regulation of the *BTF3* gene was measured after siRNA-BTF3 application (Figure 2B). The BTF3 transcription in siRNA transfected cells was 12.4% of the negative control transfected cells ($P = 0.0066$). The BTF3 protein levels in SGC7901 cells, detected by Western blotting 48 h after siRNA-BTF3 transfection, were reduced compared to the control (Figure 2C).

BTF3 silencing effect in SGC7901 cells

In order to identify the effect on gastric tumor cells, proliferation, cell cycle, apoptosis and colony forming assays were performed with siRNA-BTF3 transfected SGC7901 cells. The silencing of BTF3 significantly slowed down the SGC7901 cell growth in the proliferation assay (Figure 3). On day 1, the siRNA transfected cells already started to show an 18% slower proliferation rate compared with the negative controls. The difference became increasingly significant and peaked at the end of the proliferation assay on day 5, on which the proliferation rate in the siRNA transfected cells was only 44% of the negative control. From days 1 to 5, the relative proliferation rates of siRNA transfected SGC7901 were 82%, 70%, 57%, 49% and 44%, respectively. In summary, an obvious effect on proliferation of SGC7901 was induced by silencing BTF3 (Figure 3, BTF3-siRNA). A cell cycle profile of the siRNA-BTF3 transfected cells using PI-FACS and Annexin V showed that the percentage of cells arrested in the G_1 phase was significantly decreased, while the percentages of cells at the S and G_2/M phases were significantly increased (Figure 4). The knockdown of BTF3 in SGC7901 cells also induced an increased level of apoptosis. Cells with good transfection efficiency were harvested on day 5 for flow cytometry (Figure 5). The negative control group which was transfected with empty vectors showed 5.73% and 0.76% apoptosis, while the apoptosis rate of siRNA-BTF3 transfected group was 8.59% and 1.1% (Figure 5B and D). The down-regulation of BTF3 increased the apoptosis rate by nearly 2 folds. To detect the long-term effect on SGC7901 cells following BTF3 silencing, a colony forming assay was performed. Colonies were scanned and analyzed using the Cellomic Assay System. Cells after BTF3 silencing showed a significantly lower colony forming ability compared with the negative controls (Figure 6A). In the silenced group, 14 d following the initiation of the assay in average 10 colonies were detected, whereas a significantly higher number of 22 colonies (mean) was detected in the negative group ($P = 0.014$) (Figure 6B). In addition, the number of cells in each siRNA-BTF3 transfected SGC7901 cell colony was also reduced compared with the control.

DISCUSSION

In cells, multiple protein complexes are required to be

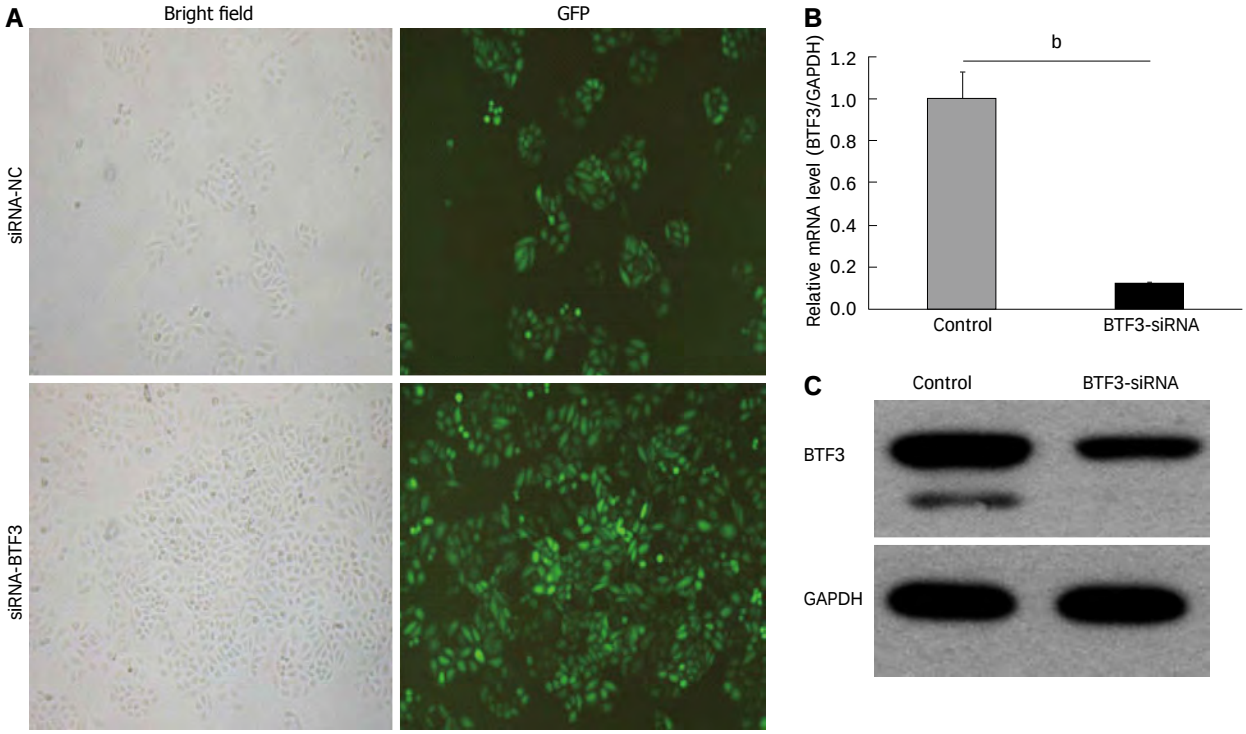


Figure 2 Silencing of basic transcription factor 3 using small interfering RNA. A: Transfection rates of small interfering RNA (siRNA)-normal control (NC)/siRNA-basic transcription factor 3 (BTF3)-green fluorescent protein (GFP) vectors in human SGC7901 cells, estimated by bright field and fluorescence microscope; B: Bar charts represent quantitative real-time polymerase chain reaction data of BTF3 mRNA levels in control and BTF3-siRNA transfected SGC7901 cells ($^{b}P < 0.01$ between groups, unpaired *t* test); C: Immuno-blotting analysis using BTF3-specific antibodies to detect BTF3 protein expressions in control and BTF3-siRNA transfected SGC7901 cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control.

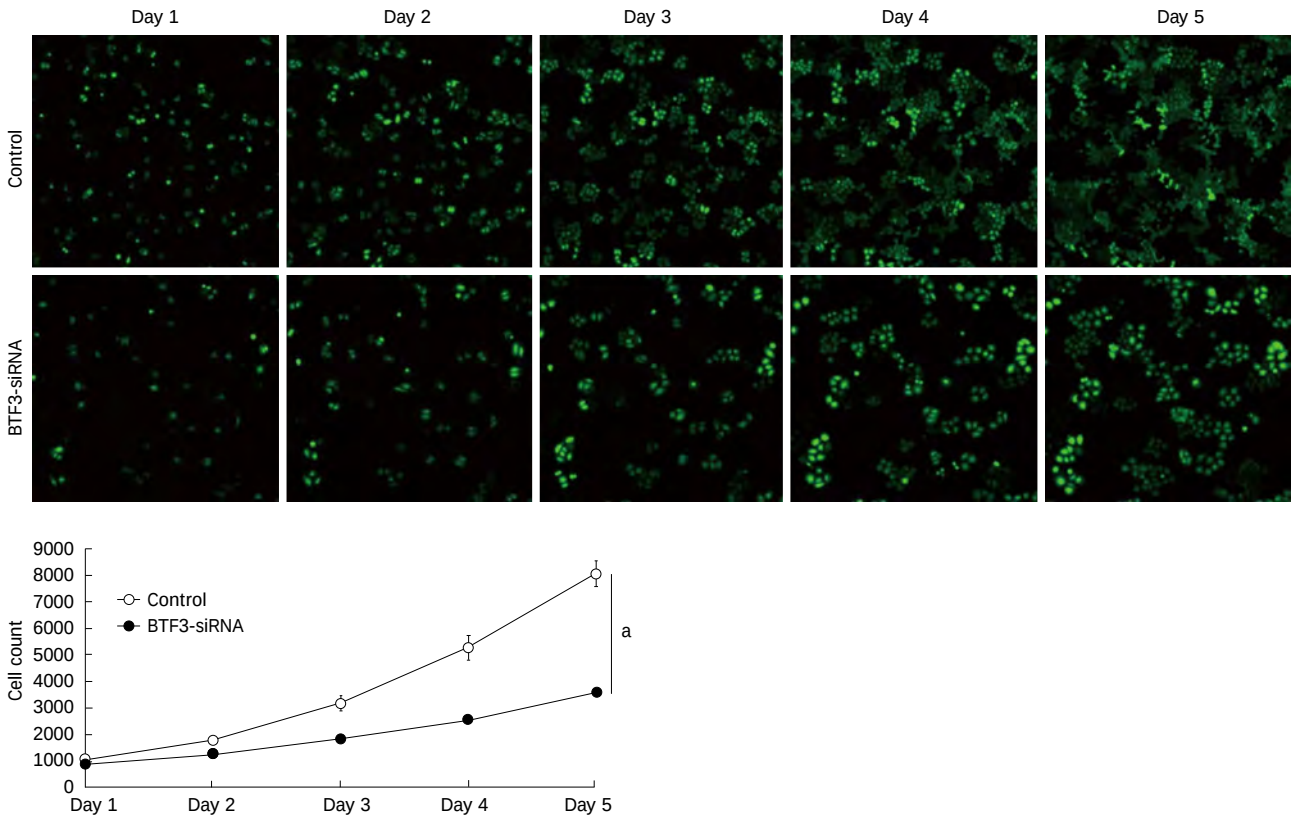
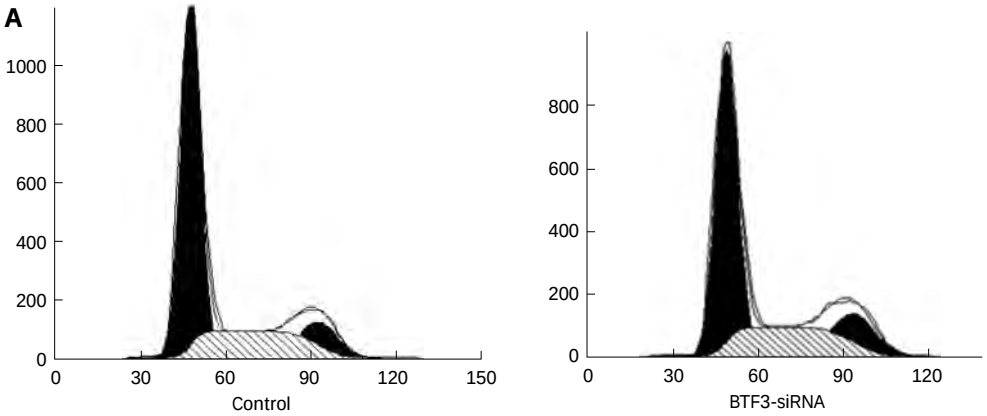


Figure 3 Cell proliferation assay. Fluorescent images of control and small interfering RNA (siRNA)-basic transcription factor 3 (BTF3) transfected cell proliferation assays. Cells were counted based on the green fluorescent protein signals during 5 d. BTF3-siRNA chart of the cell proliferation assay using two-way analysis of variance. $^{a}P < 0.05$ between groups.



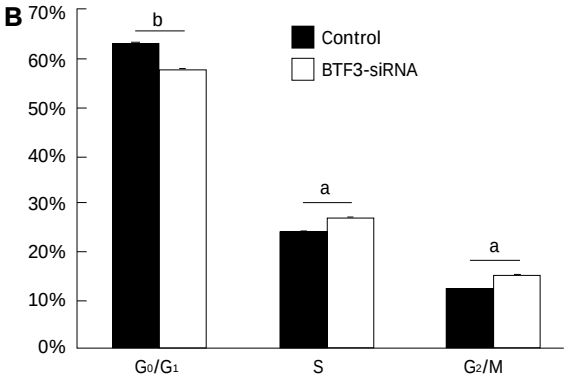


Figure 4 Flow cytometric assays. A: Cell-cycle stages of control and basic transcription factor 3 (BTF3)-small interfering RNA (siRNA) transfected cells were analyzed by flow cytometry. Data are presented as a histogram, with cell number (y-axis) plotted against DNA content (x-axis); B: Cells arrested at different cell-cycle stages were plotted as bar charts. $^{a}P < 0.05$, $^{b}P < 0.01$ between groups was assessed by unpaired *t* test and comparisons were considered significantly different.

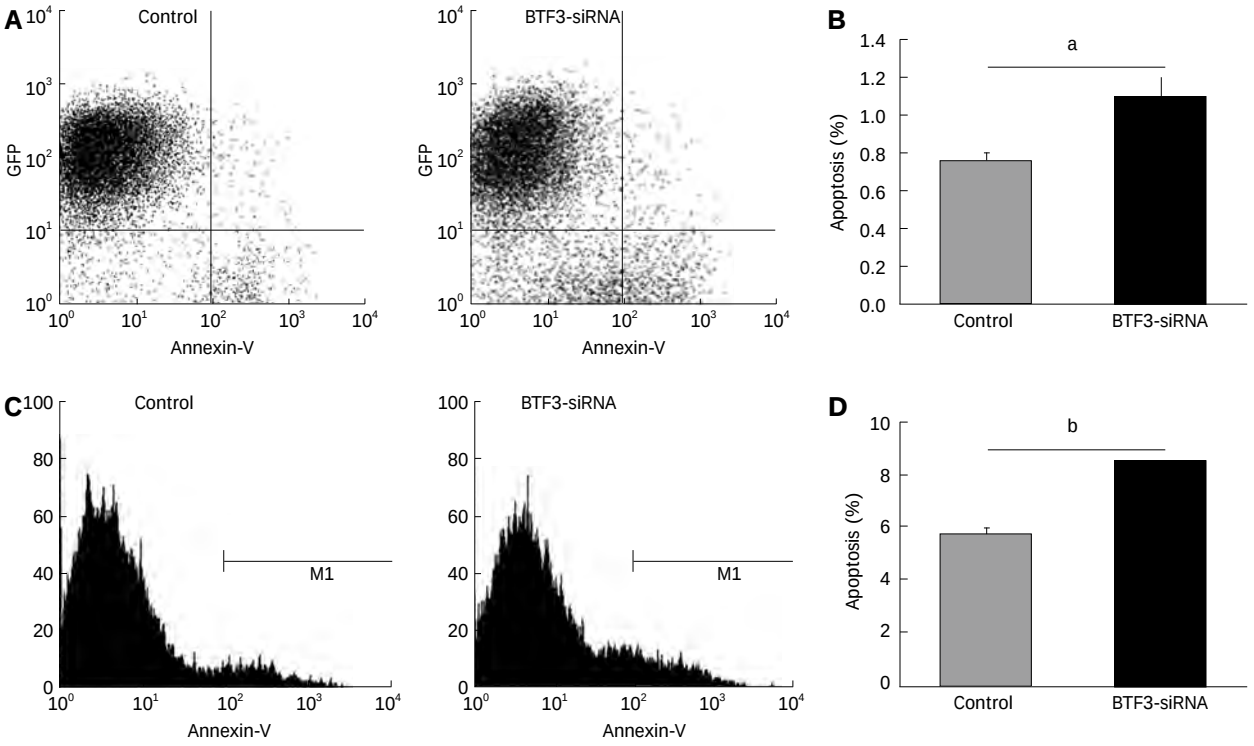


Figure 5 Apoptosis analyses by flow cytometry. A and C: Flow cytometric analysis of control and basic transcription factor 3 (BTF3)-small interfering RNA (siRNA) transfected cells; B and D: Apoptosis rates plotted as bar charts. $^{a}P < 0.05$, $^{b}P < 0.01$ between groups. GFP: Green fluorescent protein.

orderly assembled on proximal promoter elements such as the TATA box and CAAT box sequences in order to initiate the gene transcription. At the very early stage of the transcription initiation, transcription factor class Ⅱ D (TFⅡD), which belongs to the transcription factor class Ⅱ (TFⅡ) family is required to be stably bound to the transcription factor assembling TATA box. The BTF3 protein, which works as an additional TFⅡ re-

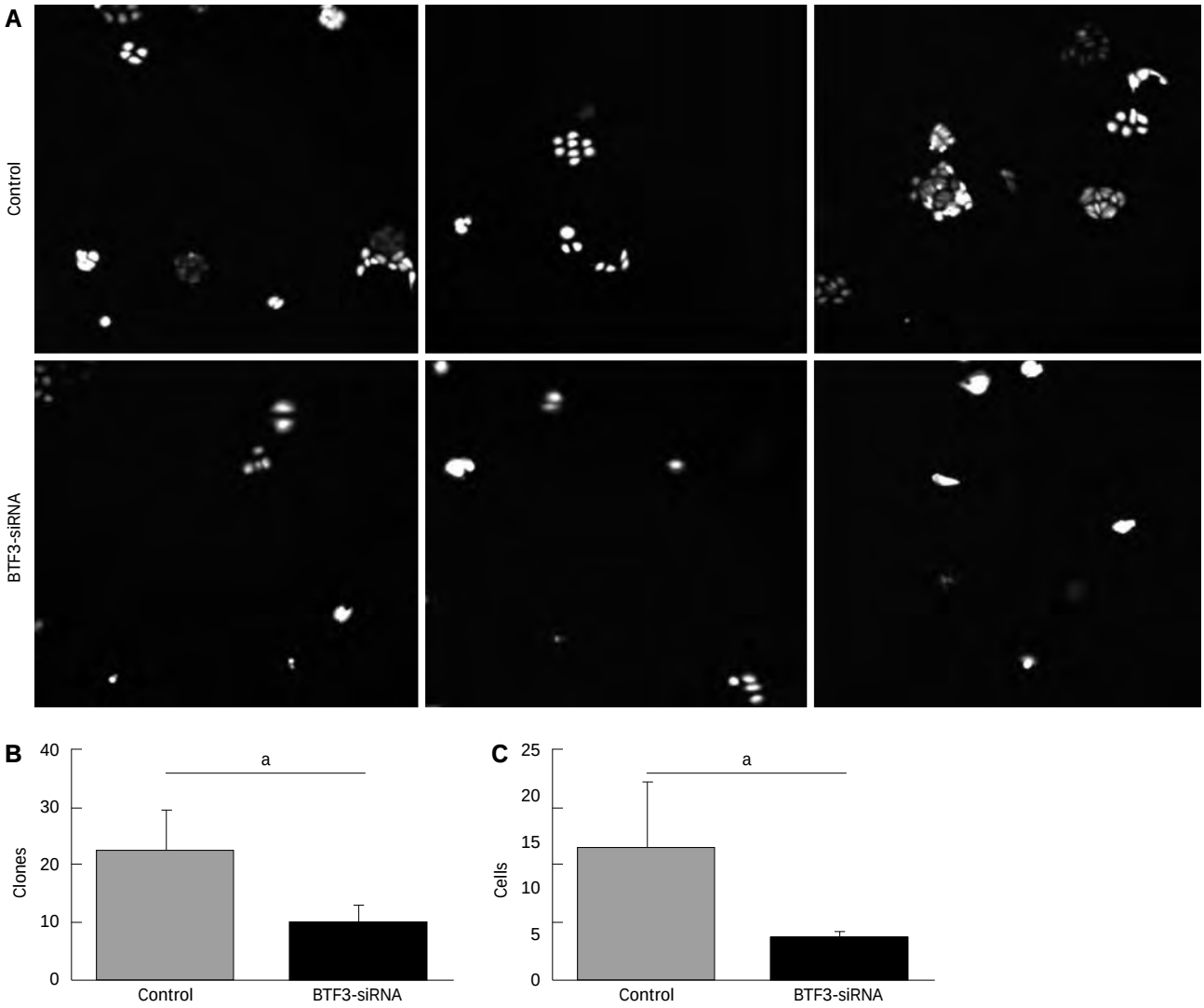


Figure 6 Colony forming abilities identified by Cellomic Assay System assays. A: Colonies formed from control and basic transcription factor 3 (BTF3)-small interfering RNA (siRNA) transfected cells were imaged *via* the Cellomic Assay System; B: Number of clones plotted in a bar chart; C: Colony cell numbers of control and BTF3-siRNA transfected cells plotted in a bar chart. $^{a}P < 0.05$ between groups were analysed with unpaired *t* test.

lated protein, does not directly associate with the proximal promoter, but forms a stable complex with RNA polymerase Ⅱ and is part of the gene transcription initiation complex[4,5]. Several studies have reported that expression pattern of transcription factors is frequently changed in gastric tumors. For example, the transcription factor Sp1 has been reported to be higher in malignant gastric tissues, and might serve as an independent prognostic factor, by influencing the tumor infiltration and progression[21]. Another transcription factor, the E2F transcription factor 1, which plays a critical role in cell cycle regulation and other biological processes of the cells, is also up-regulated in human gastric cancer tissues, but further analysis revealed that its overexpression slowed down the cancer cell growth rate[22]. Other transcription related genes were reported to be differentially expressed in gastric tumors compared to normal tissues. Some genes like the antitumor genes *GATA-4* and *GATA-5* were down-regulated[23,24], whereas others like the oncogenes Forkhead box protein M1[25], and phosphorylated Forkhead box protein O1, which are related to angiogenesis[26] were up-regulated. In addition, the activating transcription factor 4[27], which is cell protective and thereby leading to multidrug resistance, also had enhanced expression. In the present study, we found that the expression of BTF3 is up-regulated in 20 gastric tumor samples compared with normal tissues. We further investigated the expression levels of BTF3 in different malignant gastric tumor cell lines and found uniform expression levels among them (Figure 2). This is in agreement with previous information, because high BTF3 protein levels in gastric cancers have been reported in the Human Protein Atlas[28]. Hence BTF3 is a transcription factor and related to apoptosis[29-32], we thought that the expression level of BTF3 might be important for cell cycle check points, proliferation and further potentially linked with human tumor development and progression. In order to elucidate this, we silenced BTF3 in SGC7901 cells (Figure 3). As a result, the cell cycle arrest shifted from the G_1 to the G_2/M and S phases with a significantly increased apop-

tosis rate, which was also reflected in our colony forming assay with significantly less cell growth after BTF3 silencing (Figure 6). This data indicated that BTF3 silencing might induce G2/M check point failure, which led to the inhibition of cell cycle completion and enhanced apoptotic activity.

In summary, our data indicated that BTF3 has a potential link with gastric cancer development and progression. Low expression or silencing of BTF3 might inhibit tumor growth and be beneficial for cancer treatment. However, the clinical data revealed that the tumors had different BTF3 levels possibly due to the different stages of the gastric tumors examined.

COMMENTS

Background

It has been reported that the basic transcription factor 3 (*BTF3*) gene has been overexpressed in colorectal cancer, glioblastomas as well as pancreatic ductal adenocarcinoma. Down-regulation of the BTF3 expression using small interfering RNA (siRNA) resulted in changed expression of several cancer associated genes involved in tumor cell survival, cell adhesion and cell cycle.

Research frontiers

BTF3 was upregulated in all gastric cancer samples measured in this study. The authors demonstrated that silencing of BTF3 in SGC7901 cells led to a significant decline of proliferation due to enhanced cell cycle arrest and a concomitant higher apoptosis rate. The long-term effect of BTF3 silencing led to a lower amount of colony forming with less cell survival.

Innovations and breakthroughs

In gastric cancer tissues, enhanced expression of BTF3 was reported in the Human Protein Atlas, but the exact role of BTF3 in gastric cancer was not further analyzed. In this study, BTF3 was found to play an essential role in gastric cancer cell proliferation.

Applications

BTF3 was upregulated in all gastric cancer tissues derived from surgical interventions. Silencing of BTF3 led to less proliferation with an enhanced apoptosis rate in SGC7901 cells. The findings may be useful for the diagnosis and treatment of gastric cancer in the future.

Terminology

BTF3 was initially discovered as a member of the general transcription machinery and functions as a transcriptional initiation factor from proximal promoter elements by forming a stable complex with RNA polymerases, and mouse embryos, homozygous for a loss of function mutation in the *BTF3* gene, died at the early stage of development. In later developmental stages, the role of BTF3 has been described as transcriptional regulator and modulator of apoptosis.

Peer review

The authors analyzed the BTF3 expression in human gastric cancer tissues and compared the rates with expression in adjacent non-tumor tissues. As a result, all cancer tissues showed enhanced BTF3 expression. Further analysis *via* stable silencing of BTF3 in gastric cancer SGC7901 cells revealed that inhibiting BTF3 activity led to lower proliferation and enhanced apoptosis rates. The results are interesting and BTF3 might be a target gene for gastric cancer treatment.

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BRIEF ARTICLE

Neurotensin receptor 1 overexpression in inflammatory bowel diseases and colitis-associated neoplasia

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Author contributions: Gui X is the guarantor of the work as well as the main/lead researcher who was instrumental in the design, planning, selecting tissue samples, conducting the study, data review and analysis of the results, and writing the manuscript; Liu S was the major contributor to the laboratory work; Yan Y assisted with the laboratory work; Gao Z contributed partly to the results review/analysis, comments, and review of the manuscript.

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Abstract

AIM: To explore the association of neurotensin receptor 1 (NTSR1) with inflammatory bowel diseases (IBD) and colitis-associated neoplasia.

METHODS: NTSR1 was detected by immunohistochemistry in clinical samples of colonic mucosa with IBD colitis, colitis-associated raised low-grade dysplasia (LGD) including dysplasia-associated lesions or masses (DALMs, *n* = 18) and adenoma-like dysplastic polyps (ALDPs, *n* = 4), colitis-associated high-grade dysplasia (HGD, *n* = 11) and colitis-associated colorectal carcinoma (CACRC, *n* = 13), sporadic colorectal adenomatous polyp (SAP, *n* = 17), and sporadic colorectal carcinoma (SCRC, *n* = 12). The immunoreactivity of NTSR1 was semiquantitated (as negative, 1+, 2+, and 3+) and compared among different conditions.

RESULTS: NTSR1 was not detected in normal mucosa but was expressed similarly in both active and inactive colitis. LGD showed a significantly stronger expression as compared with non-dysplastic colitic mucosa, with significantly more cases showing > 2+ intensity (68.75% in LGD *vs* 32.26% in nondysplastic mucosa, *P* = 0.001). However, no significant difference existed between DALMs and ALDPs. CACRC and HGD showed a further stronger expression, with significantly more cases showing 3+ intensity than that in LGD (61.54% *vs* 12.50% for CACRC *vs* LGD, *P* = 0.022; 58.33% *vs* 12.50% for CACRC/HGD *vs* LGD, *P* = 0.015). No significant difference existed between colitis-associated and non-colitic sporadic neoplasia.

CONCLUSION: NTSR1 in colonic epithelial cells is overexpressed in IBD, in a stepwise fashion with sequential progress from inflammation to dysplasia and carcinoma.

Key words: Neurotensin; Neurotensin receptor; Inflammatory bowel diseases; Dysplasia; Colitis-associated neoplasia; Dysplasia-associated lesion or mass; Sporadic adenoma; Colorectal carcinoma

Core tip: Neurotensin receptor 1 (NTSR1) in colonic epithelial cells is overexpressed in inflammatory bowel diseases, in a stepwise fashion with the sequential progress from inflammation to low-grade dysplasia, high-grade dysplasia, and carcinoma. Both colitis-associated and sporadic dysplasia/carcinoma showed a similar pattern of NTSR1 overexpression. NTSR1 could be a potential pharmacological target in the treatment of inflammatory bowel diseases and prevention of colitis-associated neoplasia.

Gui X, Liu S, Yan Y, Gao Z. Neurotensin receptor 1 overexpression in inflammatory bowel diseases and colitis-associated neo-

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INTRODUCTION

Neurotensin (NTS) is a 13-amino-acid peptide secreted by neurons and specialized endocrine cells (N-cells) in the small intestine, which acts as a paracrine and endocrine modulator of various gut functions. The biological activities of NTS are mediated mainly through the high-affinity neurotensin receptor 1 (NTSR1), a member of the G-protein-coupled receptor family[1].

Many studies have suggested a possible role of NTS/NTSR-1 in the pathogenesis of inflammatory bowel disease (IBD) and colitis-associated neoplasia. The NTS/NTSR-1 signaling pathway has a complex dual effect (both proinflammatory and proregenerative) in the regulation of intestinal mucosal inflammation. NTS/NTSR-1 enhances the progression of acute colonic inflammation. NTS enhances mast cell degranulation and neutrophil recruitment[2-4]. NTSR-1 expression is increased in the human colonic mucosa with active ulcerative colitis (UC)[5,6] as well as in rodent colitis induced by *Clostridium difficile* toxin A[4] or by dextran sulfate sodium (DSS)[6], whereas pretreatment of NTSR-1 antagonist SR48692 inhibits the inflammatory changes[4]. Both NTS and NTSR1 expression are also increased in the mesenteric fat of mice during trinitrobenzenesulfonic-acid-induced colitis[7]. Moreover, NTS/NTSR1 activation in colonocytes stimulates interleukin (IL)-8 secretion from colonocytes through activating GTPase-mediated nuclear factor κ-light-chain-enhancer of activated B cells, mitogen-activated protein kinase, and protein kinase C[8-11]. NTS/NTSR-1 augments mucosal healing and regeneration following chronic colitis[5,6]. Pretreatment with NTSR1 antagonist worsens the severity of experimentally induced colitis and delays mucosal healing, whereas coadministration of exogenous NTS exerts the opposite effect[5,6,12,13]. NTS also stimulates colonic epithelial cell migration and proliferation through *COX-2* gene expression[5,6].

As a part of their diverse bioactivities, NTS/NTSR1 signaling is also involved in the early carcinogenesis and progression of colonic carcinoma. First, NTSR-1 is overexpressed in colonic neoplasms. A previous investigation of one of the present authors (Gui X) showed a stepwise increase in NTSR-1 mRNA with progression of colonic adenoma to adenocarcinoma[14]. Second, NTS is an epidermal growth factor (EGF)-like factor in a number of tumors[15-18]; it transactivates EGF receptor, hence it works synergistically with EGF[19,20]. Third, *NTSR-1* gene activation is linked with the *Wnt/APC/Tcf/β-catenin* pathway in colonic neoplasia. Upregulation of NTSR-1 in colorectal adenocarcinoma is the result of *Wnt/APC* pathway activation, and the increased NTSR-1 expression correlates with β-catenin cytosolic or nuclear accumulation. Additionally, *NTSR-1* gene can be activated by agents that cause β-catenin cytosolic accumulation[21]. Recently, it was also found that NTSR1 activation stimulates the expression of miRNAs 21 and 155 in colonocytes, in the experimentally induced colonic cancer (HCT-116 xenograft tumors) in mice as well as in human colonic carcinoma tissues[22].

It is well known that long-standing colitis (IBD) predisposes to the development of colorectal carcinoma (CRC). The relative risk of the development of CRC in IBD patients is 10-40-fold higher compared to that in the general population[23]. This so-called colitis-associated colorectal cancer (CACRC) is the most serious complication and the major cause of death of IBD patients. The carcinogenesis of CACRC is believed to be initiated and/or promoted by persistent active inflammation of colorectal mucosa[24,25] in the inflammation-dysplasia-carcinoma sequence, which differs from the adenoma-carcinoma sequence in sporadic CRC.

The bidirectional effect of NTS/NTSR1 on colonic mucosal inflammation-particularly the stimulation of cytokines/chemokines production and promotion of epithelial cell growth-makes NTS/NTSR1 signaling a possible unique link between chronic mucosal inflammation and carcinogenesis. For example, IL-8 [or chemokine CXC ligand (CXCL)8], an inflammatory component as a chemotactic factor for leukocytes, affects cancer (including colon cancer) progression through mitogenic, angiogenic, and motogenic effects[26]. The secretion of IL-8 can be stimulated by NTSR1 activation in colonic epithelial cells under either inflammatory or neoplastic conditions[27].

Taken together, it seems reasonable to postulate that NTSR1 in colonic epithelial cells is upregulated in IBD and that NTSR1 overexpression may play a role in the development of colitis-associated dysplasia/neoplasia. In this study, we analyzed the expression of NTSR1 in human colonic mucosa with various pathological changes characteristic of IBD and IBD-associated dysplasia and carcinoma. In order to demonstrate whether the change in NTSR1 was specific to IBD-associated neoplasia, we compared it to the sporadic colorectal neoplasia that developed in non-colitic patients.

MATERIALS AND METHODS

Study subjects

All cases were retrieved retrospectively from the surgical pathology files of the Calgary Laboratory Services in 2009 and 2010. The study was approved by the Research Committee of Calgary Laboratory Services, and ethical approval was granted by the University of Calgary Conjoint Health Research Ethics Board. The classification and grading of IBD, dysplasia and carcinoma were carried out on hematoxylin and eosin (HE)-stained slides, based on the morphological features and according to the standardized histological consensus criteria used widely

in clinical pathology practice. An attempt was made to distinguish, based on the strict morphological criterion currently available, between dysplasia-associated lesions or masses (DALMs) and adenoma-like dysplastic polyps (ALDPs) for the raised dysplastic lesions developed in a background of IBD. DALMs were defined as lesions that met all of the following criteria: (1) location within areas of chronic colitis; (2) irregularly elevated and broad-based with indistinct boundaries; and (3) surrounding mucosa also dysplastic, and in a full-thickness or "bottom-up" pattern of dysplasia. ALDPs were defined by the presence of well-circumscribed typical adenoma-looking polyps seen in the colitic regions; mostly in a "top-down" pattern of dysplasia. ALDPs are considered most likely to be sporadic adenoma.

Four separate groups of cases were included in the study.

Group 1: Eighteen colectomy cases of long-standing IBD complicated by colorectal neoplasia (14 males, 4 females, aged 26-84 years), including 13 UC, three Crohn's disease, and two indeterminate colitis. In this group, we looked for sequential histological changes of inflammation/dysplasia/carcinoma. In each case, the tissue blocks were selected from those with proven histology of normal/unremarkable colonic mucosa (n = 16), active colitis (n = 16), inactive colitis (n = 14), raised low-grade dysplasia (LGD, n = 16), high-grade dysplasia (HGD, n = 11), and adenocarcinoma (CACRC, n = 13). For the LGD lesions, 12 DALMs and four ALDPs were subgrouped.

Group 2: Eighteen colonoscopic biopsies of DALMs detected in longstanding IBD patients (10 males, 8 females, aged 23-68 years) for neoplasia surveillance.

Group 3: Seventeen randomly selected biopsies of sporadic colorectal adenomatous polyps detected in non-IBD patients (10 males, 7 females, aged 35-79 years) for colon cancer screening.

Group 4: Twelve randomly selected cases of colectomy for sporadic colorectal adenocarcinoma (SCRC) detected in non-IBD patients (7 males, 5 females, aged 42-85 years).

Detection of NTR-1 in colonic epithelial cells

The expression of NTSR1 was detected by immunohistochemistry of deparaffinized sections using the avidin-biotin-peroxidase complex method. The formalin-fixed paraffin-embedded tissue sections were pretreated in CINTec Epitope Retrieval Solution (10 mmol/L Tris/1 mmol/L EDTA, pH 9.0) for 20 min at 95-100 ℃, and then cooled down slowly to room temperature. The NTSR1 antibody was a rabbit polyclonal antibody (Imgenex, San Diego, CA, United States) against the third cytoplasmic domain of human NTSR1. All slides were stained with Ventana Nexes IHC autostainer at 1:40 dilution using UltraView Universal DAB Detection (Ventana 760-500). Immunoreactivity of NTSR1 appeared in a cytoplasmic pattern. Only the surface and cryptal epithelial cells of colonic mucosa were analyzed (in order to eliminate the variability of mononuclear cells in lamina propria). The positivity and intensity of the immunoreactivity of NTSR1 were semiquantitated independently by two pathologists as absent (negative), weak (1+), moderate (2+), and strong (3+). If the signal intensity was heterogeneous, the level assigned was based on the intensity in at least 50% of tumor cells or epithelial cells. A comparison of NTSR1 expression was analyzed between the different groups (active *vs* inactive colitis, dysplastic *vs* non-dysplastic lesions, DALMs *vs* ALDPs, colitis-associated dysplasia *vs* sporadic non-colitic dysplasia, and colitis-associated CRC *vs* sporadic non-colitic CRC).

Table 1 Neurotensin receptor 1 expression in 18 cases of inflammatory bowel diseases with colectomy *n* (%)

Histology	Case				*P* value
	Negative	1+	2+	3+	
Normal	12 (100.00)	0 (0.00)	0 (0.00)	0 (0.00)	
Colitis	1 (3.23)	20 (64.52)	10 (32.36)	0 (0.00)	
Active	0 (0.00)	12 (75.00)	4 (25.00)	0 (0.00)	
Inactive	1 (6.67)	8 (53.33)	6 (40.00)	0 (0.00)	0.338[1]
LGD	0 (0.00)	3 (18.75)	11 (68.75)	2 (12.50)	0.007[2]
DALM	0 (0.00)	3 (25.00)	7 (58.33)	2 (16.67)	
ALDP	0 (0.00)	0 (0.00)	4 (100.00)	0 (0.00)	0.298[1]
HGD	0 (0.00)	1 (9.09)	4 (36.36)	6 (54.55)	0.063[3]
CACRC	0 (0.00)	1 (7.69)	4 (30.77)	8 (61.54)	0.022[3]

[1]Within two subgroups (active *vs* inactive); [2]As compared with colitis; [3]As compared with low-grade dysplasia (LGD). HGD: High-grade dysplasia; DALM: Dysplasia-associated lesions or masses; ALDP: Adenoma-like dysplastic polyps; CACRC: Colitis-associated colorectal carcinoma.

Statistical analysis

Analysis of variance test was used to determine the statistical significance of the NTSR1 expression intensity between different groups. Differences were considered significant if P was < 0.05.

RESULTS

NTSR1 expression in colonic mucosa is associated with stepwise progression from colitis to LGD, HGD and carcinoma

Within the 18 colectomy specimens, relatively normal (uninvolved/colitis-spared) mucosa was identified in 12 cases. NTSR1 was not detected in any of the samples of normal mucosa. In the colitic mucosa, however, NTSR1 was detected in 30 out of 31 representative tissue samples and expressed similarly in both active and inactive colitis, as shown in Table 1 and Figure 1.

LGD lesions were identified in 16 of the 18 cases. The epithelium with LGD showed a significantly stronger expression of NTSR1 as compared to the non-dysplastic colitic mucosa, with most cases showing a $\geqslant$ 2+ intensity (68.75% in LGD *vs* 32.26% in non-dysplastic mucosa, P = 0.001) but fewer cases showing a 1+ intensity (18.75%

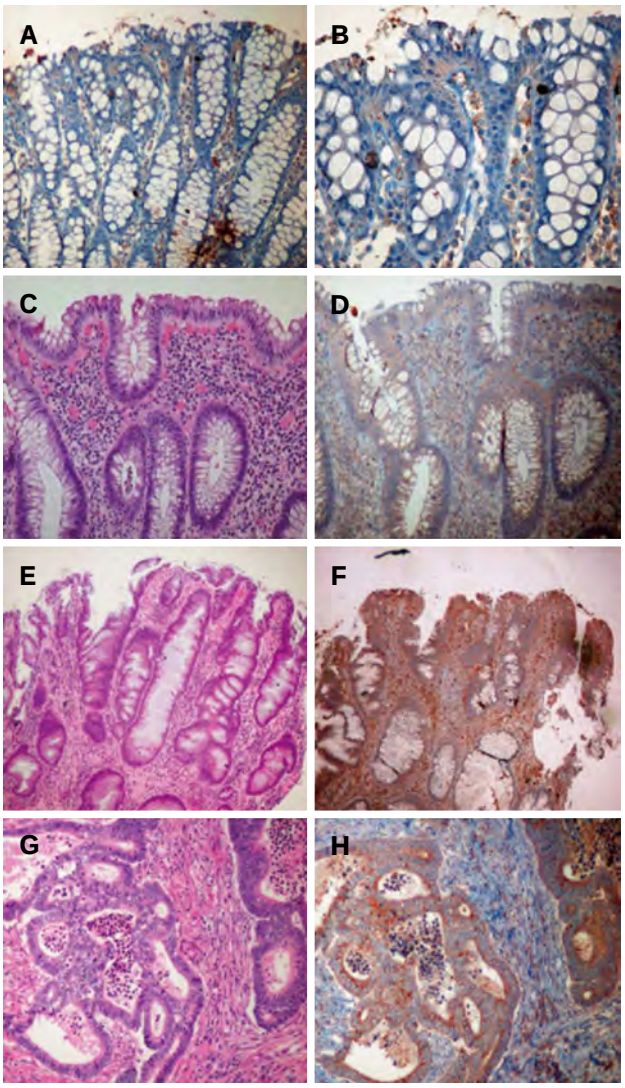

Figure 1 Neurotensin receptor 1 expression in colonic mucosa under different conditions. A and B: Normal colonic mucosa; C and D: Active chronic colitis; E and F: Dysplasia-associated lesions or masses with low-grade dysplasia; G and H: Invasive adenocarcinoma (hematoxylin and eosin histology and neurotensin receptor 1 expression immunohistochemistry).

in LGD *vs* 64.52% in non-dysplastic mucosa, $P = 0.007$). However, no significant difference existed between DALMs and ALDPs, as shown in Table 1 and Figures 1 and 2.

HGD was identified in 11 cases, and CACRC was identified in 13. As shown in Table 1 and Figures 1 and 2, expression of NTSR1 in the CACRC and HGD samples was stronger, with significantly more cases showing a 3+ intensity than in LGD of both DALMs and ALDPs (61.54% *vs* 12.50% for CACRC *vs* LGD, $P = 0.022$; 58.33% *vs* 12.50% for CACRC/HGD *vs* LGD, $P = 0.015$). However, no significant difference existed between CACRC and HGD ($P = 0.942$).

NTSR1 expressed similarly between DALMs and non-colitic sporadic adenoma

In the cases of colitis-associated DALMs ($n = 18$) and non-colitis sporadic adenomas ($n = 17$), the increased expression of NTSR1 showed a similar pattern, as shown in Table 2 and Figure 2.

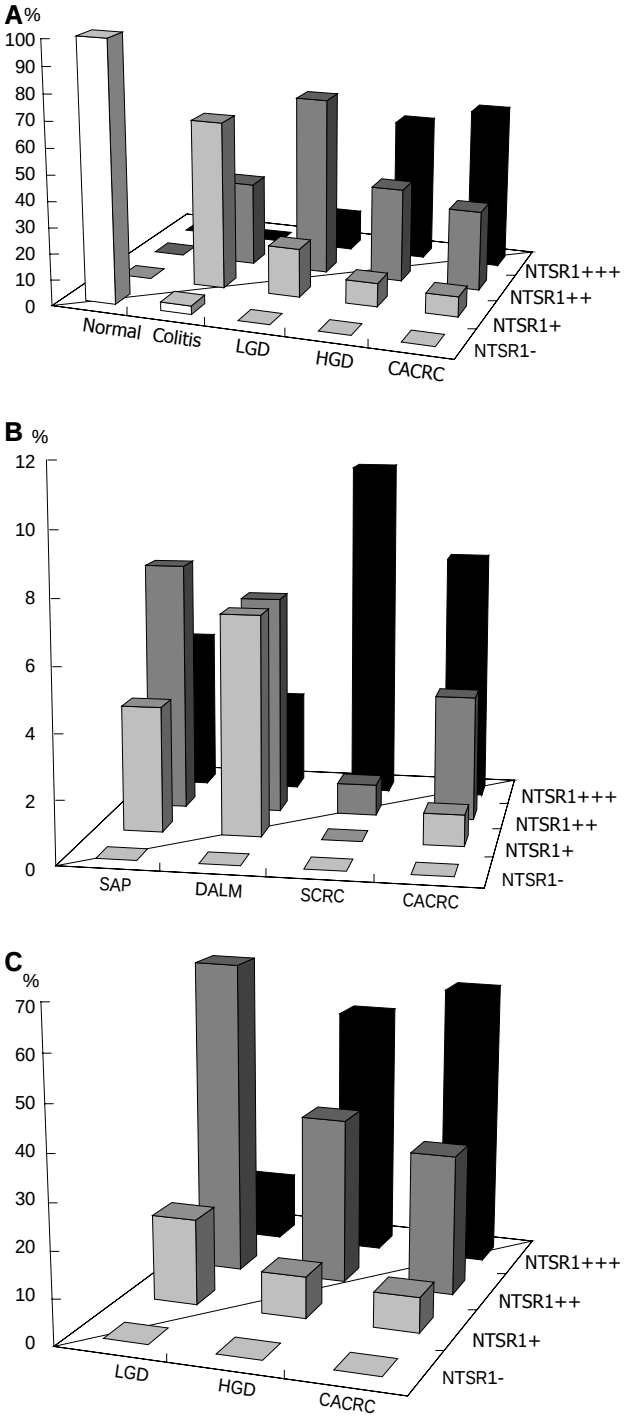

Figure 2 Neurotensin receptor 1 expression. A: Neurotensin receptor 1 (NTSR1) expression in colonic mucosa under different conditions in cases of colectomy for inflammatory bowel diseases (IBD) (percentage of cases in each subgroup); B: NTSR1 expression in colonic mucosa with dysplasia and carcinoma in cases of colectomy for IBD (percentage of cases in each subgroup); C: Comparison of colonic mucosal NTSR1 expression in sporadic neoplasia and colitis-associated neoplasia (percentage of cases in each subgroup). LGD: Low-grade dysplasia; HGD: High-grade dysplasia; DALM: Dysplasia-associated lesions or masses; CACRC: Colitis-associated colorectal carcinoma; SAP: Sporadic colorectal carcinoma; SCRC: Sporadic colorectal adenomatous polyp.

Table 2 Neurotensin receptor 1 expression *n* (%)

	Negative	1+	2+	3+
SAP	0	4 (23.53)	8 (47.06)	5 (29.41)
DALM	0	7 (41.42)	7 (41.42)	3 (17.65)
SCRC	0	0	1 (8.33)	11 (91.67)
CACRC	0	1 (7.69)	4 (30.77)	8 (61.54)

Comparison between colitis-associated dysplasia/carcinoma and sporadic dysplasia/carcinoma. Sporadic colorectal adenomatous polyp (SCRC) *vs* colitis-associated colorectal carcinoma (CACRC), $P = 0.198$; Dysplasia-associated lesions or masses (DALMs) *vs* CACRC, $P = 0.028$; DALMs *vs* sporadic colorectal carcinomas (SAPs), $P = 0.50$.

NTSR1 expressed similarly between colitis-associated and sporadic CRC

The increased expression of NTSR1 also showed a similar pattern to that in CACRC and in conventional sporadic colorectal carcinoma (SCRC, $n = 12$), as shown in Table 2 and Figure 2.

DISCUSSION

Through the detection of NTSR1 expression directly in human colonic mucosa with various IBD-related pathologies and those with sporadic colonic neoplasia in non-IBD patients, the present study demonstrated that both active and inactive IBD colitis upregulated NTSR1 in colonic epithelial cells; colitis-associated LGD to HGD and carcinoma was associated with stepwise higher expression of NTSR1; and the overexpression of NTSR1 showed a similar pattern in colitis-associated and non-colitic sporadic dysplasia/neoplasia, which suggests that NTSR1 is commonly unregulated in colonic neoplasia with or without a background of colitis.

The first two findings support the hypothesis that the upregulation of NTSR1 is involved in IBD inflammation and colitis-associated neoplasia. The findings corroborate various studies carried out in the past in animal models and *ex vivo* systems. In a similar study reported by Bossard *et al*[28], identical findings were shown with a slightly different methodology. Their study also demonstrated that coexpression of NTS/NTSR1 is present in a majority of the inflammatory and neoplastic/dysplastic lesions, suggestive of a self-activation of NTSR1 secondary to increased production of ligands; and β-catenin nuclear translocation is seen in a minority of the dysplastic and carcinomatous lesions in which no NTS was detected, suggestive of a different pathway.

To the best of our knowledge, the finding that NTSR1 is similarly overexpressed in colitis-associated dysplasia/neoplasia and sporadic dysplasia/neoplasia in non-IBD patients has not been reported previously. This finding indicates that NTSR1 is a neoplastic marker irrespective of its underlying etiology. In other words, the NT/NTSR1 signaling pathway is intrinsic and common to the tumorigenesis of all colorectal carcinomas, regardless of the tumor-promoting factors or the predisposing/initiation processes. This finding and interpretation are supported by an animal study reported recently by Bugni *et al*[29]. They developed a chemical-carcinogen-induced colonic adenoma by administration of azoxymethane to mice with or without DSS-induced colitis. NTSR1-deficient (gene knockout) mice had a < 50% chance, compared to wild-type mice, of developing colonic adenoma in the absence of colitis (*i.e.*, a model of sporadic colonic neoplasia). The difference, however, disappeared in the mice that had colitis (*i.e.*, a model of colitis-associated colonic neoplasia), even though significantly higher levels of IL-6 and CXCL2 (the mouse homolog of IL-8, both known as tumor-promoting cytokines) were seen in the latter. Our study, as well as that of Bugni *et al*[29], suggests that NTS/NTSR1 are not particularly responsible for the link between chronic inflammation and neoplasia in IBD, although it is commonly involved in the entire multistep process as an intrinsic regulator. The tumorigenetic process in colitis-associated dysplasia/neoplasia appears far more complex. However, it is still possible that increased NTSR1 expression associated with pre-existent or coexistent chronic colitis may further enhance the carcinogenesis.

It was noted in a minority of cases that NTSR1 expression was less upregulated, which occurred nonspecifically for each of the conditions and in different cases. We have no solid explanation for these relative negative cases. It is possible that NTSR1 expression in the epithelium is regulated by multiple factors, including a variety of cytokines and other gut peptides in the local mucosa or circulation that are not always the same in different patients.

Overall, our findings further provide a rationale for exploring the anti-NTSR1 approach in the treatment of IBD and in the chemoprevention of IBD-associated colorectal neoplasia as well as the treatment of sporadic colonic neoplasia. Hopefully, the development of clinically useful NTSR1 blockers will become a reality in the near future with the recent better understanding of the chemical structure of NTSR1[30].

ACKNOWLEDGMENTS

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COMMENTS

Background

Inflammatory bowel diseases (IBD) are chronic and debilitating inflammatory conditions of the colon, which also increase the relative risk of colorectal cancer. Identification of the factors associated with both mucosal inflammation and colitis-associated carcinogenesis could lead to novel treatments.

Research frontiers

Neurotensin (NTS) is a 13-amino-acid peptide that acts as a paracrine and endocrine modulator of various gut functions. Its bioactivities are mediated mainly through the high-affinity neurotensin receptor 1 (NTSR1). The NTS/NTSR1 signaling pathway has dual effects (both proinflammatory and proregenerative) in the regulation of intestinal mucosal inflammation. NTS/NTSR1 signaling is also involved in the carcinogenesis and progression of colonic carcinoma. It is suggested that NTSR1 in colonic epithelial cells is upregulated in IBD and NTSR1 overexpression may play a role in the development of colitis-associated dysplasia/neoplasia.

Innovations and breakthroughs

The present study used colonic tissue samples to detect the expression of NTSR1 in the context of various pathological conditions of IBD, with a focus on NTSR1 expression in the progressive changes from active inflammation to low-grade dysplasia, high-grade dysplasia, and carcinoma. A stepwise increase in NTSR1 expression was identified in the sequential progression. Moreover, a similar pattern of NTSR1 overexpression in colitis-associated and sporadic dysplasia/neoplasia was also determined for the first time.

Applications

The findings provide a rationale for exploring the anti-NTSR1 approach in the treatment of IBD and in the chemoprevention of IBD-associated colorectal neoplasia, a clinically useable anti-NTSR1 agent becomes available in the near future.

Terminology

Colitis-associated dysplasia and carcinoma develop in association with the longstanding chronic colitis in IBD patients. It is now well recognized that this type of colorectal carcinogenesis is clearly initiated and/or promoted by chronic, persistent and repetitively active mucosal inflammation. This type of colorectal carcinoma (CRC) develops and progresses in an inflammation-dysplasia-carcinoma sequence and therefore differs from the adenoma-carcinoma sequence in the sporadic CRC.

Peer review

The study investigates the relationship between NTSR1 expression in IBD and the possibility of its association with mucosal inflammation and colitis-associated neoplasia. They found a strong correlation with the progression from normal mucosa to colitis, degree of dysplasia, and carcinoma. The methodological approach was correct and the findings are interesting.

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BRIEF ARTICLE

First-line erlotinib and fixed dose-rate gemcitabine for advanced pancreatic cancer

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Author contributions: Milella M designed clinical protocol; Vaccaro V, Gelibter A, Bria E and Milella M enrolled and treated patients and analyzed the results; Moscetti L, Mansueto G, Ruggeri EM, Gamucci T and Cognetti F enrolled and treated patients; Sperduti I analyzed data; Vaccaro V wrote the paper.
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Abstract

AIM: To investigate activity, toxicity, and prognostic factors for survival of erlotinib and fixed dose-rate gemcitabine (FDR-Gem) in advanced pancreatic cancer.

METHODS: We designed a single-arm prospective, multicentre, open-label phase Ⅱ study to evaluate the combination of erlotinib (100 mg/d, orally) and weekly FDR-Gem (1000 mg/m^2, infused at 10 mg/m^2 per minute) in a population of previously untreated patients with locally advanced, inoperable, or metastatic pancreatic cancer. Primary endpoint was the rate of progression-free survival at 6 mo (PFS-6); secondary endpoints were overall response rate (ORR), response duration, tolerability, overall survival (OS), and clinical benefit. Treatment was not considered to be of further interest if the PFS-6 was < 20% (p0 = 20%), while a PFS-6 > 40% would be of considerable interest (p1 = 40%); with a 5% rejection error (α = 5%) and a power of 80%, 35 fully evaluable patients with metastatic disease were required to be enrolled in order to complete the study. Analysis of prognostic factors for survival was also carried out.

RESULTS: From May 2007 to September 2009, 46 patients were enrolled (male/female: 25/21; median age: 64 years; median baseline carbohydrate antigen 19-9 (CA 19-9): 897 U/mL; locally advanced/metastatic disease: 5/41). PFS-6 and median PFS were 30.4% and 14 wk (95%CI: 10-19), respectively; 1-year and median OS were 20.2% and 26 wk (95%CI: 8-43). Five patients achieved an objective response (ORR: 10.9%, 95%CI: 1.9-19.9); disease control rate was 56.5% (95%CI: 42.2-70.8); clinical benefit rate was 43.5% (95%CI: 29.1-57.8). CA 19-9 serum levels were decreased by > 25% as compared to baseline in 14/23 evaluable patients (63.6%). Treatment was well-tolerated, with skin rash being the most powerful predictor of both longer PFS (P < 0.0001) and OS (P = 0.01) at multivariate analysis (median OS for patients with or without rash: 42 wk *vs* 15 wk, respectively, Log-rank P = 0.03). Additional predictors of better outcome were: CA 19-9 reduction, female sex (for PFS), and good performance status (for OS).

CONCLUSION: Primary study endpoint was not met. However, skin rash strongly predicted erlotinib efficacy, suggesting that a pharmacodynamic-based strategy for patient selection deserves further investigation.

Key words: Pancreatic cancer; Gemcitabine; Fixed dose-rate; Erlotinib; Prognostic factors; Cutaneous rash; Phase Ⅱ trial

Core tip: The most important finding reported in this study is the strong predictive value of the appearance of skin rash, related to epidermal growth factor receptor (EGFR)-pathway inhibition. Our data suggest that patients developing any grade of skin rash during the treatment, can achieve disease control and survival comparable to those obtained with more intensive and more toxic chemotherapy. These findings underline the relevance of further investigation of the biological mechanisms related to the occurrence of skin rash upon EGFR blockade in order to identify clinical/molecular biomarkers predicting toxicity and efficacy and to prospectively select a subset of patients who could potentially benefit from Gem/erlotinib.

Vaccaro V, Bria E, Sperduti I, Gelibter A, Moscetti L, Mansueto G, Ruggeri EM, Gamucci T, Cognetti F, Milella M. First-line erlotinib and fixed dose-rate gemcitabine for advanced pancreatic cancer. *World J Gastroenterol* 2013; 19(28): 4511-4519 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i28/4511.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i28.4511

INTRODUCTION

Pancreatic adenocarcinoma (PDAC) is arguably the most aggressive solid malignancy, with nearly as many deaths as the number of newly diagnosed cases each year. In 2012 an estimated 43920 new cases and an estimated 37390 deaths are expected to occur, making pancreatic carcinoma the fourth leading cause of cancer-related death in the United States. The prognosis of pancreatic cancer is extremely poor due to difficulties in early detection and early metastatic dissemination, with a 5-year survival rate of only 6%[1].

The majority of PDAC patients present with metastatic or inoperable disease. In this setting, systemic chemotherapy remains the treatment of choice, with a palliative objective and a disappointing, marginal, survival advantage. Single-agent gemcitabine (Gem), administered as weekly 30-min *iv* infusions, has become the standard care for advanced PDAC based on a small but statistically significant advantage over bolus 5-fluorouracil (5-FU), in terms of both clinical benefit (CB) and survival[2].

Until recently, efforts to improve on single-agent Gem efficacy[3], by combining Gem with either a second cytotoxic drug or a molecularly targeted agent, have failed[4,5]. The addition of erlotinib, an oral epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, to Gem has produced a minimal, albeit statistically significant, improvement in overall survival (OS), leading to FDA approval of the Gem/erlotinib combination in the setting of advanced, inoperable PDAC[6]. On the other hand, pharmacokinetic Gem modulation, achieved by prolonging its infusion time, is justified by the observation that deoxycytidine kinase, the enzyme converting Gem into its active triphosphate metabolite, is rapidly saturated at plasma concentrations achieved with the standard 30-min infusion. Consequently, the infusion of Gem over a prolonged period at the constant dose rate of 10 mg/m^2 per min (FDR-Gem) avoids enzyme saturation and permits greater intracellular accumulation, possibly increasing Gem antitumor activity. This strategy has proven promising in a randomized phase Ⅱ trial, in which FDR-Gem significantly improved time to treatment failure as compared with the standard 30-min infusion[7]. Although formally negative, a phase Ⅲ trial comparing standard Gem with either FDR-Gem or the GEMOX combination, produced a clear signal in favor of FDR-Gem, which was as effective as the GEMOX combination[8].

Recently, a four-drug combination including 5-FU, folinic acid, oxaliplatin and irinotecan (FOLFIRINOX regimen) has demonstrated to improve objective response rate (ORR), progression-free survival (PFS) and OS over single-agent Gem administered by standard 30-min infusion in metastatic PDAC patients[9]. However, such improved efficacy comes at the price of significantly higher toxicity (both hematological and non-hematological), which restricts the use of such regimen to accurately selected, young and fit patients.

Based on our previous experience with a modified FDR-Gem regimen, which utilizes a lower Gem dose (1000 mg/m^2) as compared with the original FDR-Gem described by Tempero *et al*[7] (1500 mg/m^2) resulting in reduced hematological toxicity[10,11], we prospectively investigated the activity and tolerability of FDR-Gem in combination with erlotinib in advanced, inoperable PDAC patients.

MATERIALS AND METHODS

Patient population

Patients with cytologically or histologically proven, treatment-naïve, unresectable or metastatic PDAC and measurable disease were eligible for the study. Prior radiation for the management of local disease was allowed, provided that disease progression had been documented, all toxicities had resolved and treatment was completed at least 4 wk before study enrollment. Prior chemotherapy was not permitted, except for fluorouracil or Gem given concurrently with RT for radiosensitization purposes. Other inclusion criteria included: age > 18 years; Eastern Cooperative Oncology Group (ECOG) performance status (PS) ≤ 3; life expectancy > 12 wk; adequate hematological, renal, hepatic function; absence of other relevant medical conditions, potentially precluding the delivery of the planned treatment. The study was

Table 1 Patients' characteristics *n* (%)

Characteristics	Categories	Data
Age (yr)	Median (range)	64 (35-81)
Gender	Male	25 (54)
	Female	21 (46)
Stage	Locally advanced	5 (11)
	Metastatic	41 (89)
ECOG PS	0	10 (22)
	1	26 (56)
	2	9 (20)
	3	1 (2)
Basal CA 19-9 (U/mL)	Median (range)	897 (1-49, 483)
Interval between symptoms and treatment (wk)	Median (range)	12 (2-179)
Clinical benefit	Evaluable	33 (75)
	Not evaluable	13 (25)
Follow-up (wk)	Median (range)	21.5 (2-91)
Number of administrations	Median (range)	9 (1-29)

PS: Performance status; ECOG: Eastern Cooperative Oncology Group; CA 19-9: Carbohydrate antigen 19-9.

Table 2 Objective response, clinical benefit response and carbohydrate antigen 19-9 reduction in the overall population *n* (%)

Parameter	Patients (*n* = 46)
CR/PR	5 (10.9)
SD	21 (45.7)
PD	20 (43.4)
CB	
Pos	15 (42.9)
Neg	20 (57.1)
CA 19-9 reduction > 25%	14 (63.6)[1]

[1]In 23 evaluable patients. CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; CB: Clinical benefit; Pos: Positive; Neg: Negative; CA 19-9: Carbohydrate antigen 19-9.

reviewed and approved by the institutional review board of the Regina Elena National Cancer Institute (Rome, Italy), and written informed consent, according to Institutional requirements, was obtained from all patients before entering the study.

Treatment and study design

This was a single-arm, open-label, multicenter phase II study, evaluating the activity and tolerability of the combination of FDR-Gem and erlotinib in patients with advanced PDAC. Study patients received Gem at the dose of 1000 mg/m^2, administered as a 10 mg/m^2 per min FDR *iv* infusion (100 min total infusion time)[10,11], weekly for 7 consecutive weeks and on days 1, 8, and 15 of a 4-wk cycle thereafter for a maximum of 6 cycles in the absence of progressive disease (PD) or unacceptable toxicity; erlotinib was administered as a daily oral dose of 100 mg from day 1 until PD or unacceptable toxicity. Toxicities were recorded according to the National Cancer Institute-Common Toxicity Criteria Version 3.0. Appropriate dose reductions of each study agent were planned in case of severe toxicities. Tumor assessments were performed

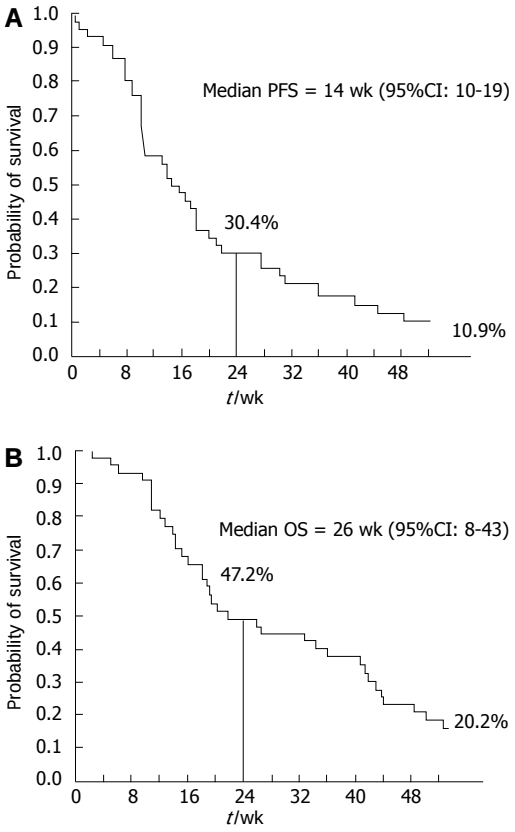

Figure 1 Kaplan-Meier analysis of progression-free survival and overall survival in the intent to treat population. A: Progression-free survival (PFS); B: Overall survival (OS).

at the end of cycle 1 and every 2 cycles thereafter.

Response and progression were evaluated using the Response Evaluation Criteria in Solid Tumours (RECIST 1.0)[12]. All patients who had measurable lesions and who had at least one objective tumour assessment after baseline were considered evaluable for response. The composite end point of CB was evaluated according to the criteria established by Burris *et al*[2] and included the assessment of pain (pain intensity and analgesic consumption) and functional impairment (assessed by Karnofsky PS) as primary measures and weight change (assessed by body weight) as a secondary measure. Each patient was classified as positive, stable, or negative for each of the primary CB measures (pain intensity or PS)[2]. For all patients, positive indicated a sustained (≥ 4 wk) improvement over baseline. If the patient was stable on both primary measures of clinical benefit, the patient was then classified as either positive or non-positive on the basis of the secondary clinical benefit measure of weight. For patients to achieve an overall rating of a positive CB, they had to be positive for at least one parameter without being negative for any of the others.

Statistical analysis

PFS rate at 6 mo (PFS-6) was selected as the primary study endpoint. Secondary endpoints were ORR, response duration, tolerability, OS, and CB. Sample size

Table 3 Toxicity (maximum toxicity per patient) *n* (%)

Variables	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Haemoglobin	15 (32.6)	17 (37.0)	10 (21.7)	4 (8.7)	-
Leucopenia	29 (63.0)	3 (6.5)	9 (19.6)	5 (10.9)	-
Neutropenia	25 (54.3)	2 (4.3)	9 (19.6)	8 (17.4)	2 (4.3)
Febrile neutropenia	44 (95.7)	-	2 (4.3)	-	-
Platelet	32 (69.6)	6 (13.0)	2 (4.3)	6 (13.0)	-
Fever	35 (76.1)	9 (19.6)	2 (4.3)	-	-
Bleeding	45 (97.8)	-	-	1 (2.2)	-
Alopecia	41 (89.1)	4 (8.7)	1 (2.2)	-	-
Anorexia	38 (82.6)	7 (15.2)	1 (2.2)	-	-
Asthenia	22 (47.8)	12 (26.1)	11 (23.9)	1 (2.2)	-
Cardiac	45 (97.8)	-	1 (2.2)	-	-
Skin	24 (52.2)	15 (32.6)	5 (10.9)	2 (4.3)	-
Diarrhoea	18 (39.1)	16 (34.8)	11 (23.9)	1 (2.2)	-
Constipation	46 (100.0)	-	-	-	-
Stomatitis	42 (91.3)	1 (2.2)	3 (6.5)	-	-
ALT	24 (52.2)	13 (28.3)	6 (13.0)	3 (6.5)	-
AST	19 (41.3)	12 (26.1)	10 (21.7)	4 (8.7)	1 (2.2)
Bilirubine	42 (91.3)	2 (4.3)	2 (4.3)	-	-
Renal	43 (93.5)	3 (6.5)	-	-	-
Neurological	45 (97.8)	1 (2.2)	-	-	-
Nausea	39 (84.8)	3 (6.5)	4 (8.7)	-	-
Vomiting	40 (87.0)	3 (6.5)	3 (6.5)	-	-

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

Table 4 Multivariate analysis for progression-free survival and overall survival

Variables	PFS		OS	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
PS	NA		8.78 (1.60-48.2)	0.01
Gender	2.99 (1.13-7.90)	0.03	2.66 (0.85-8.35)	0.09
CB	NA		3.07 (0.87-10.8)	0.08
Skin rash[1]	8.66 (2.65-28.32)	< 0.0001	5.10 (1.41-18.4)	0.01
CA 19-9 decrease[2]	2.64 (0.93-7.45)	0.07	3.36 (1.05-10.6)	0.04

[1]None *vs* any grade; [2]Carbohydrate antigen 19-9 (CA 19-9) decrease of ⩾ 25%. PFS: Progression-free survival; OS: Overall survival; PS: Performance status according to Eastern Cooperative Oncology Group (PS 0-1 *vs* PS 2-3); CB: Clinical benefit; NA: Not applicable.

was computed according to the exact single-stage Phase II design described by A'Hern[13]. The treatment was not considered to be of further interest if the PFS rate at 6 mo was < 20% (p0 = 20%). The alternate hypothesis assumed that a PFS rate at 6 mo of > 40% would be of considerable interest (p1 = 40%). With a 5% rejection error (α = 5%) and a power of 80%, a total of 35 fully evaluable patients were needed to complete the study. In order to have enough power to also analyze the 'pure' metastatic sub-population separately, 46 patients were planned to enter the study, taking into account a dropout rate of approximately 15%. The Kaplan-Meier method was used to estimate PFS and OS[14]. PFS was defined as the time from the first day of treatment to the first observation of disease progression or death due to any cause and OS was defined as the time from the first day of treatment to death from any cause. ORR was estimated as the proportion of patients evaluable for response who met RECIST criteria for complete or partial response (CR or PR). Response duration was calculated for all patients achieving a PR or CR as the time from first objective status assessment of CR/PR to the first time documented PD or death. Cox proportional hazards models were used to compare survival among different patient/disease characteristics and treatment response groups[15]; hazard ratios were appropriately derived from these models. The SPSS statistical software package version 20.0 (SPSS, Inc, Chicago, IL, United States) was used for all statistical analyses.

RESULTS

Between May 2007 and September 2009, 46 patients with advanced-stage PDAC were enrolled in the study from 3 institutions. Patient characteristics are shown in Table 1.

Treatment outcome

All 46 patients were evaluable for response according to RECIST criteria. PR and stable disease (SD) were observed in 5/46 (10.9%) and 21/46 (45.7%) patients, respectively, for an overall disease control rate (DCR), defined as the percentage of patients who had CR, PR or SD as their best response, of 56.5% (95%CI: 42.2-70.8); PD was documented at the first response assessment in 20 patients (43.5%). Median response duration was 27.4 wk (range 11-45 wk); median duration of stable disease was 27.6 wk (range 10-85 wk). Fifteen out of 35 evaluable patients (42.9%) experienced a positive CB. CA 19-9 serum levels were decreased by > 25% as compared to baseline in 14/23 evaluable patients (63.6%). Similar results were obtained in the pure metastatic population

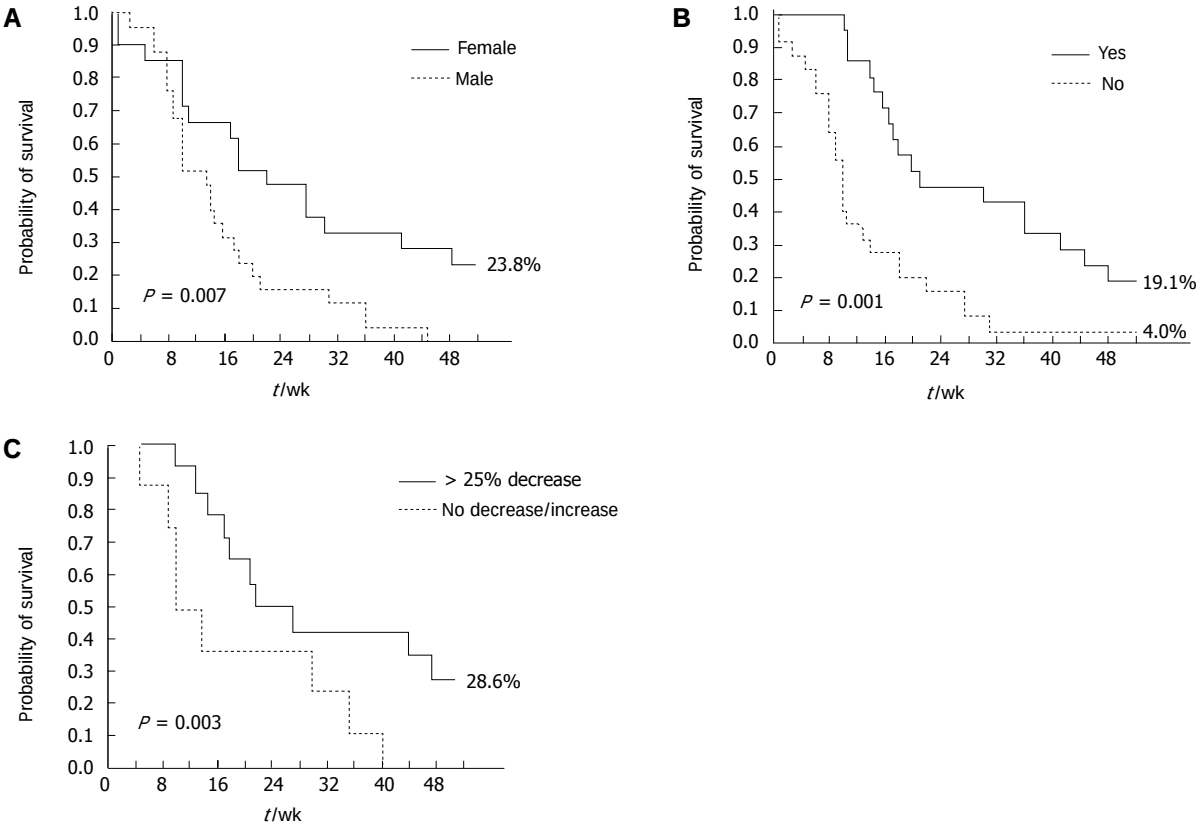

Figure 2 Kaplan-Meier analysis of independent progression-free survival predictors. A: Progression-free survival (PFS) by sex; B: PFS by skin rash; C: PFS by carbohydrate antigen 19-9 decrease.

(data not shown). At a median follow-up of 23.6 wk (range 2-139 wk), the median PFS and 1-year PFS rate were 14 wk (95%CI: 10-19) and 10.9%, respectively; PFS-6 (primary study endpoint) was 30.4% (Figure 1A). The median OS and 1-year OS rate were 26 wk (95%CI: 8-43 wk) and 20.2%, respectively (Figure 1B). In the pure metastatic population the corresponding figures were: median PFS: 14 wk, PFS-6: 24.4 %, median OS: 20 wk, 1-year OS: 12.7% (Table 2, data not shown).

Toxicity

All patients were evaluable for toxicity. Main hematological and non-hematological toxicities are summarized in Table 3. Treatment protocol was well tolerated, with only 3 serious adverse events that required hospitalization: 2 episodes of GI bleeding and 1 duodenal perforation. Three patients (7%) reported grade 4 toxicities (neutropenia in 2 patients and asymptomatic transaminase elevation in 1 patient). Grade 3 hematological toxicity was also rare: anemia in 4 patients (9%), neutropenia in 8 patients (18%), thrombocytopenia in 5 patients (11%); only 1 patient (2%) experienced febrile neutropenia. The main non-hematological toxicity were: asymptomatic serum transaminase elevation and hyperbilirubinemia (grade 3 in 9% of patients); grade 3 diarrhea in 1 patient (2%); grade 2 and 3, erlotinib-related skin rash in 11% and 4% of patients, respectively. Median time to rash was 7 d. Gem and erlotinib doses were reduced in 14 and 3 patients, respectively. No toxic deaths were recorded.

Clinical predictors of response and survival

A positive CB and skin rash (any grade) were significant, independent predictors of DCR at multivariate analysis, in both the overall and pure metastatic populations. Female gender ($P = 0.03$) and skin rash (any grade, $P < 0.0001$) were significant, independent predictors of longer PFS (Table 4). ECOG PS (0-1, $P = 0.01$), skin rash (any grade, $P = 0.03$), and carbohydrate antigen (CA 19-9) decrease (> 25% relative to baseline, $P = 0.04$) were significantly associated with longer OS at multivariate analysis (Table 4). Conversely, the occurrence of other Gem- or erlotinib-related toxicities, such as hematological toxicity and diarrhea, did not significantly impact on survival outcomes. The impact of these factors on PFS and OS was further confirmed by Kaplan-Meier analysis (Figures 2 and 3): in particular, median PFS and median OS were both significantly longer in patients experiencing any grade of skin rash (21 wk *vs* 10 wk, Log-rank $P = 0.001$, and 42 wk *vs* 15 wk, Log-rank $P = 0.03$, respectively) (Figures 2A and 3A). In the "pure metastatic" population, gender and skin rash ($P = 0.007$ and $P = 0.002$), and gender, PS, CB and skin rash ($P = 0.01$, $P = 0.06$, $P = 0.02$ and $P = 0.02$) were significant, independent predictors of PFS and OS, respectively, at multivariate analysis (data not shown).

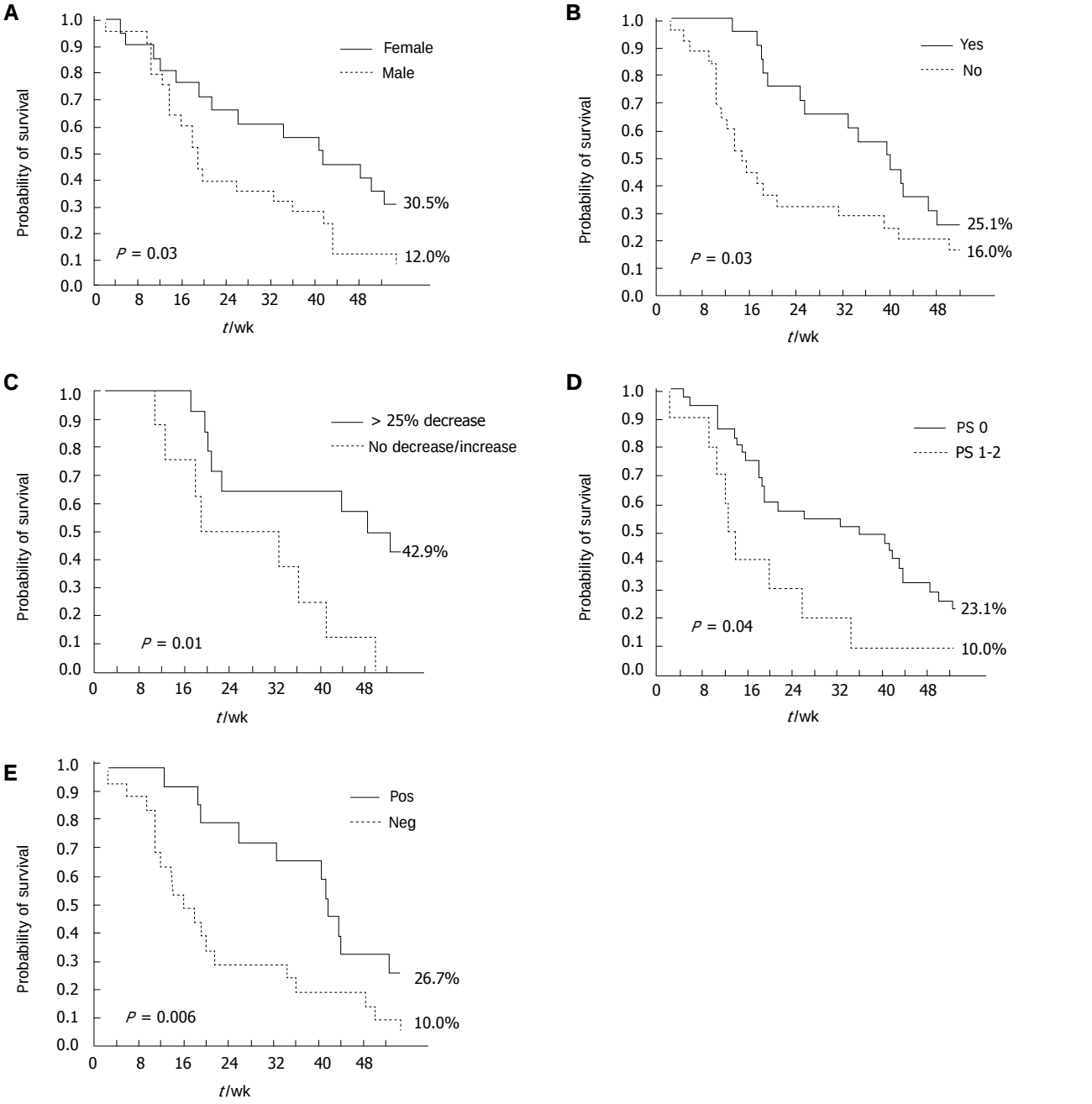

Figure 3 Kaplan-Meier analysis of independent overall survival predictors. A: Overall survival (OS) by sex; B: OS by skin rash; C: OS by carbohydrate antigen 19-9 decrease; D: OS by Eastern Cooperative Oncology Group performance status (PS); E: OS by clinical benefit. Pos: Positive; Neg: Negative.

DISCUSSION

In this study, performed in an unselected patient population, the administration of FDR-Gem in combination with erlotinib proved to be feasible, well tolerated, and moderately active. However, the planned goal to obtain a PFS-6 > 40% was not reached (PFS-6: 31.8%). Thus, the addition of erlotinib to an FDR-Gem backbone in unselected patients is unlikely to improve on historical result; indeed 1-year OS (21.6%), median OS (26 wk, 95%CI: 9-43), and activity in terms of responses, with a DCR of 59% are within the ranges reported with single-agent Gem, administered either as a 30-min *iv* infusion or as FDR, or with the combination of Gem and erlotinib[2,6-8,10].

The safety profile of the tested FDR-Gem/erlotinib combination is extremely manageable, an important issue in advanced PDAC patients, who are often frail and at a high risk of an adverse impact of treatment on quality of life. In particular, we confirm here that administering FDR-Gem at 1000 mg/m^2, as in previous experiences from our group[10,11], decreases hematological toxicity in comparison with the original FDR-Gem schedule developed by Tempero *et al*[7], where FDR-Gem was administered at the 1500 mg/m^2 dose level (grade 3-4 neutropenia 23% in the present trial *vs* 48.8% in Tempero's trial).

Other experiences with a different EGFR-TKI (gefitinib) have also confirmed an extremely safe and manageable toxicity profile of Gem-FDR at a lower dose (1200 mg/m^2), thus suggesting these combinations as feasible platforms for associations with additional chemotherapeutics or different targeted agents[16].

Though the combination under study proved feasible and well tolerated, the question remains as to whether such a strategy (*i.e.*, adding an EGFR kinase inhibitor to a FDR-Gem backbone in unselected patients) is worthy pursuing if it does not improve efficacy. As the results of the trial are technically negative (primary endpoint was not met), the easiest answer would be that this combination does not merit further investigation, particularly in a scenario, such as that of advanced PDAC treatment, where novel polychemotherapy strategies (FOLFIRINOX and Gem/nab-paclitaxel combinations) are moving the field forward and, for the first time in almost 20 years, show increased efficacy and improved survival as compared with single-agent Gem. However, survival analysis of the present trial and of two other recently reported experiences[17,18] clearly show that, at least in some patients, the addition of erlotinib to Gem has both biological and clinical activity: indeed, the most relevant finding reported herein is the strong predictive value of the appearance of skin rash. Patients developing erlotinib-related skin toxicity experience a more than doubled median OS (42 wk *vs* 15 wk, $P = 0.03$), and PFS (21 wk *vs* 10 wk, $P = 0.001$); conversely, the occurrence of other toxicities, such as hematologic toxicity or diarrhea, has no impact on treatment activity and/or survival outcomes. A similar predictive effect had already been described in the registration trial of erlotinib in PDAC, where patients experiencing grade 2 skin rash had a 1-year survival of 43%[6] and is shared by other agents targeting the EGFR pathway, either small molecules or monoclonal antibodies, regardless of the disease setting[19-25]. The trial exploring the addition of bevacizumab to Gem and erlotinib, also showed a significantly better outcome for patients developing skin rash, regardless of the treatment arm[26]. A more recent randomized trial showed that skin rash is able to dichotomize patients receiving erlotinib between good and poor prognosis[27].

In addition to skin rash, survival analysis of the current study also underlines the importance of two other treatment-modified factors to guide the management of advanced PDAC patients: clinical benefit and decline in CA 19-9 levels. Though chosen as the primary end-point in the Gemcitabine registration trial by Burris *et al*[2], the relationship between CB and OS has never been validated. Interestingly, in the present trial the occurrence of CB was a significant independent predictor of longer OS, while objective response, as assessed by RECIST criteria, was not, a finding of great clinical relevance in the context of a disease with dismal prognosis, where symptom control represents a real issue for clinical practice. A reduction in CA 19-9 levels > 25% from baseline was also an independent prognostic factor for survival, thus adding to the numerous evidence supporting the prognostic role (and clinical utility) of a CA 19-9 reduction, regardless of the chosen cut-off point[28-31].

In conclusion, although the study reported herein failed to meet its primary endpoint of prolonging PFS with the addition of erlotinib to FDR-Gem, intriguing data on skin rash do suggest that a subset of advanced PDAC patients could actually achieve disease control and survival comparable to those obtained with more intensive (and more toxic) polychemotherapy approaches, such as FOLFIRINOX and Gem/nab-paclitaxel combinations, with a well tolerated and easily manageable regimen, potentially also suitable for elderly and unfit patients. However, in order for this strategy to become a concrete treatment option, an in-depth investigation of the biological mechanisms underlying the occurrence of skin rash upon EGFR blockade is required to identify clinical/molecular biomarkers predicting toxicity and efficacy and to prospectively select patients who could potentially benefit from Gem/erlotinib combinations.

COMMENTS

Background

Pancreatic adenocarcinoma has a dismal prognosis. Although the disappointing survival advantage obtained in many studies, chemotherapy is the only treatment option for the majority of patients, and single agent gemcitabine (Gem) remains standard care for many of them. Recently, the polychemotherapy regimen named FOLFIRINOX has produced an improvement in survival over single agent Gem but require an accurate selection of young and fit patients to limit treatment-related toxicities. In order to improve Gem efficacy, pharmacokinetic Gem modulation and combination with other chemotherapeutic agent has been proposed. To this regard, the prolonged infusion at constant dose rate has shown promising results in phase Ⅱ and Ⅲ trials and the addition of erlotinib to Gem has provided a minimal, albeit statistically significant, improvement in survival.

Research frontiers

In the field of advanced pancreatic adenocarcinoma (PDAC), the research hotspot is to find active regimen, for patients not suitable for aggressive combination chemotherapy, able to improve survival over single agent Gem, without worsening tolerability. In the context of targeted therapies, applied to PDAC, but also any other malignancy, the opportunity of prospectively select patients who could benefit from targeted therapy plays a fundamental role.

Innovations and breakthroughs

The combination of fixed dose-rate (FDR)-Gem at 1000 mg/m^2 and erlotinib appears feasible, well tolerated and extremely manageable. In comparison to other FDR-Gem schedules with different doses (1500 or 1200 mg/m^2), the regimen shows a reduced hematological toxicity profile. This suggests that the schedule is a feasible platform for combining targeted therapies. In the study, a strong predictive value of the appearance of skin rash is demonstrated. Patients developing erlotinib-related skin toxicity experienced a more than doubled median overall survival, comparable to that obtained with more intensive polychemotherapy approaches. This relation has not been reported for other toxicities (hematologic toxicities or diarrhea). Moreover, occurrence of clinical benefit and reduction in carbohydrate antigen levels > 25% from baseline also proved to be an independent prognostic factor for survival. All these data confirm these factors as an important guide for the management of advanced PDAC patients.

Applications

The study results suggest the importance of investigating the biological mechanisms underlying the occurrence of skin rash upon epidermal growth factor receptor blockade. The identification of clinical/molecular biomarkers is strongly required to predict toxicity and efficacy and to prospectively select patients who

could potentially benefit from Gem/erlotinib combinations.

Peer review

This study investigated activity, toxicity, and prognostic factors for survival of erlotinib and FDR-Gem in advanced pancreatic cancer. They highlighted the correlation between the rash and efficacy. The similar studies were published in the past and they had the similar results, furthermore there were randomized controlled trials among them. This study is the confirmation of result of those studies, but it has reference to clinical practice.

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BRIEF ARTICLE

Negative capsule endoscopy in patients with obscure gastrointestinal bleeding reliable: Recurrence of bleeding on long-term follow-up

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Abstract

AIM: To assess the rate of recurrent bleeding of the small bowel in patients with obscure bleeding already undergone capsule endoscopy (CE) with negative results.

METHODS: We reviewed the medical records related to 696 consecutive CE performed from December 2002 to January 2011, focusing our attention on patients with recurrence of obscure bleeding and negative CE. Evaluating the patient follow-up, we analyzed the recurrence rate of obscure bleeding in patient with a negative CE. Actuarial rates of rebleeding during follow-up were calculated, and factors associated with rebleeding were assessed through an univariate and multivariate analysis. A *P* value of less than 0.05 was regarded as statistically significant. The sensitivity, specificity, and positive and negative predictive values (PPV and NPV) of negative CE were calculated.

RESULTS: Two hundred and seven out of 696 (29.7%) CE studies resulted negative in patient with obscure/overt gastrointestinal bleeding. Overall, 489 CE (70.2%) were positive studies. The median follow-up was 24 mo (range 12-36 mo). During follow-up, recurrence of obscure bleeding was observed only in 34 out of 207 negative CE patients (16.4%); 26 out of 34 with obscure overt bleeding and 8 out of 34 with obscure occult bleeding. The younger age (< 65 years) and the onset of bleeding such as melena are independent risk factors of rebleeding after a negative CE (OR = 2.6703, 95%CI: 1.1651-6.1202, *P* = 0.0203; OR 4.7718, 95%CI: 1.9739-11.5350, *P* = 0.0005). The rebleeding rate (CE+ *vs* CE-) was 16.4% *vs* 45.1% (χ^2 test, *P* = 0.00001). The sensitivity, specificity, and PPV and NPV were 93.8%, 100%, 100%, 80.1%, respectively.

CONCLUSION: Patients with obscure gastrointestinal bleeding and negative CE had a significantly lower rebleeding rate, and further invasive investigations can be deferred.

Key words: Capsule endoscopy; Enteroscopy; Anemia; Obscure gastrointestinal bleeding; Rebleeding

Core tip: Although capsule endoscopy (CE) is widely used as a first-line diagnostic modality for obscure gastrointestinal bleeding after the execution of a work-out negative for gastrointestinal bleeding properly done by following the guidelines proposed by American Gas

Association, the rebleeding rate after negative CE varies according to different studies. We tried to elucidate the outcomes after a negative CE for obscure gastrointestinal bleeding (OGIB) and to determine the risk factors associated with rebleeding. Based on the results of our study patients with OGIB and negative CE had a significantly lower rebleeding rate, and further invasive investigations can be deferred.

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INTRODUCTION

Obscure gastrointestinal bleeding (OGIB) remains a major clinical challenge. Many instances of OGIB originate from the small bowel, which is beyond the reach of an ordinary endoscope, including an esophagogastroduodenoscope (EGD) and colonoscope. The scene was recently revolutionized by the availability of the capsule endoscopy (CE), which is noninvasive and well tolerated by patients.

CE is currently indicated as part of the workup for OGIB (obscure-overt or obscure-occult), undiagnosed iron deficiency anemia, Crohn's disease, polyposis syndromes and cancer, celiac disease, for monitoring after small bowel transplant and, occasionally, for undiagnosed abdominal pain or diarrhea[1-5]. Since its development, several studies have compared the diagnostic yield of CE with other modalities commonly used to investigate the small bowel. Many studies have shown CE to be more sensitive and more effective compared with either push enteroscopy or small bowel follow-through[6-20]. The sensitivity and specificity of CE have been cited to be as high as 89% and 95%, respectively[20]. Although many papers have attempted to estimate the effectiveness of CE based on its diagnostic yield, few studies have considered the utility of a negative CE. In fact, only one of them[21-23] has considered a negative CE as a failure.

A great amount of data about a positive CE and its therapeutic and prognostic implications are available in literature. However, few data on the outcomes of patients with a negative CE are available. It is also somewhat more difficult to ascertain the value of a negative test. Previous studies have considered objective measures such as whether a negative test leads to other tests or therapeutic interventions. The impact on patients' overall outcome remains poorly defined, particularly in patients with OGIB. It remains uncertain that CE findings predict rebleeding.

A negative CE, though it does not confirm a specific diagnosis, may still be useful, because it allows the physician to quit a certain line of investigation, thereby impacting patient care.

MATERIALS AND METHODS

We reviewed the medical records of all patients referred to the Digestive Endoscopy Unit of the Catholic University in Rome to undergo a CE analysis for the investigation of OGIB between December 1st, 2002 and January 30th, 2011. All of them presented an overt or occult gastrointestinal bleeding as clinical presentation according to the guidelines of the American Gastroenterological Association (AGA)[24]. All patients had undergone both EGD and ileo-colonoscopy resulted negative before to referral for CE.

All patients, opportunely consented, underwent a CE with the PillCam capsule endoscopy system (Given Imaging, Yoqneam, Israel), according to the standard protocols endorsed by the American Society for Gastrointestinal Endoscopy[25]. All the procedures were performed in an out patient setting, after fasting for 8 h without any bowel preparation. The PillCam small bowel (Given Imaging) was then administered. The patients had a light breakfast 2 h after and a light meal 4 h after the administration of the PillCam as recommended in the standard protocol. After 8 h, they returned to the Endoscopy Unit, data recorder was removed and images were downloaded on the computer. The recordings of CE were reviewed by 2 experienced endoscopists/gastroenterologists independently (Riccioni ME, Urgesi R) at 8-10 frames per second using the Rapid® Reader (version 5.0). When possible, the stomach and the colon were also observed. The interobserver difference in interpretation about any findings was less than 5% and if and when it existed, it was resolved by reexamination.

A positive CE was defined as the presence of CE findings that may account for the clinical bleeding (angiodysplasia, ulcers or erosions, tumor, Crohn's disease, and active bleeding with no identifiable source), whereas a negative CE was defined as the absence of abnormalities on CE as reason of the bleeding.

In all cases in which CE did not reach the valve or with inadequate small bowel cleansing the examination was repeated. The analysis was considered negative when the second procedure rule out any GI abnormalities[26].

The median follow-up for all patients, strictly monitored for rebleeding, was 24 mo (range 12-36 mo). Patients' records, including blood tests, hospital admissions (especially for anemia and/or recurrent gastrointestinal bleeding), blood transfusions, need of iron supplementation, additional endoscopies (including push endoscopies), and surgery were considered from the date of the CE. Overt clinical rebleeding was defined as passing melena or fresh blood per rectum with a drop in hemoglobin of 2 g/dL or more. Occult rebleeding was defined as an unexplained hemoglobin drop of more than 2 g/dL in the absence of melena or hematochezia.

We defined patients with no recurrent obscure gastrointestinal bleeding or anemia during follow-up as "negative for rebleeding" and those with a confirmed bleeding source identified by an invasive interventions, clinical rebleeding, or recurrent unexplained anemia (using standardized and published criteria: blood haemoglobin level

of < 13.8 g/dL for men, < 11.5 g/dL for postmenopausal women, and < 11 g/dL for pre-menopausal women, with a plasma ferritin level of < 30 μg/L and a mean corpuscular volume of < 80 fL[26]) as "positive for rebleeding".

Statistical analysis

The Statistical Package for Social Science (version 13.0; SPSS Inc., Chicago, IL, United States) was used for all statistical computation. Actuarial rates of rebleeding during follow-up were calculated, and factors associated with rebleeding were assessed through an univariate and multivariate analysis. A *P* value of less than 0.05 was regarded as statistically significant.

The sensitivity, specificity, and positive and negative predictive values (PPV and NPV) were calculated[27] using as "gold standard" the patients negative for obscure bleeding.

RESULTS

CE indications included obscure overt bleeding (532), obscure occult bleeding (164) and several other indications (282). CE studies resulted negative in 207 out of 696 (29.7%) with obscure/overt gastrointestinal bleeding: 110 male (53.1%) and 97 female (46.8%) with a median age of 61.4 years (range 8-92 years). Overall, 489 patients (70.2%) were positive patients. The flowchart of the selection process of patients involved in the study and characteristics of the patients with negative CE are showed in Figure 1 and Table 1 respectively.

Follow-up

The median follow-up for all patients closely monitored for rebleeding was 24 mo (range 12-36 mo). Clinical recurrence of bleeding was observed during follow-up in 34 (16.4%) out of 207 patients with CE "negative for bleeding". In details, 2 out of 34 patients had a new episode of melena, otherwise 32 patients had a recurrence of obscure occult bleeding with anemia and positive fecal occult blood test.

A Meckel diverticulum and a gastrointestinal stromal tumor were respectively diagnosed in the two patients presenting a new episode of obscure overt bleeding. In the group of 32 patients affected by a recurrence of obscure occult bleeding, the cause was undiagnosed in 13 cases (40.6%), whereas an extra-intestinal disease was diagnosed in 11/32 (34.3%) patients, a parasitic infestation in 3/32 (9.37%) and the chronic use of NSAIDs was indicated as the probable cause of the recurrence of bleeding in 5/32 (15.62%) patients. Moreover, we note that 2 out of 34 (5.8%) patients with negative CE and episodes of early rebleeding were receiving chronic therapy with oral anticoagulation, at 10 mo of follow-up.

Data analysis

The results of univariate and multivariate analysis about factors associated with rebleeding in patients with negative CE and reccurence re-bleeding are summarized in Table 2. Patients with OGIB and negative CE have a low percentage of probability of rebleeding (34/207). The statistical analysis of our data shows that the age of onset of the first episode of bleeding < 65 years and the type of bleeding (melena) are the only factors that statistically significantly influence the risk of re-bleeding (OR = 2.6703, 95%CI: 1.1651-6.1202, *P* = 0.0203; OR = 4.7718, 95%CI: 1.9739-11.5350, *P* = 0.0005) (Table 3). Other parameters considerated during our survey such as gender, number of blood transfusions, hemoglobin level, intake of anticoagulants, number of hospitalizations didn't show a statistically significant correlation with rebleeding episodes. The final diagnosis and treatment of these patients are summarized in Table 3.

The patients "negative for rebleeding" with CE negative didn't need a hospital admission at the time of rebleeding and no blood transfusions were required.

Regarding CE "positive" patients, clinical rebleeding was observed in 221 out of 489 (45.1%) patients. Rebleeding rate was 16.4% *vs* 45.1% (χ^2 test, *P* = 0.00001). The sensitivity, specificity, and PPV and NPV were 93.8%, 100%, 100%, 80.1%, respectively. Most rebleedings occurred within the first 12 mo after the CE examinations (18/34; 52.9%).

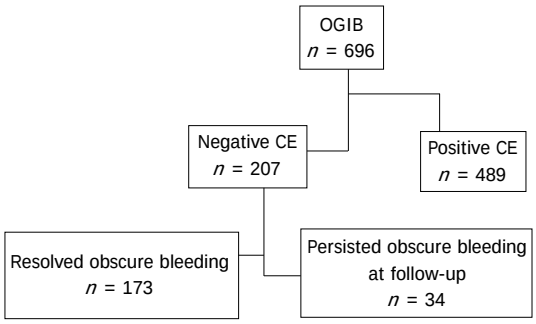

Figure 1 Flow chart of the selection process of patients involved in the study. CE: Capsule endoscopy; OGIB: Obscure gastrointestinal bleeding.

Table 1 Characteristics of patients with negative capsule endoscopy

Variables	Total patients	Negative for rebleeding	Rebleeding patients
n	207	173	34
Age (yr)	56.8	56.8	57.1
Sex (M/F)	110/97	70/59	22/12
Obscure-overt bleeding	157	121	2
Obscure-occult bleeding	50	52	32
Median Hb level	8.2	8.8	8.2
OAT/LMWH	36	32	4
Rebleeding time			
At 10 mo of follow-up			28
At 24 mo of follow-up			34

OAT: Oral anticoagulant therapy; LMWH: Low molecular weight heparin. M/F: Male/female.

DISCUSSION

Despite the wide diagnostic yield of CE compared to

Table 2 Results univariate analysis *n* (%)

Variables	No rebleeding	Rebleeding	*P* value	Odds ratio	95%CI	*P* value
Patients	173	34	-			
Sex (M/F)	88/85	22/12	0.1403	1.8447[1]	0.8175-4.1625[1]	0.1403[1]
Age < 65 yr	23 (67.6)	89 (51.4)	0.0838	2.6703	1.1651-6.1202	0.0203
CE indications (melena)	77 (44.5)	26 (76.5)	0.0006	4.7718	1.9739-11.5358	0.0005
Blood transfusion	64 (36.9)	14 (41.2)	0.6463			
Hb < 8 g/dL	48 (27.7)	14 (41.2)	0.1189	2.0064	0.8891-4.5274	0.0935
Use of FANS	30 (17.3)	5 (14.7)	0.7085			
Oral anticoagulant therapy	13 (7.5)	4 (11.9)	0.4104			
Hospitalizations (*n*)	62 (35.8)	14 (41.2)	0.5559			

[1]Variables with $P < 0.05$ are significant. Variables with $P < 0.125$ entered in the multivariate analysis (logistic regression): (χ^2 test). Likelihood ratio: 22.1037; $P = 0.0002$. The younger age (< 65 yr) and the onset of bleeding such as melena are an independent risk factor of rebleeding after negative capsule endoscopy (CE). M/F: Male/female.

Table 3 The final diagnosis and treatment of the 34 negative capsule endoscopy patients with rebleeding

Type of rebleeding	*n*	Final diagnosis	Treatment
Obscure overt bleeding	2		Surgery
		Extraluminal GIST	
		Meckel's diverticulum	
Obscure occult bleeding	32		
	13	Causes not found	SR
	5	Myelodisplastyc syndrome	MT
	3	Uterine fibroma	Surgery
	1	Metastatic breast cancer	Surgery + MT
	3	Giardia lamblia infection	MT
	5	Chronic use of NSAID	
	2	Erosive gastritis	MT

GIST: Gastrointestinal stromal tumor; SR: Spontaneous resolution; MT: Medical therapy.

conventional diagnostic techniques, the impact of this relatively new investigation on patient outcome remains poorly defined. In particular, few CE studies used long-term rebleeding as the primary outcome. In this study, we determined the long-term clinical outcome and characteristics of patients with OGIB after negative CE. As reported previously[22,23,28,29], CE could not identify all bleeding lesions in patients with OGIB. Up to 36.7% of patients in this study had a negative CE despite overt clinical bleeding at presentation. Notably, this group of patients with negative CE had a low (19.8%) rebleeding rate in a more than 1 year follow-up. The rebleeding rate was significantly lower in patients with negative CE than in cases with positive CE. Moreover, considering the group of patients with negative CE, chronic therapy with oral anticoagulation and NSAIDs seems to be related to a higher risk of rebleeding. In accordance with our findings, Neu *et al*[30] found that rebleeding occured in 20% of patients with negative CE after a median follow-up of 13 mo. In a more recent study Lorenceau-Savale *et al*[31] showed as in 35 patients with a history of OGIB and negative CE and a minimum follow-up duration of one year (median: 15.9 mo) eight patients presented a recurrence of bleeding, with an overall rebleeding rate of 23%. Four women with recurrence before new investigations. In the four remaining patients, repeat endoscopy work-ups after negative CE were performed and revealed previously missed lesions with bleeding potential, mainly in the stomach. Overall, 13 patients, with or without rebleeding, had repeated endoscopy work-ups after a negative CE, leading to a definitive diagnosis in nine patients, with lesions located in the stomach and colon in eight of them.

Since the patients with OGIB and a negative CE had a low rate of rebleeding, further interventions or investigations could be deferred until clinical rebleeding occurs. In these cases, after ruling out a gastrointestinal lesions as causes of the recurrence of bleeding after the execution of a work-out negative for gastrointestinal bleeding properly done by following the guidelines proposed by AGA, the search for causes of obscure bleeding outside of the digestive system should be "necessarily" done.

Otherwise, patients with positive CE had a significantly higher rebleeding rate on long-term follow-up. Recently, Kim *et al*[32] performed CE in 125 patients with OGIB. The complete visualization of the small bowel was achieved in 93 patients (74.4%). Of the 63 patients (50.4%) with negative CE results, 60 patients did not receive any further specific treatment for OGIB. Rebleeding episodes were observed in 16 out of 60 patients (26.7%). Substantial rebleeding events were observed with similar frequency both after negative CE without subsequent treatment (26.7%) and after positive CE without specific treatment (21.2%) ($P = 0.496$). The Authors conclude that in some cases despite a negative CE, approach such as double balloon endoscopy (DBE) should be considered as complementary procedures for further evaluation.

Our study confirms, also in accordance with Lai *et al*[33] that patients with positive CE have a high rebleeding rate, a longer hospital stay and require more units of blood transfused than those with negative CE; in accordance with Kim *et al*[32], the device-assisted enteroscopy (DBE or single-balloon enteroscopy) could be helpful in patients with a high index of suspicion for small bowel pathologies and with a high risk of rebleeding and negative CE.

The limits of the present study are discussed below. First, the lack of a gold standard for small bowel diagnosis limits the accuracy in the determination of CE performance. It is highly possible that some lesions may be

missed despite an extensive investigation. However, unlike many published studies, we used long-term clinical rebleeding instead of small bowel lesions as the primary end point for the determination of CE performance. It may be interesting to determine, in future studies, whether the use of the recently available methods of device-assisted enteroscopy[34,35] and their future technical developments could overcome this problem. Secondly, all our "negative for rebleeding" patients refused to undergo further endoscopic examinations for various reasons. Third, we only recruited patients with "genuine" OGIB, meaning that these patients had undergone multiple upper and lower gastrointestinal endoscopies by experienced endoscopists to rule out other possible sources of bleeding. Consequently, these results could not necessarily be generalized to all patients with suspected small bowel bleeding.

In conclusion, even if additional studies are warranted to confirm these results, we found that in a follow-up of a mean 24 mo, patients with OGIB and negative CE had a significative low long-term rebleeding rate, suggesting that further invasive investigations could be deferred and may not be necessary in this group of patients. Only an accurate and careful clinical observation can help us to identify false negative patients at CE.

COMMENTS

Background

Obscure gastrointestinal bleeding (OGIB) remains a major clinical challenge to gastroenterologists. Many instances of OGIB originate from the small bowel, which is beyond the reach of an ordinary endoscope, including an esophagogastroduodenoscope and colonoscope.

Research frontiers

The scene was recently revolutionized by the availability of capsule endoscopy (CE), which is noninvasive and well tolerated by patients. In this study the authors assess the rate of recurrent bleeding of the small bowel in patients with obscure bleeding already undergone CE with negative results.

Innovations and breakthroughs

Recent reports have highlighted the importance of CE in the clinical assessment of patients with presumed small bowel diseases. For OGIB, CE is recommended as an investigation modality for the detection of a bleeding source after traditional endoscopy. However, even after full evaluation of the small bowel, CE is not able to highlight the bleeding focus. In the present study, they sought to reveal the outcomes after negative CE for OGIB and the risk factors associobscure bleeding already undergone CE with negative results.

Applications

By understanding the value of negative CE and the value of long-term of follow-up in these patients. Patients with OGIB and negative CE had a significantly lower rebleeding rate, and further invasive investigations can be deferred. However, when a confirmatory diagnosis is made by CE study, specific treatments can be applied according to the diagnosis.

Terminology

CE is the most innovative and less invasive resource for the study of the small bowel playing an essential role in the diagnosis of small bowel diseases until now disregarded and the setting of therapeutic decisions.

Peer review

The authors retrospectively present information on 696 patients who underwent CE for OGIB with negative standard tests. They excluded for detailed analysis 489 patients an instead concentrated on outcome in 207 patients in whom the CE proved negative. They found a statistically lower rebleed rate over a median of 24 mo in these CE negative patients compared to the CE positive group. The CE-patients had various other explanations found later in 60%. No explanation was found in the rest. In multivariate analysis age < 65 years and melena on presentation were found to be predictors of rebleed in CE-patients.

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BRIEF ARTICLE

Gallbladder polyps: Factors affecting surgical decision

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Abstract

AIM: To determine the factors affecting the decision to perform surgery, and the efficiency of ultrasonography (USG) in detecting gallbladder polyps (GP).

METHODS: Data for 138 patients who underwent cholecystectomy between 1996 and 2012 in our clinic with a diagnosis of GP were retrospectively analyzed. Demographic data, clinical presentation, principal symptoms, ultrasonographic and histopathological findings were evaluated. Patients were evaluated in individual groups according to the age of the patients (older or younger than 50 years old) and polyp size (bigger or smaller than 10 mm) and characteristics of the polyps (pseudopolyp or real polyps). χ^2 tests were used for the statistical evaluation of the data.

RESULTS: The median age was 50 (26-85) years and 91 of patients were female. Of 138 patients who underwent cholecystectomy with GP diagnosis, only 99 had a histopathologically defined polyp; 77 of them had pseudopolyps and 22 had true polyps. Twenty-one patients had adenocarcinoma. Of these 21 patients, 11 were male, their median age was 61 (40-85) years and all malignant polyps had diameters > 10 mm (P < 0.0001). Of 138 patients in whom surgery were performed, 112 had ultrasonographic polyps with diameters < 10 mm. Of the other 26 patients who also had polyps with diameters > 10 mm, 22 had true polyps. The sensitivity of USG was 84.6% for polyps with diameters > 10 mm (P < 0.0001); however it was only 66% in polyps with diameters < 10 mm.

CONCLUSION: The risk of malignancy was high in the patients over 50 years old who had single polyps with diameters > 10 mm.

Key words: Gallbladder; Polyps; Ultrasound; Cholecystectomy; Malignancy

Core tip: Early stage gallbladder cancers can often be detected as polyps in imaging studies. The aim of this study was to determine the factors affecting surgery by analyzing the incidence of malignancy of gallbladder polyps (GP) and the efficiency of ultrasonography in detecting GP. Of 138 patients with GP on imaging, 99 had polyps and 21 had histopathologically confirmed adenocarcinoma. Of these 21 patients, all malignant polyps were solitary and had a diameter > 10 mm. In our study, the risk of malignancy correlated with age over 50 years old, solitary polyp and polyp diameter > 10 mm.

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INTRODUCTION

Gallbladder polyps (GP) present as masses protruding from the gallbladder mucosa. They are found in 0.3% to 12% of healthy individuals[1]. The actual prevalence is

unknown; however, at present, GPs are diagnosed more frequently because of the widespread use of abdominal imaging techniques. GPs are usually asymptomatic and are diagnosed incidentally during radiological examinations done for other reasons. GPs are classified as pseudopolyps or true polyps. Pseudopolyps consist of cholesterol polyps/cholesterolosis, adenomatous polyps, adenomyoma, inflammatory polyps and hyperplastic polyps; these are all benign lesions. True polyps are grouped into benign (adenoma), premalignant (dysplastic polyps) and malignant (adenocarcinoma)[2]. Cholesterol polyps are the most frequently observed GPs. Therefore, most GPs are benign lesions. Occasionally, early stage gallbladder cancers can be detected as a polyp in imaging studies.The prevalence of malignant polyps of GPs can reach 27%[3]. In patients older than 50 years old, the presence of polyps larger than 10 mm has been reported as a risk factor for malignancy[3-8]. The most commonly used imaging modality for diagnosis is ultrasonography (USG). However, USG is poor at differentiating benign and malignant polyps. Additional diagnostic tools comprise computed tomography and endoscopic USG.

In this study, patients in our clinic diagnosed with-GPs who had surgery were examined; and indication for surgery, frequency of polyp types, malignancy rates of polyps and reliability of USG in identification and differentiation of polyps were investigated.

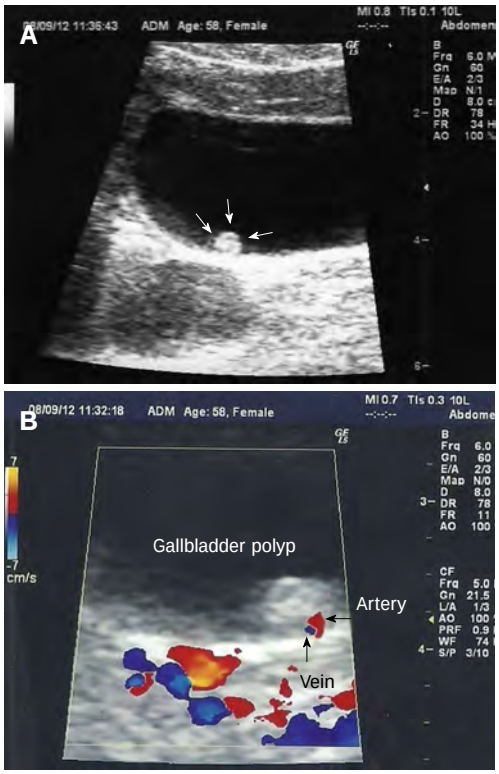

Figure 1 The ultrasonographic image of a 6-mm gallbladder polyp (A) and the same polyp with a feeding artery in Doppler ultrasonography (B).

MATERIALS AND METHODS

Patients

Age, sex, clinical signs and symptoms, preoperative ultrasound and histopathological diagnoses of patients were analyzed retrospectively in patients admitted to our clinic with GP and underwent cholecystectomy from 1996-2012. All the patients were evaluated with USG before surgery in the Radiology Department of Uludag University Faculty of Medicine. Hyperechoic lesions that had no acoustic shadowing and didnot move with position change represented a confirmed diagnosis of GP[8]. Detection of polyps > 10 mm, suspicious findings in USG (such as a vascularization pattern, Figure 1), growth during follow-up, and personal request of the patient were indications for surgery.

Polyp size, number and presence of stones were evaluated in preoperative USG reports, and compatibility of these findings with histopathological data was analyzed.

According to histopathological diagnoses, cholesterol polyps/cholesterolosis, hyperplastic and adenomatous polyps were assembled under the title of "pseudopolyp"; adenoma and adenocarcinoma were assembled under the title of "real polyps". In addition, patients were evaluated in individual groups according to the age of the patients (older or younger than 50 years old) and polyp size (bigger or smaller than 10 mm).

Statistical analysis

The χ^2 test was used, when appropriate, to calculate the statistical significance of the different demographic and clinical variables. *P* values of < 0.05 were deemed significant.

RESULTS

Demographic and clinical characteristics of patients with PLG

Cholecystectomy was performed in 5832 patients between 1996-2012 and surgical indication of 138 patients (2.3%) was GPs. Ninety-one of the patients were female and 47 of them were male, with a median age of 55 (26-85) years. Polyps were detected in 99 of the 138 patients (71.7%) undergoing surgery for GPs; gallbladder stones were detected in the remaining 39. Thus, the false positive rate was 28% in ultrasound evaluation of polyps. Remarkably, the polyps in all of these cases were < 10 mm.

Sixty-six patients (66.6%) did not have any symptoms at the time of presentation; however, 33 patients with polyps were symptomatic. Sixty-two of 66 asymptomatic patients elected to have surgical treatment because of possible future risks. Three of four asymptomatic patients had a cholecystectomy because their polyp increased to > 10 mm in 6 mo; the remaining patient had a cholecystectomy because of their age and sex (65 years old male). On pathological examination, the polyps of these four patients were detected as cholesterol polyps and adenomatous polyps. The 33 symptomatic patients presented with complaints of right upper quadrant pain and dyspepsia, and had surgery upon detection of polyps in USG (Table 1). Gallstones were accompanied with

Table 1 Characteristics of 99 patients diagnosed with gallbladder polyps by histopathological examination

Characteristics		Pseudopolyp	True polyp (n = 22)		P value[1]
Pathology result		(n = 77)	Adenocarcinoma	Adenoma	
Sex	Woman	51	10	1	0.33
	Man	26	11	0	
Age (yr)	< 50	52	1	1	< 0.0001
	⩾ 50	25	20	0	
Symptoms	Yes	24	9	0	0.62
	No	53	12	1	
Number	Multiple	23	0	0	0.01
	Single	54	21	1	
Size (mm)	⩽ 10	73	0	1	< 0.0001
	> 10	4	21	0	

[1]In terms of true polyp incidence between data.

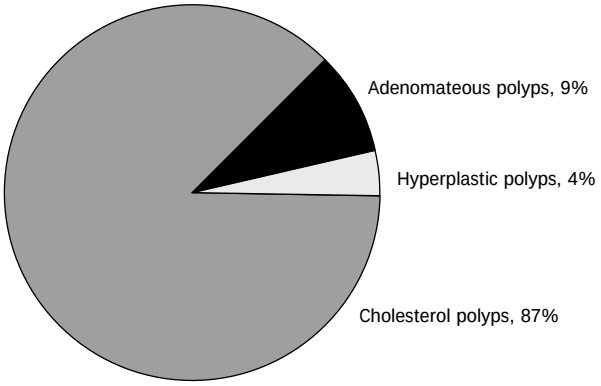

Figure 2 Distribution of pseudopolyp cases.

polyps in 18 (54.5%) of these symptomatic patients. Only in two of the malignant cases was a polypoid structure accompanied by gallstones.

As shown in Table 1, 54.5% of patients were under the age of 50 and 90% of true polyps were seen in patients over 50 years old. In addition, the incidence of polyps was 3.7% under 50 years of age, rising to 44% in patients over 50 years of age ($P < 0.0001$).

Sonograhic characteristics of the patients

While gallstones were detected only in 1 of 26 patients who had lesions of 10 mm diameter in preoperative USG, postoperative diagnoses was true gallbladder polyp in 21 (84.6%) of the remaining 25 patients. The other four patients were reported to have pseudopolyps. Preoperative USG diagnosed 96% of lesions over 10 mm accurately and 84% of them were found to be true polyps (adenoma/adenocarcinoma). Histopathological diagnoses reported polyps only in 74 of 112 patients who had lesions < 10 mm. Thus, the accuracy of USG for polyps < 10 mm decreased to 66% and only one of these 74 cases was a true polyp (adenoma). There was a statistically significant difference in the diagnosis of true polyps between polyps < 10 mm or > 10 mm ($P < 0.0001$).

Histopathological examinations of GP

The mean polyp diameter of the polyps from 99 patients (histopathologically defined as 77 pseudopolyps and 22 true polyps) was 8.8 mm (range 3-19 mm). The most commonly seen GP was a cholesterol polyp (Figure 2). Twenty-one of 22 patients with true polyps were diagnosed with adenocarcinoma, and the other one was adenoma. All the malignant polyps were > 10 mm and solitary. Eleven patients with malignancy were male and the median age was 61 (40-85) years. In our series, the incidence of malignant GP was 21.2% (21 of 99 cases). The incidence dropped to 15.2% when all 138 patients with preoperative diagnoses of polyps were taken into consideration.

Results of malignant patients

Cholecystectomy only was performed in 16 of 21 patients with malignancy, and cholecystectomy with liver S-5 resection and lymph node dissection was performed in the remaining five patients. No additional treatment other than cholecystectomy was performed in 10 patients with T1 tumors limited to the mucosa and submucosa. The other 7 patients did not accept additional treatment. Eight patients received chemotherapy treatment after surgery. Ten of these patients were still alive and 11 of them died. Survival was 14.8 mo (range 4-38 mo).

DISCUSSION

GPs are common gallbladder lesions and should not be ignored because of their association with malignancy. In the literature, the incidence of GP has been reported as between 0.3% and 12.0%[9,10]. In our clinical series of 5832 patients undergoing cholecystectomy, GPs were an indication for cholecystectomy in 2.3% (138 patients) of cases. Thirty-nine of these patients were diagnosed with only cholelithiasis; therefore, the true incidence of GPs was 1.7%.

There are different concepts about the effect of demographic factors such as age and gender on the incidence of GPs. Some studies reported that GPs are more frequently seen in males[2,11-14] or females[9-15], and some studies even suggest that there is no effect of gender on GPs[14-21]. Approximately 2/3 of cases in our study were-women and the true polyp ratio was 29.7% in men and 17.7% in women (Table 1). Ito *et al*[17] reported that the mean age was 59 years in their 417 patients series. Although 53% of patients in our study were under the age of 50, 90% of true polyps were detected in patients over 50 years old. As demonstrated in Table 1, the incidence of true polyps was 3.8% under the age of 50 years and 44% over the age of 50 years ($P < 0.05$).

Ultrasonography is the most frequently used and most valuable diagnostic tool for preoperative evaluation of gallbladder pathologies[8]. One hundred thirty eight patients in our series were diagnosed with GP using USG. Considering that 39% of them were also diagnosed with cholelithiasis, the accurate diagnosis rate of USG was 71.7%. The sensitivity of USG for GPs has beenreported to be between 32% and 90%[5,18]. While USG can

usually detect polyps > 5 mm, it becomes more accurate if the polyp is > 10 mm[19]. Indeed, USG detected almost all polyps > 10 mm accurately (25 of 26 cases) and these polyps were true polyps (adenoma/adenocarcinoma). However, the accuracy of USG diagnosis lesions < 10 mm was 66%. In addition, GPs were detected as < 10 mm in 39 patients who were thought to have GPs preoperatively but in whom no polyps were detected postoperatively. Postoperatively, the pathological diagnoses of these 39 patients were chronic cholecystitis and cholelithiasis. Cholesterolosis occurs as a result of accumulation of esterified cholesterol and triglycerides in macrophages of the lamina propria, and they are often mistaken as small polyps in USG[17]. Gallbladder stones attached to the wall of the gallbladder can easily be interpreted as a polyp in USG[18]. The presence of stones in the gallbladder reduces the success rate of USG in the diagnosis of GPs; USG diagnosis of GPs is to 99% accurate in the absence of any stones. On the other hand, in our patients, GPs did not usually cause any symptoms. Association of stones with GPs may cause symptoms and prompts the patient to consult a doctor, making the diagnosis easier. In our study, gallstones accompanied GPs in only 18.1% (18 patients) of patients. All patients with stones were symptomatic. However, there were no stones in 15 of the 33 symptomatic patients and GPs caused the symptoms in these patients. In our series of patients, being symptomatic did not have any impact on detection of true polyps ($P = 0.71$).

Another important factor associated with malignancy in GPs is the diameter of the polyps[6,20]. Kozuka *et al*[21] reported that the critical limit for differentiation of benign and malignant GPs was 12 mm and suggested cholecystectomy for GPs larger than 12 mm. Kubota *et al*[22] compared postoperative pathological data of 72 patients with GPs and preoperative ultrasound. They reported 22% of neoplastic polyps of the gallbladder as > 10 mm. They also reported that evaluation of the polyp shape may be beneficial, but it is not enough to distinguish cholesterol polyps from adenoma and cancer. Sugiyama *et al*[23] tried to make a distinction between benign and malignant polyps using preoperative USG and endoscopic USG. They detected adenoma or cancer in 14% of polyps with diameters of 6-10 mm in preoperative USG. Zielinski *et al*[2] emphasized that there is a significant increase in the risk of neoplasia in polypoid lesions > 6 mm; they suggest performing cholecystectomy in these patients. In our study, the majority of polyps (73 of 74 cases) < 10 mm were pseudopolyps, and the remaining polyps were adenomas. None of the malignant polypswere < 10 mm. Eight-four percent of polyps > 10 mm were true polyps (adenoma/adenocarcinoma) and all of the these true polyps were found to be adenocarcinoma. This suggests that a limit of 10 mm is very important ($P = 0.0001$). Similarly, no true polyps were detected in the setting of multiple polyps. Remarkably, 28% of single polyps were diagnosed as adenocarcinoma.

The literature suggests that patients over 50 years old, polyps > 10 mm, polyps with a broad base or long pedicle, polyps associated with cholecystitis or cholelithiasis, or irregular thickening of the gallbladder in the setting of biliary colic are indications for cholecystectomy[4,23,24]. In our study, 21 (21.2%) of 99 patients with GPs were diagnosed with malignancy, all of whom were older than 50 years with single polyps > 10 mm. In addition, the success rate of USG for diagnosing GPs > 10 mm was more evident and an important point. Patients had surgery mostly because of their extreme sensitivity and anxiety. We found that surgery was not beneficial in patients with multiple polypoid lesions or polyps < 10 mm. For this reason, the surgical team should reassure and relax the patients and avoid unnecessary cholecystectomies.

In conclusion, being male and over 50 years old with a solitary polyp > 10 mm benefited most from cholecystectomy.

COMMENTS

Background

Gallbladder polyps (GP) are frequently detected incidentally. They are usually misdiagnosed as gallstones in sonographic examinations. There is no consensus for treatment and follow-up of GP because of its particularly rare incidence of malignancy. There are some risk factors associated with high risk of malignancy. Early diagnosis and surgical treatment of GP affects survival of gallbladder carcinomas.

Research frontiers

Many studies have investigated risk factors that increase the incidence of malignancy of GP. Age, gender, polyp size, polyp number, accompanying gallstones and the inflammatory status of the gallbladder are significant risk factors.

Innovations and breakthroughs

In this study, all the malignant polyps were solitary and over 10 mm in size. Malignant polypsweredetermined in 44% of the patients aged over 50. The authors failed to show an association between gender and malignancy for GP. Ultrasonography (US) was more sensitive for polyps over 10 mm. US was more helpful in showing malignancy for cases with polyps under 10 mm.

Applications

This study will facilitate surgeons' decision making for treatment and follow-up of patients with GP.

Terminology

Histopathologically, cholesterol polyps/colestherolosis, hyperplastic and adenomateous polyps are defined as pseudopolyps, while adenomas and adenocarcinomas are defined as true polyps.

Peer review

This manuscript, which was written on a subject of considerable controversy in general surgery, has been generally well designed.

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BRIEF ARTICLE

Newly designed J-shaped tip guidewire: A preliminary feasibility study in wire-guided cannulation

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Abstract

AIM: To perform wire-guided cannulation using a newly designed J-shaped tip guidewire, and to verify feasibility and safety for use.

METHODS: The study was conducted on endoscopic retrograde cholangiopancreatography (ERCP) patients with naïve papilla undergoing diagnosis and treatment of biliary diseases between September 2011 and July 2012. We performed ERCP in a succession of 50 cases with a J-shaped tip guidewire. The first insertion attempt began with a trainee who had 5 min to complete cannulation, followed if necessary by the trainer for another 5 min. We assessed the primary success rate of selective biliary cannulation within 10 min and adverse events such as post-ERCP pancreatitis (PEP), bleeding or perforation.

RESULTS: The primary success rate was 90% (45/50) within 10 min, the initial success rate within 5 min by trainee staff was 76% (38/50). The rate of PEP was 6% (3/50), but all 3 cases were mild pancreatitis. All patients were managed successfully with conservative treatment. There was no bleeding or perforation.

CONCLUSION: A newly designed J-shaped tip guidewire has the possibility to facilitate selective biliary cannulation for ERCP and appears to be safe.

Key words: J-shaped tip guidewire; Wire-guided cannulation; Endoscopic retrograde cholangiopancreatography; Biliary tract; Cannulation technique; Perforation

Core tip: We conducted a feasibility study that performed endoscopic retrograde cholangiopancreatography (ERCP) with a newly designed J-shaped tip guidewire. This new guidewire has a strongly-flexed atraumatic tip with hydrophilic coating; therefore, it may contribute to the improvement of the passage through the intra-duodenal biliary segment and to the decrease of adverse events such as post-ERCP pancreatitis. We assessed the primary success rate of selective biliary cannulation within 10 min and rate of post-ERCP pancreatitis. The primary success rate was 90% (45/50); the rate of post-ERCP pancreatitis was 6% (3/50), but all 3 cases were mild. The J-shaped tip guidewire may facilitate selective biliary cannulation in ERCP.

Omuta S, Maetani I, Shigoka H, Gon K, Saito M, Tokuhisa J, Naruki M. Newly designed J-shaped tip guidewire: A preliminary feasibility study in wire-guided cannulation. *World J Gastroenterol* 2013; 19(28): 4531-4536 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i28/4531.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i28.4531

INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) is used to diagnose and treat biliary disease. Deep cannulation of the common bile duct is required for this procedure, and the current success rate for the relatively difficult conventional contrast-guided cannulation (CGC) technique ranges from 50%-90%[1-5]. Wire-guided cannulation (WGC) is a recently developed alternative to CGC that has been shown to increase primary biliary cannulation rate[6-11], chiefly by reducing incidence of post-ERCP pancreatitis (PEP)[12-20]. However, despite efficiency improvements, the sharp tips of guidewires are sometimes associated with perforation[21-23]. Even without perforation, complications can occur when the guidewire tip hits the fold and flexion of the intra-duodenal biliary segment. While a looped tip guidewire has been developed, its utility in avoiding perforation has not sufficiently been evaluated[24].

Here, we assessed the efficiency of ERCP using a newly designed J-shaped tip guidewire with a strongly flexed atraumatic tip and hydrophilic coating designed to improve passage through intra-duodenal biliary segments and decrease the adverse events, such as PEP, bleeding or perforation.

MATERIALS AND METHODS

Patients

Fifty patients with naïve papilla undergoing diagnosis and treatment for biliary diseases between September 2011 and July 2012 received ERCP using J-shaped tip guidewires. Patients were excluded if only their pancreatic ducts were diagnosed or treated, if they had previously undergone endoscopic sphincteroplasty, or if they had duodenal stenosis or Billroth Ⅱ or Roux-en-Y anastomosis, or refused to provide informed consent.

Patients were sedated *via* intravenous administration of midazolam (5-10 mg) and buprenorphine (0.2 mg). Scopolamine butylbromide (20 mg) or glucagon (1 mg) was injected intravenously to inhibit gastrointestinal peristalsis, and each patient received nafamostat mesilate (20 mg/d) prior to ERCP. Blood samples collected 2 h after ERCP were used to determine complete blood counts and serum amylase levels, and those collected after 18-24 h also measured hepatobiliary enzymes and C-reactive protein. We did not place a pancreatic duct stent for the prevention of pancreatitis in either procedure.

J-shaped tip guidewire

The guidewire (RWHJ-2545A, 0.025-inch; Paiolax Medical Devices, Inc., Kanagawa, Japan) tip was bent to attain a 1-mm radius, and a hydrophilic coating was applied starting 50 mm from the tip. The shaft was covered by a sheath and the jacket coated with water-repellent material (Figure 1).

Endoscopic procedure

Endoscopy was performed with JF-260V (Olympus, Tokyo, Japan) or ED-530XT8 (Fujinon, Tokyo, Japan) endoscopes, after catheters were preloaded with guidewires. In the present study, in general, a regular catheter was chosen except for the case undergoing sphincterotomy. First, catheters (CleverCut3 V, Olympus, Tokyo, Japan; Tamdem XL, Boston Scientific, Natick, MA, United States) were preloaded with guidewires, the guidewire tip was extended 5 mm from the catheter, bent into a "J" shape, and then the guidewire was pulled back into a stand-by position (Figure 2A). Endoscopists controlled the direction parallel to the axis of bile duct of the catheter by inches. Assisting endoscopists participated in the guidewire manipulation in all cases. An assisting endoscopist moved the guidewire back and forth in small motions by using a tactile feedback (in-and-out movement method). No fluoroscope was used during attempts of insertion, but once the guidewire was inserted without resistance then fluoroscopy was used only after insertion to confirm success (Figure 2B). The catheter was then inserted into the biliary system along the guidewire, and contrast medium was injected (Figure 2C). No test injection was performed before successful cannulation.

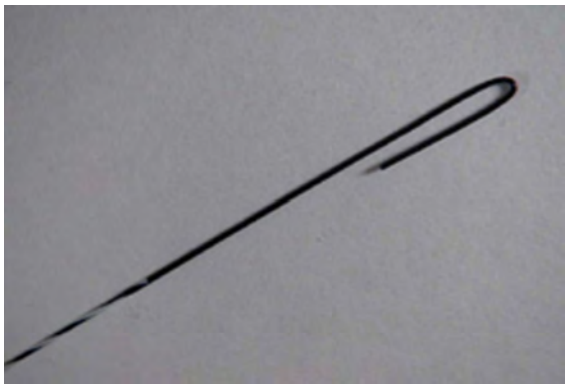

Figure 1 Newly designed J-shaped tip guidewire. The shape of the tip is a radius of 1 mm; 50 mm from the tip is the start of a hydrophilic coating.

The first insertion attempt began with a trainee who had 5 min to complete cannulation, followed if necessary by a trainer with career experience of over 500 ERCPs (Maetani I or Shigoka H or Omuta S) for another 5 min. If both attempts failed, efforts continued with a standard biliary guidewire (Jagwire 0.035 angle type, Boston Scientific) for another 10 min (second attempt) and were repeated as necessary according to the trainers' recommendations (exchange of endoscopist or guidewire, pancreatic duct guidewire placement method, or pre-cutting sphincterotomy).

Definitions

Success was defined as completing cannulation with the J-shaped tip guidewire and obtaining a cholangiogram within 10 min. Cannulation time was defined as from when a tip of the guidewire first touched the orifice of the papilla to the obtainment of cholangiogram. PEP was defined as continued abdominal pain ⩾ 24 h after ERCP, with more than 3 times the normal (upper limit) serum-

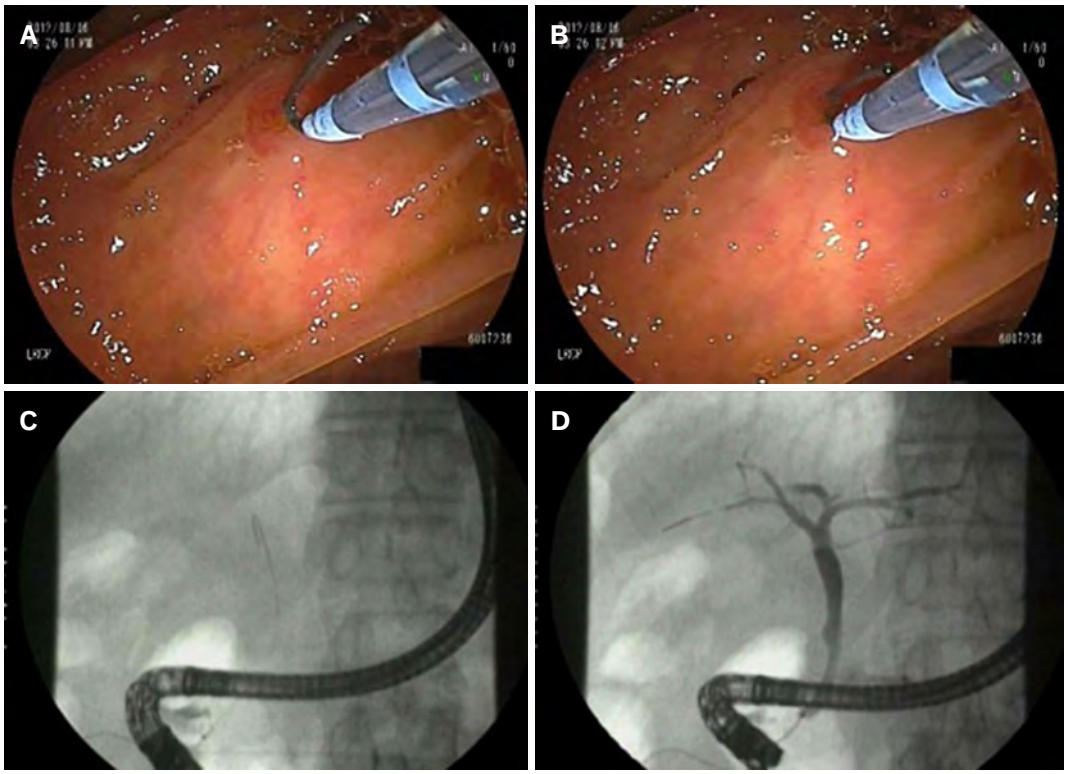

Figure 2 Endoscopic and fluoroscopic images showing the technique with J-shaped tip guidewire. A: Assistant endoscopist extended approximately 5 mm of the guidewire tip and restored it to the original "J" configuration (stand-by position); B: Selective biliary cannulation was attempted under endoscopic control without fluoroscopy; C: The guidewire was moved in an in-and-out motion by an assisting endoscopist. Once the guidewire was advanced without resistance, fluoroscopy was used to confirm successful cannulation; D: Contrast medium was injected after confirmation of successful biliary cannulation.

Table 1 Baseline patient characteristics and indications

Item (n = 50)	Value
Age, yr [median (IQR)]	75.3 (68-83)
Sex (male)	24
Periampullary diverticulum, n (%)	22 (44)
Indications	
Choledocolithiasis (including suspicion)	27
Cholangiocarcinoma	7
Pancreatic cancer	6
Gallbladder cancer	4
Other malignant disease	2
Cholangiocellular carcinoma	1
Suspected biliary SOD	1
Mirrizi syndrome	1
Biliary leak after cholecystectomy	1

IQR: Interquartile rage; SOD: Sphincter of Oddi dysfunction.

amylase level[25]. Pancreatitis severity was classified using the Atlanta International Symposium criteria[26]. Suspected sphincter of Oddi dysfunction was defined according to the revised Milwaukee classification[27]. Sphincter of Oddi manometory was not performed. Hyperamylasemia was defined as 3 times the normal (upper limit) amylase level 18-24 h after ERCP.

Ethics

The protocol adhered to the Helsinki Declaration and was approved in advance by the Institutional Ethical Review Board. The trial was registered with the University hospital Medical Information Network Clinical Trials Registry (UMIN000007526). All participants gave written informed consent beforehand.

Outcome measurement

The primary study endpoint was the success rate of cannulation with the J-shaped tip guidewire performed within 10 min. The secondary endpoints were as follows: (1) the rate of the occurrence of PEP; (2) time to selective biliary cannulation; (3) number of attempts for selective biliary cannulation; and (4) number of accidental pancreatic duct insertions. Data are presented as median and interquartile ranges (IQR).

RESULTS

Baseline characteristics and indications are summarized in Table 1, and details of the endoscopic procedure are given in Table 2.

The overall success rate of endoscopy was 90% (45/50, Table 3), with cannulation achieved within the first 5 min in 38 patients (76%). Cannulation was achieved on the second attempt in 3 patients. The median time to cannulation for these 48 patients was 42.5 s (IQR: 5-262 s). Of the remaining two patients, one required pancreatic duct guidewire placement and the other a pre-cutting sphincterotomy. The median number of attempts was 2.0 (IQR:

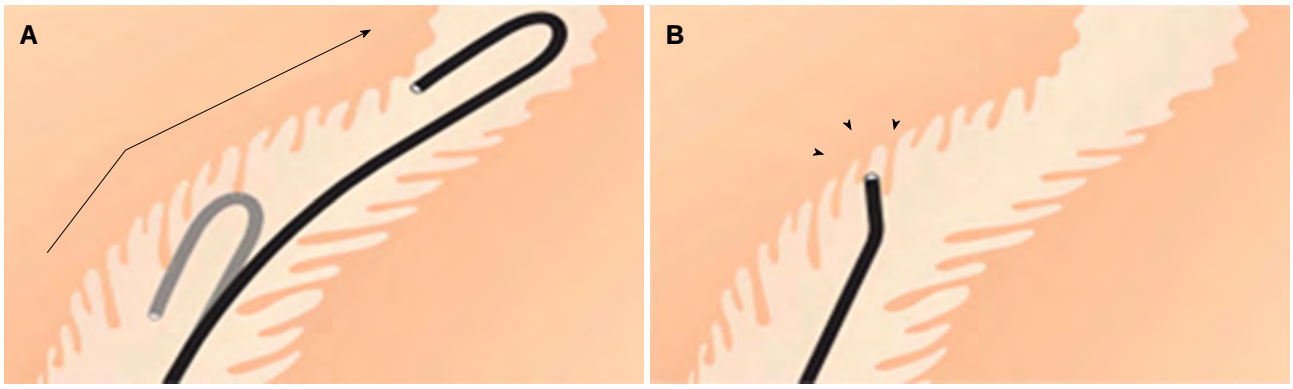

Figure 3 An image *via* intra-duodenal biliary segments of J-shaped tip guidewire (A) and standard guidewire (B). A: Blunted J-shaped tip may facilitate passage through intra-duodenal segment (arrow); B: Normal guidewire tips may be become stuck in epithelial folds or flexion of intra-duodenal biliary segments (arrowheads).

Table 2 Number of patients receiving different procedures

Procedure	*n*
Endoscopic papillary (large) balloon dilation	24
Endoscopic sphincterotomy	19
Endoscopic nasobiliary drainage	20
Endoscopic nasobiliary gallbladder drainage	2
Placement of biliary stent (plastic or metal)	18
Intraductal ultrasonography	7
Aspiration, biopsy	12
Only cholangiogram	0

Table 3 Cannulation outcomes

Item (*n* = 50)	Value
Success, *n* (%)	45 (90)
< 5 min	38 (76)
5-10 min	7 (14)
Time to selective biliary cannulation[1], s	42.5 (5.0-262.0)
No. of attempts[1]	2.0 (1.0-6.0)
No. of accidental pancreatic duct insertion[1]	1.0 (0.0-3.0)
Amylase level[1], IU/L	148 (94-331)
Post-ERCP pancreatitis, *n* (%)	3 (6)
Mild	3
Severe	0
Hyperamylasemia, *n* (%)	4 (8)

[1]Data is shown as median (IQR). IQR: Interquartile range; ERCP: Endoscopic retrograde cholangiopancreatography.

1.0-6.0), and the median number of accidental pancreatic duct insertions was 1.0 (IQR: 0.0-3.0). The median serum-amylase level was 148 IU/L (IQR: 94-331 IU/L), and hyperamylasemia occurred in 4 patients.

Mild PEP occurred in 3 patients (6%); in 2 of these, success was achieved within 5 min after endoscopic papillary large balloon dilation, while the third patient received the pre-cutting sphincterotomy mentioned above. All patients were managed successfully with conservative treatment. There were no other adverse events including bleeding or perforation.

DISCUSSION

The success rate for selective biliary cannulation using a J-shaped tip guidewire was comparable to that found in previous studies[6-16,28,29], and no guidewire-related adverse events such as bleeding or perforation occurred. Although ours was a preliminary study, the atraumatic and blunt tip of the new guidewire may facilitate selective biliary cannulation (Figure 3A) and reduce instances of perforation and bleeding.

Although straight and angled tips are the most common types used in WGC[1-20,28,29], these sharp tips often stick in the intra-duodenal biliary segment (Figure 3B). While the superiority of the J-shaped tip cannot be definitively shown without controls, the success rate, speed of cannulation, and facility of use appear improved compared to other studies. While similar procedures using standard guidewires resulted in a 77.9% overall success rate (trainees and trainer combined)[28], here we achieved a 76% success rate with trainees, and an overall success rate of 90%. Additionally, the 6% PEP rate is similar to that of other studies[14,18,19,20].

WGC was first introduced by Siegel *et al*[30]. Meta-analysis has shown that the reduction of pancreatic duct opacification is another possible advantage over CGC[14,18,19]. Further, WGC has been suggested to decrease the risk of PEP[14,18,19], facilitating its spread across the globe as a potential first-line method.

Usually, when guidewires are extended from the tip of a catheter without enough space for advancement, the wire may act like a needle and pierce the epithelium. The J-shape of the guidewire protrudes from the catheter before approaching the biliary orifice, and reduces this likelihood. We therefore believe our J-shaped design to be the aspect that improved insertion into the biliary system. Limitations to this study include small sample size, no controls, a single institution, and involvement of multiple endoscopists. A randomized comparison is warranted for objective evaluation of its performance. One drawback of the J-shaped tip guidewire is the 1-mm radius, which is wider than a standard guidewire and may hamper selective cannulation through a narrow orifice.

In conclusion, a newly designed guidewire with a

J-shaped tip may facilitate selective biliary cannulation in ERCP. However, a large prospective randomized control trial is necessary to verify the performance of this guidewire in comparison with standard guidewires.

COMMENTS

Background

Selective biliary cannulation is essential for diagnosis and therapeutic endoscopic retrograde retrograde cholangiopancreatography (ERCP) in biliary diseases. Wire-guided cannulation (WGC) increases the primary biliary cannulation rate and decreases the risk of post-ERCP pancreatitis (PEP). Therefore, WGC is now widely performed. However, even experts meet with difficulty and the possible risk of bleeding and perforation due to the guidewire.

Research frontiers

The authors performed ERCP using a newly designed J-shaped tip guidewire. A J-shaped tip guidewire with a strongly flexed atraumatic tip and hydrophilic coating was designed to improve passage through intra-duodenal biliary segments and decrease the adverse events, such as PEP, bleeding and perforation. The authors conducted a feasible study.

Innovations and breakthroughs

This is a single center pilot study. The primary success rate was 90% (45/50) within 10 min. The rate of PEP was 6% (3/50), but all 3 cases were mild pancreatitis. All patients were managed successfully with conservative treatment. There was no bleeding or perforation.

Applications

A newly designed J-shaped tip guidewire may facilitate selective biliary cannulation and the structure of the tip may contribute to decrease PEP and bleeding, or perforation. However, it is necessary to conduct a large prospective randomized control trial to verify the performance.

Peer review

This is a single center pilot study of a newly designed J-shaped tip guidewire for wire-guided cannulation. The authors hypothesized that the J-shaped tip prevented perforation or PEP during cannulation. The limitation of this study is a small sample size without a control group as the authors discussed.

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BRIEF ARTICLE

Survival outcome of patients with spontaneously ruptured hepatocellular carcinoma treated surgically or by transarterial embolization

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Abstract

AIM: To evaluate clinical outcomes of patients that underwent surgery, transarterial embolization (TAE), or supportive care for spontaneously ruptured hepatocellular carcinoma (HCC).

METHODS: A consecutive 54 patients who diagnosed as spontaneously ruptured HCC at our institution between 2003 and 2012 were retrospectively enrolled. HCC was diagnosed based on the diagnostic guidelines issued by the 2005 American Association for the Study of Liver Diseases. HCC rupture was defined as disruption of the peritumoral liver capsule with enhanced fluid collection in the perihepatic area adjacent to the HCC by dynamic liver computed tomography, and when abdominal paracentesis showed an ascitic red blood cell count of > 50000 mm^3/mL in bloody fluid.

RESULTS: Of the 54 patients, 6 (11.1%) underwent surgery, 25 (46.3%) TAE, and 23 (42.6%) supportive care. The 2-, 4- and 6-mo cumulative survival rates at 2, 4 and 6 mo were significantly higher in the surgery (60%, 60% and 60%) or TAE (36%, 20% and 20%) groups than in the supportive care group (8.7%, 0% and 0%), respectively (each, $P < 0.01$), and tended to be higher in the surgical group than in the TAE group. Multivariate analysis showed that serum bilirubin (HR = 1.09, $P < 0.01$), creatinine (HR = 1.46, $P = 0.04$), and vasopressor requirement (HR = 2.37, $P = 0.02$) were significantly associated with post-treatment mortality, whereas surgery (HR = 0.41, $P < 0.01$), and TAE (HR = 0.13, $P = 0.01$) were inversely associated with post-treatment mortality.

CONCLUSION: Post-treatment survival after surgery or TAE was found to be better than after supportive care, and surgery tended to provide better survival benefit than TAE.

Key words: Ruptured hepatocellular carcinoma; Surgery; Transarterial embolization

Core tip: We have shown here that overall survival rates of patients with ruptured hepatocellular carcinoma (HCC) is significantly higher in patients with surgery or transarterial embolization (TAE) than in those with supportive care, and tended to be higher in patients with surgery than in those with TAE. To date, there has been a dearth of reliable clinical evidence on the merits of surgical treatment versus those of TAE, in the context of survival benefit in patients with a spontaneous HCC rupture. Therefore, the present study may provide useful information for clinicians to determine the most appropriate treatment option for spontaneously ruptured HCC.

Jin YJ, Lee JW, Park SW, Lee JI, Lee DH, Kim YS, Cho SG, Jeon YS, Lee KY, Ahn SI. Survival outcome of patients with spontaneously ruptured hepatocellular carcinoma treated surgically or by transarterial embolization. *World J Gastroenterol* 2013; 19(28): 4537-4544 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i28/4537.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i28.4537

INTRODUCTION

Hepatocellular carcinoma (HCC) is the worldwide health problem and the third leading cause of cancer-related death globally[1-3]. Despite recent considerable advances in the understanding of tumor biology and the continued progression and development of diagnostic and therapeutic tools[4-7], the overall prognosis of HCC remains disappointing. In particular, due to its hypervascularity, HCC can exhibit rapid progression with direct invasion of surrounding tissues or it can invoke spontaneous tumor rupture[8]. HCC rupture is one of the life-threatening complications of HCC, and therefore, the most efficient treatment modality should be selected and rapidly applied to patients with ruptured HCC.

The incidence of spontaneous HCC rupture has decreased due to the earlier detection of HCC. Nevertheless, its incidence has been reported to be high as 3%-15% and its in-hospital mortality rate to range from 25% to 75% in the acute phase[9-12]. Open surgery was the main method used to treat HCC rupture from the 1960s to the 1980s[13-15]. Recently, survival benefit by transarterial embolization (TAE) has been reported[16-18]. However, to the best of our knowledge, no definite recommendation has been issued regarding optimal treatment of HCC rupture, and the comparative survival benefits of surgery and TAE remain unclear.

Therefore, in this retrospective study, we undertook to evaluate survival outcomes according to treatment modalities, that is, surgery, TAE, or supportive care, in patients with a spontaneously ruptured HCC, and sought to identify the factors that predispose post-treatment mortality in these patients.

MATERIALS AND METHODS

Study subjects

Between August 2003 and February 2012, 1765 consecutive patients were initially diagnosed as having HCC at Inha University Hospital. Of these 1765 patients, 61 (3.5%) patients were clinically diagnosed as having spontaneously ruptured HCC. No patient had recent history of HCC treatment such as surgery or transarterial chemoembolization (TACE) within one month prior to the diagnosis of HCC rupture. HCC was diagnosed according to the diagnostic guidelines issued by the American Association for the Study of Liver Diseases[19]. HCC rupture was defined as disruption of the peritumoral liver capsule with enhanced fluid collection in the perihepatic area adjacent to the HCC by dynamic liver computed tomography (CT)[20], and when abdominal paracentesis showed an ascitic red blood cell count of > 50000 mm^3/mL in bloody fluid[21,22].

Of the 61 patients, 4 patients were excluded because they underwent two-staged surgical treatment after TAE for ruptured HCC (n = 3) and they had concurrent malignancy (gastric cancer, n = 1). Three patients were also excluded because they did not meet the diagnostic criteria of a ruptured HCC although HCC rupture was clinically suspected based on right upper quadrant abdominal pain and a reduced serum hemoglobin level. Therefore, 54 patients finally constituted the study cohort and their retrospective database was analyzed.

Evaluation of patients with ruptured HCC

Database information at time of diagnosis of ruptured HCC was reviewed: age, gender; vital signs; medical history; white blood cell count, hemoglobin, and platelet count; international normalized ratio (INR); serum alanine aminotransferase, bilirubin, albumin, and creatinine; viral hepatitis findings including hepatitis B surface antigen, and anti-hepatitis C virus antibody findings; serologic tests for human immunodeficiency virus; alpha-fetoprotein; and vasopressor requirement. Furthermore, we evaluated HCC tumor statuses namely tumor number, size, presence of portal vein tumor thrombosis, and presence of extra-hepatic metastasis. Intrahepatic HCC lesion size was recorded as the longest diameter of the largest lesion in at least one dimension. Liver cirrhosis was diagnosed based on clinical evidence of portal hypertension (encephalopathy, esophageal varices, ascites, splenomegaly, or platelet count < 100000/mm^3)[23] or by previously performed ultrasonography[24]. Child-Turcotte-Pugh (CTP) and Model for End-stage Liver Disease (MELD) scores were assessed, and HCC staging was performed using the Barcelona Clinic Liver Cancer (BCLC) staging system[25].

Treatment of ruptured HCCs

Immediately following a diagnosis of HCC rupture, patients were transferred to an intensive care unit. At time of HCC rupture, liver functions can be much more ag-

gravated by hemorrhage or shock than after the condition was relatively well controlled. Therefore, frequent assessments of liver function were required concurrently with volume replacement and coagulopathy correction, and liver function was closely monitored before definite treatment decision making. Norepinephrine was infused intravenously as the primary vasopressor if shock event developed, and all 54 patients were administered 3rd generation cephalosporin antimicrobial therapy.

The need for emergency hemostasis, such as, open surgery or TAE, was explained to all patients in the absence of a contraindication and their family members. They were informed of the risks and benefits of emergency surgery or TAE in detail. To avoid any coercion, written informed consent was obtained from all patients and a family member before intervention of hemostasis. The follow were viewed as surgical contraindication: the presence of poorly controlled chronic ascites; the presence of poorly controlled chronic hepatic encephalopathy; the presence of a poor liver function; or a poor performance status. Of the 54 patients enrolled, 6 (11.1%) underwent surgery and 25 (46.3%) TAE, and the remaining 23 (42.6%) patients received supportive care without hemostatic intervention (Figure 1). Successful control of hemorrhage was defined as hemodynamic stabilization, a normal hemoglobin level, and no requirement for further transfusion. During follow-up period after treatment, dynamic liver CT images and serum alpha-fetoprotein levels were obtained every 1-3 mo.

TAE group: In hemodynamically unstable patients with an obvious continuous hemorrhage, TAE was considered if reserved liver function was relatively good regardless of the correction of coagulopathy. Briefly, the tumor location, the active bleeding site, and portal vein patency were determined angiographically. Thereafter, embolization of the feeding artery was performed with gelfoam, which is the small cube of approximately 1 mm^3 sized absorbable gelatin sponge particles. Two patients who underwent TACE were included in the TAE group. However, TACE/TAE was not performed if the main portal vein was completely occluded by tumor thrombus.

Surgical group: After stabilizing hemodynamic status by volume replacement and transfusion, patients underwent a full clinical assessment to evaluate the possibility of surgical treatment. Segmentectomy with perihepatic packing (n = 3, 50%), lobectomy (n = 2, 33.3%), or liver wedge resection with feeding artery ligation (n = 1, 16.7%) were performed depending on circumstances (Figure 1).

Supportive care group: Patients contraindicated for surgery or TACE/TAE received only vigorous and careful conservative treatments with replacement of blood or albumin, correction of coagulopathy, antimicrobial therapy, and analgesics, diuretics, *etc*.

The study protocol was approved by the Institutional Review Board at Inha University Hospital, Incheon 400-711, South Korea.

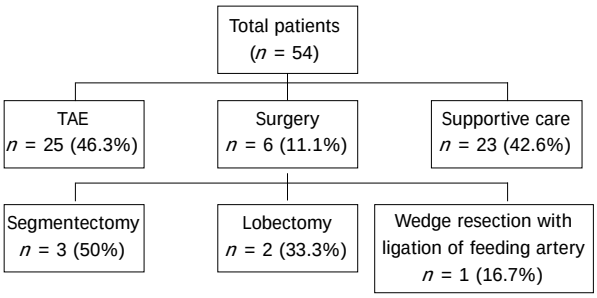

Figure 1 Flow diagram showing the treatment of 54 patients. Of the 54 patients enrolled, 6 (11.1%) underwent surgery and 25 (46.3%) transarterial embolization (TAE), and the remaining 23 (42.6%) patients received supportive care without hemostatic intervention.

Statistical analysis

The baseline characteristics of patients are expressed as medians (ranges) and frequencies. Differences between categorical or continuous variables were analyzed using the χ^2 test, Fisher's exact test, or the Student's t test. Post-treatment cumulative mortality rates were analyzed using Kaplan-Meier survival curves, and group differences were compared using the log-rank test. In patients that received supportive care, survival was defined from diagnosis of HCC rupture to patients' death. Multivariate analysis was performed using a Cox regression hazard model to identify predictors of post-treatment mortality in patients with spontaneously ruptured HCC. Two-tailed P values of less than 0.05 were considered statistically significant in all analyses. Statistical analysis was performed using SPSSv18.0 (SPSS Inc, Chicago, IL, United States).

RESULTS

Baseline characteristics of patients

The baseline characteristics of the 54 patients are summarized in Table 1. Median age was 54 years (range, 30-87 years) and 47 (87.0%) were male. The most common etiology of HCC was hepatitis B virus infection, which was observed in 36 (66.7%) patients. Of the 54 patients, 6 (11.1%) were of CTP class A, 23 (42.6%) were of CTP class B, and 5 (46.3%) were of CTP class C. Eleven (20.4%) of the 54 patients had a single HCC and 43 (79.6%) patients had multiple HCC. Median tumor size was 8.5 cm (range, 2.9-25.5 cm), and 4 (7.4%) patient had HCCs within Milan criteria. Forty-seven (87.7%) patients had nodular type HCC. Before treatment, 0 (0%), 5 (9.3%), 9 (16.7%), 15 (27.8%), and 25 (46.3%) patients were found to have BCLC 0, A, B, C, or D stage HCC, respectively. Median alpha-fetoprotein concentration at diagnosis of HCC rupture was 1158 ng/mL (range, 3.0×10^4-6.1×10^4 ng/mL). Thirteen (24.1%) patients required a vasopressor due to shock at presentation. Ruptured HCC was located on the surface of liver in all the patients.

Comparison of clinical parameters by treatment modality

Clinical variables in the three treatment groups are summarized in Figures 2 and 3, and Table 2. Mean tumor

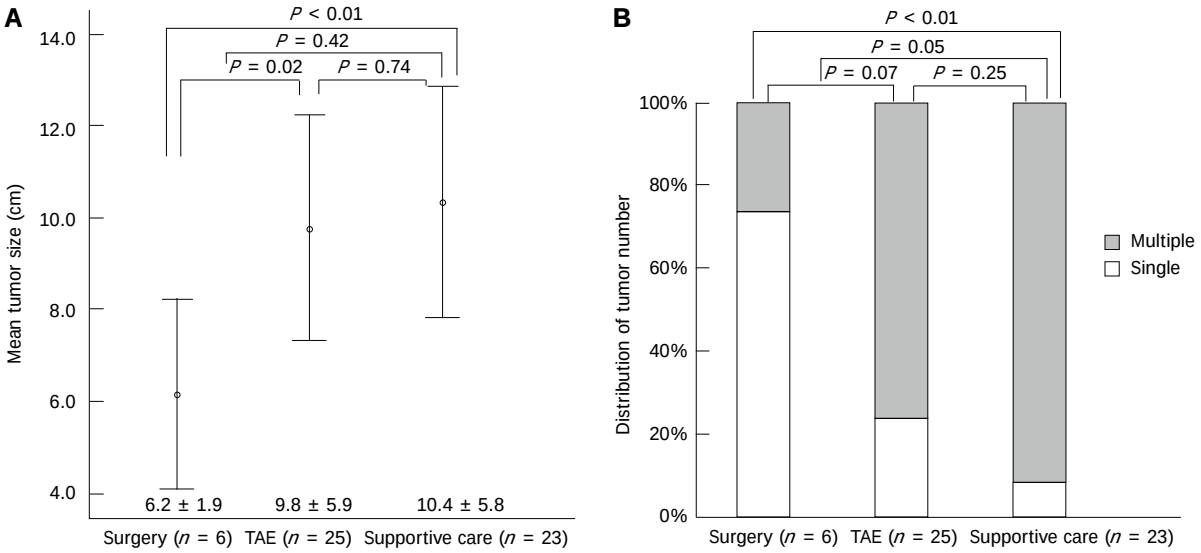

Figure 2 Comparison of clinical parameters in the three treatment groups. A: Mean tumor size was significantly smaller in the surgical group than in the transarterial embolization (TAE) (P = 0.02) or supportive care (P < 0.01) groups; B: The single tumor rate was significantly higher in the surgical group than in the supportive care group (P < 0.01).

Table 1 Baseline clinical characteristics of the 54 patients with ruptured hepatocellular carcinoma *n* (%)

Variable	Total (n = 54)
Age[1], yr	54 (30-87)
Gender (male)	47 (87.0)
Etiology	
HBV/HCV/alcohol/others	36 (66.7)/6 (11.1)/7 (13.0)/5 (9.3)
CTP classification	
A/B/C	6 (11.1)/23 (42.6)/25 (46.3)
Tumor size[1], cm	8.5 (2.9-25.5)
Tumor number	
Single/multiple	11 (20.4)/43 (79.6)
Tumor type	
Nodular/infiltrative	47 (87.0)/7 (13.0)
Within Milan criteria	4 (7.4)
BCLC stage	
0/A/B/C/D	0 (0.0)/3 (5.6)/8 (14.8)/15 (27.8)/28 (51.9)
Vasopressor requirement	13 (24.0)
Alpha-fetoprotein[1], ng/mL	1158 (3.0×10^4-6.1×10^4)

[1]Median (range). HBV: Hepatitis B virus; HCV: Hepatitis C virus; CTP: Child-Turcotte-Pugh classification; BCLC: Barcelona Clinical Liver Cancer.

size was significantly smaller in the surgical group than in the TAE (P = 0.02) or supportive care (P < 0.01) groups (Figure 2A). The single tumor rate was significantly higher in the surgical group than in the supportive care group (P < 0.01) (Figure 2B). Furthermore, the surgical group had better reserve hepatic function (CTP class) than the other two groups (both P < 0.01) (Figure 3A). Mean MELD score was higher in the supportive care group than in the other two groups (both P = 0.01), but was not different in the surgical and TAE groups (P = 0.24) (Figure 3B).

Serum platelet count (P = 0.03), total bilirubin (P < 0.01), and creatinine levels (P < 0.01) were significantly lower in surgical group than supportive care group (Table 2). The other clinical parameters including age, gender, and tumor type showed no difference among three treatment groups. Incomplete hemostasis occurred in 1 (16.7%) patient in the surgical group and in 5 (20%) patients in the TAE group (P = 1.00), and post-treatment liver failure occurred in 0 (0%) patient in the surgical group and in 1 (5%) patient in the TAE group (P = 0.31). Rebleeding after complete hemostasis was observed in 0 (0%) patients in the surgical group and in 1 (5%) patient in the TAE group (P = 1.00), and they received supportive care (Table 2).

Cumulative overall survival of ruptured HCC patients according to treatment types

One-month overall cumulative mortality for the 54 study subjects was 63.8%. Cumulative survival rates at 2-, 4- and 6-mo were 60.0%, 60.0% and 60.0%, respectively, in the surgical group and 36.0%, 20.0% and 20.0%, respectively in the TAE group, and 8.7%, 0% and 0%, respectively in the supportive care group (each, P < 0.01). Cumulative survival rates at 2-, 4- and 6-mo were tended to be higher in the surgical group than in the TAE group despite the statistical insignificance (P = 0.14) (Figure 4A). Cumulative survival rates at 2-, 4- and 6-mo were 50.2%, 40.1% and 33.1%, respectively in the intervention group such as surgery or TAE, and 8.7%, 0% and 0%, respectively, in the supportive care group (P < 0.01) (Figure 4B).

Multivariate analysis for predictors of post-treatment mortality

Multivariate analysis showed that surgery (HR = 0.41, P < 0.01), and TAE (HR = 0.13, P = 0.01) were inversely associated with post-treatment mortality in ruptured HCC patients. Serum bilirubin (HR = 1.09, P < 0.01), creatinine (HR = 1.46, P = 0.04), and vasopressor use (HR = 2.37, P = 0.02) were positively associated with post-treatment mortality. Age, gender, INR, albumin, tumor

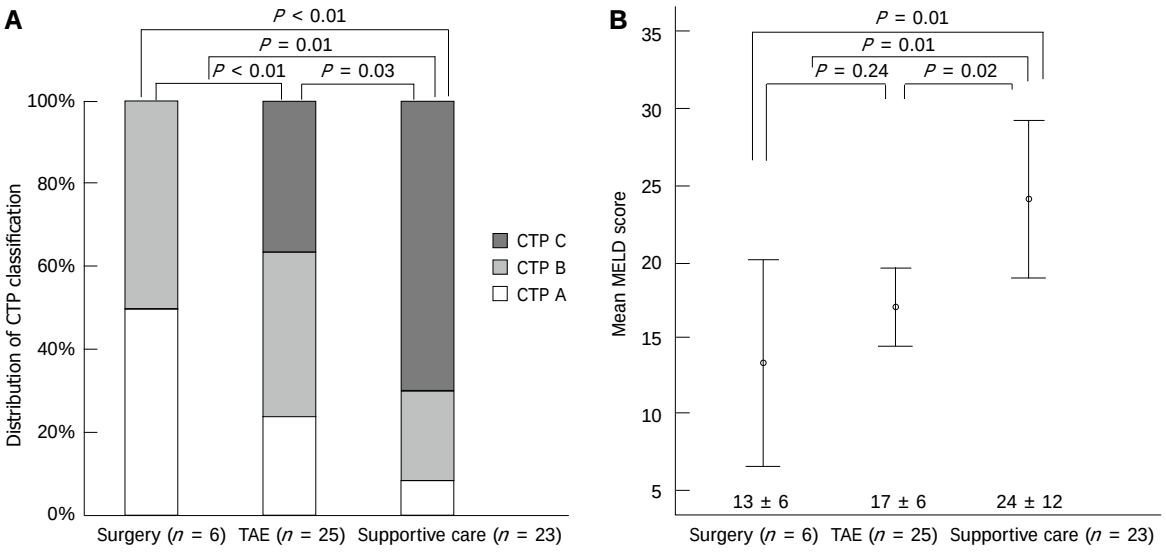

Figure 3 Comparison of Child-Turcotte-Pugh and Model for End-stage Liver Disease scores in the three treatment groups. A: The surgical group had better reserve hepatic function [Child-Turcotte-Pugh (CTP) class] than the other two groups (both $P < 0.01$); B: Mean Model for End-stage Liver Disease (MELD) score was higher in the supportive care group than in the other two groups (both $P = 0.01$), but was not different in the surgical and transarterial embolization (TAE) groups ($P = 0.24$).

Table 2 Clinical parameters of patients according to treatment modality *n* (%)

Variables	Resection	TAE	Supportive	*P* value
	6 (11.1)	25 (46.3)	23 (42.6)	
Age, yr	59 (42-79)	54 (30-83)	53 (36-87)	NS
Gender (male)	5 (83.3)	22 (88.0)	20 (86.9)	0.95
Hb[1], g/dL	8.2 (5.1-12.1)	7.6 (4.5-11.2)	8.1 (2.9-13.6)	NS
Platelet[1], × 10^3/mm^3	127.5 (80-236)	139 (11-534)	191 (86-606)	0.03[4], NS
Prothrombin time[1], INR	1.4 (0.7-3.0)	1.3 (1.0-2.3)	2.2 (1.0-6.6)	NS
Albumin[1], mg/dL	2.6 (0.4-3.5)	2.9 (1.8-3.7)	2.6 (1.2-4.3)	NS
Total bilirubin, mg/dL	2.3 (0.5-6.2)	1.2 (0.4-5.4)	6.7 (0.6-23.0)	< 0.01[4], NS
Creatinine[1], mg/dL	1.2 (0.8-2.0)	1.1 (0.6-1.2)	1.9 (0.9-4.9)	< 0.01[4], 0.01[5]
Tumor type				
Nodular/infiltrative	5/1 (83.3/16.7)	21/4 (84/16)	21/2 (91.3/8.7)	0.72
BCLC stage A/B/C/D	3/2/1[2]/0 (50.0/33.3/16.7/0)	0/4/9/12 (0/16/36/48)	0/2/5/16 (0/8.7/21.7/69.6)	< 0.01
Alpha-fetoprotein[1], ng/mL	33.9 (3-3.6 × 10^4)	1345 (3-6.1 × 10^4)	1389 (19-6.1 × 10^4)	NS
Vasopressor requirement	1 (16.7)	7 (28.0)	5 (21.7)	0.79
Incomplete hemostasis	1 (16.7)	5 (20)	NA	1.00[3]
Post-treatment liver failure	0 (0)	6 (24)	NA	0.31[3]
Rebleeding	0/5 (0)	1/20 (5.0)	NA	1.00[3]

[1]Median (range); [2]1 patient received palliative resection; [3]*P* value between resection and TAE group; [4]*P* value between resection and supportive group; [5]*P* value between TAE and supportive group. TAE: Transarterial embolization; BCLC: Barcelona Clinical Liver Cancer; NA: Not available; NS: Not significant.

size, and tumor number were not found to be associated with post-treatment mortality (Table 3).

DISCUSSION

We have shown here that overall survival rates of patients with ruptured HCC is significantly higher in patients with surgery or TAE than in those with supportive care, and tended to be higher in patients with surgery than in those with TAE. Furthermore, high serum bilirubin and creatinine levels, and vasopressor requirement were found to be significantly associated with post-treatment mortality. To our knowledge, this is the first study to investigate the survival benefit of surgery *vs* TAE. Although several previous studies have evaluated the therapeutic efficacy of surgery or TAE, in patients with spontaneous HCC rupture, direct comparative information is little available regarding survival outcomes after surgery and TAE in such patients.

Spontaneous HCC rupture is likely to occur in patients with advanced staged HCC with reported incidences of 10.0% in Japan[15], 12.4% in Thailand[10], and about 3.0% in the United Kingdom[26]. In the present study, the estimated incidence of spontaneous HCC rupture was 3.5%, and the overall 1-mo mortality was a high as 64% in our cohort, which are the similar to the outcomes of previous studies[10,11,27]. However, despite its high mortality, survival benefits for surgical treatment[13-15] and for TAE[16-18] have been reported in patients with a spontaneous HCC rupture. Likewise, in the present study, the

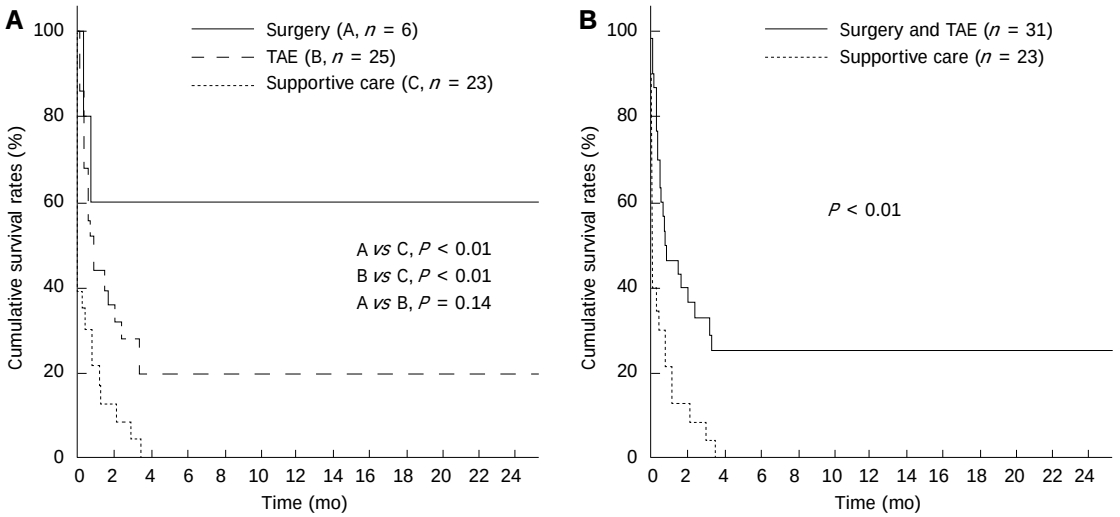

Figure 4 Cumulative overall survival according to treatment types. A: Cumulative survival rates at 2-, 4- and 6-mo in the surgical group or in the transarterial embolization (TAE) group were significantly higher in the supportive care group (each, $P < 0.01$); B: Cumulative survival rates at 2-, 4- and 6-mo were significantly higher in the intervention group such as surgery and TAE than in the supportive care group ($P < 0.01$).

Table 3 Significant predictive factors of post-treatment mortality in spontaneously ruptured hepatocellular carcinoma patients

Variables	Univariate analysis			Multivariate analysis[1]		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age, yr	0.99	0.96-1.01	0.18	0.98	0.95-1.01	0.14
Gender (male)	0.88	0.37-2.09	0.77	-	-	-
INR	1.77	1.30-2.41	< 0.01	0.92	0.49-1.70	0.79
Total bilirubin, mg/dL	1.13	1.07-1.19	< 0.01	1.09	1.13-1.15	< 0.01
Albumin, mg/dL	0.73	0.42-1.03	0.07	0.81	0.49-1.32	0.39
Creatinine, mg/dL	1.86	1.33-2.61	< 0.01	1.46	1.01-2.13	0.04
AFP, ng/mL	1.00	1.00-1.01	0.21	-	-	-
Tumor number multiple *vs* single	2.45	1.09-5.54	0.03	1.14	0.46-2.83	0.78
Tumor size, cm	1.01	0.96-1.05	0.88	-	-	-
Vasopressor requirement	1.96	1.87-3.29	0.01	2.37	1.13-4.96	0.02
Treatment type						
Supportive care (control)	-	-	-	-	-	-
TACE/TAE	0.44	0.03-0.64	0.01	0.13	0.03-0.66	0.01
Surgery	0.15	0.24-0.80	< 0.01	0.41	0.21-0.79	< 0.01

Subjects, $n = 54$; event, patient death after treatment ($n = 47$). [1]Cox proportional hazards model. CTP: Child-Turcotte-Pugh classification; AFP: Alpha-fetoprotein; TACE: Transarterial chemoembolization; TAE: Transarterial embolization; INR: International normalized ratio.

overall survivals of patients with surgery or TAE were significantly higher than that in those with supportive care, especially in those in a hemodynamically stable state with a low serum bilirubin level, and good renal function. However, to date, there has been a dearth of reliable clinical evidence on the merits of surgical treatment versus those of TAE, in the context of survival benefit in patients with a spontaneous HCC rupture. Therefore, the present study may provide useful information for clinicians to determine the most appropriate treatment option for spontaneously ruptured HCC.

Ruptured HCC is a catastrophic disorder characterized by fatal complications, such as, coagulopathy, hemodynamic instability, or liver insufficiency. Thus, treatment should be considered carefully based on adequate information. As has been found in previous studies[13-15], surgical treatment was found to provide significant survival benefit as compared with supportive care in the current study. Furthermore, surgical group had relatively better hepatic function reserve, smaller tumors, and smaller numbers of tumors than the supportive care groups, although bias might have been introduced by selection for surgery. Nonetheless, the cumulative overall survival rate was higher in surgical group than in the supportive care group, and surgical treatment was found to be independent predictor of post-treatment survival by multivariate analysis. Although not all patients could have undergone surgery due to a poor hepatic function or an unstable vital status, surgical hemostasis can be considered if hepatic dysfunction or hemodynamic instability can be maximally corrected immediately after initial rupture of a hepatoma. Furthermore, post-surgical complications need to be considered before treatment decision-making despite the absence of an immediate severe complication after surgical intervention in the present study.

It has been reported that TAE is effective in achiev-

ing immediate hemostasis for ruptured HCC. In the present study, the overall survival rate was better in the TAE group than in the supportive care group. Advanced angiographic techniques enable the tumor location, active bleeding focus, and portal vein patency to be assessed, but life-threatening complications, such as, liver failure can developed after TAE, at rates ranging from 12% to 34%[12,17]. In the present study, post-TAE liver failure and technical failure for immediate hemostasis was observed in 6 (24%) and 5 (20%) patients of the TAE group, respectively, which suggests that TAE should be selectively administered in patients with good reserved hepatic function, tolerable coagulopathy, and a patent main portal vein.

In terms of comparison of survival benefits between the surgical and TAE groups, the current study failed to show a significant difference, although the cumulative overall survival rate tended to be higher in the surgical treatment group. Although TAE is less invasive than open surgical hemostasis, we suppose that open surgical treatment offer a higher chance of successfully achieving hemostasis by removing the bleeding focus or by allowing complete ligation of feeding artery. In previous studies, the 30-d mortality rate after TAE group has been reported to be lower than that after open surgical group[12,17,28]. However, in the present study, cumulative overall 1-mo survival rates were statistically similar between two groups. Moreover, at 1 mo after operative treatment, cumulative survival was clinically higher in the surgical group than in the TAE group despite the statistical insignificance (Figure 4). However, it should be borne in mind that this lack of significance may have been due to small patient numbers. Therefore, large number of patients who underwent surgical treatment need to be evaluated in the comparative study in the future.

Patients with a poor liver function reserve cannot tolerate surgical resection or aggressive angiographic intervention. Therefore, CTP class or MELD score, which reflect reserved hepatic function, could be important pretreatment factors. However, in the present study, of variables comprising the CTP class, only serum bilirubin was found to be independently associated with post-treatment survival. Likewise, of the variables comprising the MELD scores, only serum bilirubin and creatinine were found to be independent factors. Therefore, we estimated individual variables of CTP class or MELD score to avoid overestimation of the other factors in the multivariate analysis of post-treatment survival in patients with spontaneous HCC rupture.

Elevated serum creatinine and vasopressor requirements are likely to reflect multiorgan failure[29], and decreased effective circulating volume or the presence of superimposed infection may induce alterations in organ perfusion and in hemodynamic stability. Moreover, inflammatory reactions triggered by hepatocellular necrosis after HCC rupture may contribute to liver insufficiency, and subsequent multiorgan failure. Although decreased serum albumin level and a prolonged prothrombin time suggest reduced liver synthetic function, they can be corrected by albumin and coagulation factor replacement. On the other hand, serum bilirubin level cannot be rapidly and artificially corrected immediately after parenchymal liver damage. Therefore, serum bilirubin may be an independent factor of patient survival, unlike serum albumin or INR. Furthermore, the importance of serum bilirubin level in patients with HCC rupture has been previously reported[17,30]. However, during treatment decision-making, age, INR, albumin, and tumor size and number may also be clinically important variables despite their lack of statistical significance in the present study.

Our study has several limitations. First, the study is inherently limited by its retrospective study design. However, we enrolled all eligible patients. Second, the varied clinical and tumor statuses of patients and the critical disease status prevented randomization, and probably introduced bias. Furthermore, it takes long time to collect the prospective database of patients due to low incidence of the disease. Third, the absolute number of patients who underwent surgery was small, and therefore, there might be no significant difference in cumulative survival rate between surgery and TAE groups. Accordingly, we suggest a large-scale study be conducted to confirm our study.

In conclusions, the present study suggests that the post-treatment outcomes of surgery or TAE are better than that of supportive care in patients with spontaneous HCC rupture, and that surgical hemostasis might provide better survival benefit than TAE. However, we advise that serum bilirubin, creatinine, and hemodynamic status should be considered during treatment decision making. Regardless of its shortcomings, we believe that the present study would provide important information that aids decision making in patients with spontaneous HCC rupture.

COMMENTS

Background

Hepatocellular carcinoma (HCC) rupture is one of the life-threatening complications of HCC, and therefore, the most efficient treatment modality should be selected and rapidly applied to patients with ruptured HCC.

Research frontiers

Recently, survival benefit by transarterial embolization (TAE) has been reported. However, no definite recommendation has been issued regarding optimal treatment of HCC rupture, and the comparative survival benefits of surgery and TAE remain unclear.

Innovations and breakthroughs

The present study suggests that the post-treatment outcomes of surgery or TAE are better than that of supportive care in patients with spontaneous HCC rupture, and that surgical hemostasis might provide better survival benefit than TAE.

Applications

This study may provide useful information for clinicians to determine the most appropriate treatment option for spontaneously ruptured HCC.

Peer review

It is a rare situation specially for Western Europe, so it is good to know the experience of the group.

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BRIEF ARTICLE

Different regional distribution of *SLC25A13* mutations in Chinese patients with neonatal intrahepatic cholestasis

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Abstract

AIM: To investigate the differences in the mutation spectra of the *SLC25A13* gene mutations from specific regions of China.

METHODS: Genetic analyses of *SLC25A13* mutations were performed in 535 patients with neonatal intrahepatic cholestasis from our center over eight years. Unrelated infants with at least one mutant allele were enrolled to calculate the proportion of *SLC25A13* mutations in different regions of China. The boundary between northern and southern China was drawn at the historical border of the Yangtze River.

RESULTS: A total of 63 unrelated patients (about 11% of cases with intrahepatic cholestasis) from 16 provinces or municipalities in China had mutations in the *SLC25A13* gene, of these 16 (25%) were homozygotes, 28 (44%) were compound heterozygotes and 19 (30%) were heterozygotes. In addition to four well described common mutations (c.851_854del, c.1638_1660dup23, c.615+5G>A and c.1750+72_1751-4dup17insNM_138459.3:2667 also known as IVS16ins3kb), 13 other mutation types were identified, including three novel mutations: c.985_986insT, c.287T>C and c.1349A>G. According to the geographical division criteria, 60 mutant alleles were identified in patients from the southern areas of China, 43 alleles were identified in patients from the border, and 4 alleles were identified in patients from the northern areas of China. The proportion of four common mutations was higher in south region (56/60, 93%) than that in the border region (34/43, 79%, χ^2 = 4.621, P = 0.032) and the northern region (2/4, 50%, χ^2 = 8.288, P = 0.041).

CONCLUSION: The *SLC25A13* mutation spectra among the three regions of China were different, providing a basis for the improvement of diagnostic strategies and interpretation of genetic diagnosis.

Key words: Citrin deficiency; Mutation spectrum; Intrahepatic cholestasis; *SLC25A13*

Core tip: Genetic testing of *SLC25A13* gene was performed in individuals from southern, border and northern regions of China. The proportion of four common mutations was significant higher in southern region than in the border region and the northern region, so mutation screening for the common 4 mutations an appropriate test in the southern region. In the border and northern region, DNA sequencing is probably more practical.

Chen R, Wang XH, Fu HY, Zhang SR, Abudouxikuer K, Saheki T, Wang JS. Different regional distribution of *SLC25A13* mutations in Chinese patients with neonatal intrahepatic cholestasis. *World J Gastroenterol* 2013; 19(28): 4545-4551 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i28/4545.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i28.4545

INTRODUCTION

Citrin deficiency is estimated to be the most common urea cycle disorder in the world. It is an autosomal recessive disorder which includes adult-onset type Ⅱ citrullinemia (CTLN2; OMIM #60347)[1,2] and neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD; OMIM #605814)[3-5]. Most NICCD patients show symptoms which ameliorate by 1 year of age[6], but some patients may progress to liver failure and even require liver transplantation during infancy[7-10]. Others may develop CTLN2 more than a decade later[11,12]. Dietary treatment has shown to ameliorate symptoms and may prevent the need for transplant[13,14]. Therefore, prompt diagnosis and appropriate management are important for achieving a favorable long term prognosis for this disease.

Citrin deficiency is caused by mutations in the *SLC25A13* gene[1,15]. The protein product of the *SLC25A13* gene is citrin, a polypeptide of 675 amino acid residues with a molecular weight of 74 kDa. Citrin contains four EF-hand domains and six mitochondrial transmermbrane (TM)-spanning domains, and resides in the mitochondrial inner membrane[1]. Citrin is expressed in the liver and functions as calcium (Ca^{2+})-stimulated aspartate-glutamate carrier (AGC) for cytosolic glutamate and protons[16]. Over 60 different functional proved mutations in the human *SLC25A13* gene have been identified. These show significant differences in their racial distribution[13,17-20]. In China, the carrier frequency of 4 most common known mutations shows significant regional difference[19,20]. The estimated carries in population are 1/48 in south of the Yangtze River and 1/940 are carries of the river in the North[19].

Currently common mutation screening is used for rapid molecular diagnosis[21]. However, the appropriateness of use in specific populations needs to be established in that population. Therefore, we undertook the present study to investigate the regional distribution of *SLC25A13* mutations spectrum in Chinese patients with neonatal intrahepatic cholestasis. Our results will facilitate the design of appropriate screening strategies for this disorder in different regions.

MATERIALS AND METHODS

Subjects

Between June 2003 and December 2011, patients with cholestasis who were referred to the pediatric liver center of Children's Hospital of Fudan University for conjugated hyperbilirubinemia were enrolled. The inclusion criteria included the onset of conjugated jaundice before 6 mo of age; serum total bilirubin < 5 mg/dL and conjugated bilirubin > 1 mg/dL, or total bilirubin > 5 mg/dL and conjugated bilirubin > 20%[22]. We excluded other diseases that may affect the extrahepatic biliary system, such as biliary atresia, choledochal cyst, tumor, inspissated bile, or hemangioma, by imaging the hepatobiliary system. The imaging procedures included ultrasound scanning and hepatobiliary iminodiacetic acid (HIDA) scintigraphy in each case and laparotomic cholangiography in selected cases. Cases (n = 535) met the inclusion criteria and written informed consent was obtained from their parents.

The study protocol conforms to the ethical guidelines of the Declaration of the Helsinki of 1975 and was approved by the Ethics Committee on human research of the Children's Hospital of Fudan University.

Mutation identification

DNA was extracted from peripheral blood samples, which were obtained from each participant and his or her parents using the Tiangen Blood Genomic DNA Isolation Kit according to the manufacturer's instructions (Tiangen Biotech, Shanghai, China). Four common mutations (c.851_854del, c.1638_1660dup23, c.615+5G>A and c.1750+72_1751-4dup17insNM_138459.3:2667 also known as IVS16ins3kb)[1,20,23] were screened in all subjects. In patients for whom only one mutation was identified by the above screening or who had hyperaminoacidemia were subject to DNA sequencing as described previously[24]. Selection process of patients with mutant allele for analysis is given in Figure 1. The mutation alleles were verified in their parents by the target sequencing to establish segregation. Genomic sequences were obtained at the National Center for Biotechnology Information with RefSeq NM_014251.2 as *SLC25A13* reference. Nomenclature of *SLC25A13* variants was assigned following the guidelines of Human Genome Variation Society (http://www.hgvs.org/mutnomen)[25].

Geographical division

The population boundary between northern and southern China is drawn at the historical border of the Yangtze River during early Neolithic times (3000-7000 years ago)[26]. According to this criteria, Zhejiang, Jiangxi, Fujian, Guangdong, Hunan, Guizhou, Taiwan are classified as southern areas as they are south of Yangtze River; the provinces of Jiangsu, Shanghai, Anhui, Hubei, Sichuan and Chongqing are classified as border areas as they are in the basin of the Yangtze River; and the provinces of Henan, Liaoning, Shanxi, Jilin, Shandong, Hebei and Ningxia are classified as northern areas as they are in north of Yangtze River (Figure 2).

Patients with at least one mutated *SLC25A13* allele were selected. To calculate the mutation spectra, the mutations observed in the related family members were counted only once. If the patient was a heterozygote or a compound heterozygote and the parents were from

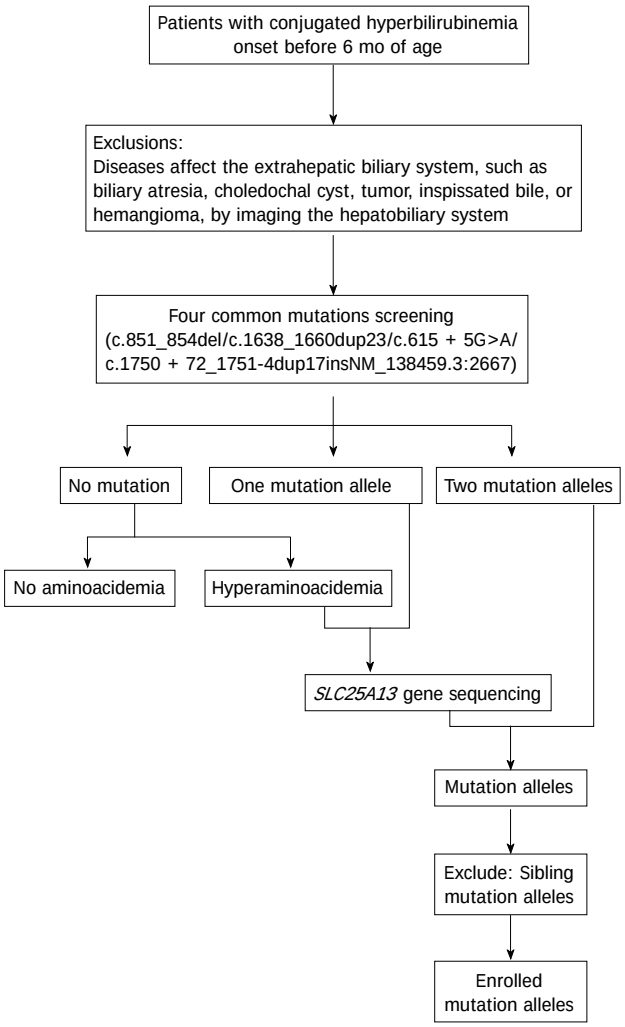

Figure 1 Selection process of patients with mutant allele for analysis.

different regions, the parent's sample was tested to determine the origin of the allele(s). Except for two patients who were born from consanguineous parents, all other infants were to our knowledge unrelated.

Homology and structural predictions

The homology between the mutated Citrin protein and the human reference, as well as Citrin from other species, were surveyed using Clustal X software (European Bioinformatics Institute, Hinxton, Saffron Walde, United kingdom). PolyPhen-2 (Polymorphism Phenotyping version2.2.2), which is available at http://genetics.bwh.harvard.edu/pph2/, was used to predict the possible impact of an amino acid substitution on the structure and function of the Citrin protein. MutationTaster was used to evaluate the disease-causing potential of sequence alterations, at http://mutationtaster.org/MutationTaster/index.html. A *P* value close to 1 indicates a high 'security' of the prediction. MutationTaster employs a Bayes classifier to eventually predict the potential of an alteration causing disease. The Bayes classifier is fed with the outcome of all tests and the features of the alterations and calculates probabilities for the alteration to be either disease causing or not.

Statistical analysis

Statistical tests on the distribution of mutant genotypes in the three areas of China were assessed by performing a 2×2 χ^2 test with the SPSS version 17.0 software (University of Chicago, Chicago, IL, United States) package. A P value < 0.05 was considered to be statistically significant. When there are small expected values in the 2×2 table, the result of fisher's exact test was used.

RESULTS

General information

Among the 535 patients, 183 originated from the southern area, 291 were from the border area and 61 were from the northern area. Sixty-nine patients with at least one *SLC25A13* gene mutation were found, including 6 sibling pairs. These sixty-three unrelated patients, including 25 females and 38 males, were further analyzed. Sixteen (25%) were found to be homozygotes for one mutation, 28 (44%) were compound heterozygotes and 19 (30%) heterozygotes for only one mutation. The distribution of carriers according to the state of origin is depicted in Figure 2.

Mutation types

A total of 17 mutations, including 14 mutations that had been previously reported by us and others (c.851_854del, c.1638_1660dup23, c.615+5G>A, c.1750+72_1751-4dup17insNM138459.3:2667, c.1019_1177del, c.1801G>A, c.550C>T, c.1078C>T, c.955C>T, c.1754G>A, c.775C>T, c.1092_5delT, c.615+1G>A, c.254T>C)[17,20,24,27-30] and 3 novel mutations (c.985_986insT, c.287T>C, c.1349A>G),were observed in the present investigation (Table 1).

Analysis of 3 previously unreported variants

The c.287T>C mutation in exon 4 is predicted to result in the substitute of phenylalanine to serine at position 96 (p.F96S). This mutation was found in a compound heterozygote state with the mutations c.851_854del. p.F96S is located between the second and third EF-hand domain, which is highly conserved in different species (Table 2). The Polymorphism Phenotyping for the variant amino acid p.F96S from Polyphen 2 is 1.000, indicating that the missense mutation has a high chance of affecting protein function. The *P* value from MutationTaster is 0.997, suggesting that is most likely a disease-causing mutation.

Mutations c.985_986insT and c.1349A>G were found in compound heterozygote state in a patient. The mutation c.985_986insT was found to be derived from this patient's paternal allele and predicted to result in a frame shift and the introduction of a premature stop codon at position 372. Mutation c.1349A>G (p.E450G) was derived from the patient's maternal allele, which is located in the loop between the TM3 and TM4 spanning regions. Conservation analysis in different species indicated that the amino acid in this position is highly conserved (Table 2). The Polymorphism Phenotyping for the variant amino

Figure 2 Distribution of mutant alleles enrolled in this study. As shown in the map, the provinces were separated into three parts by the Yangtze River. The numbers in parentheses are the number of mutation c.851_854del/c.1638_1660dup23/c.615+5G>A/c. 1750+72_1751-4dup17ins NM_138459.3:2667/other alleles.

Table 1 Regional distribution of mutant *SLC25A13* alleles and frequencies in China *n* (%)

Mutation	South	Border	North	Nucleotide change	Protein change	Ref.
Common	56 (93)	34 (79)[1]	2 (50)[2]			
851del4	41 (68)	22 (51)	2 (50)	c.851_854del	p.M285fsX286	Kobayashi *et al*[1]
1638ins23	9 (15)	4 (9)		c.1638_1660dup23	p.A554fsX570	Kobayashi *et al*[1]
IVS6+5G>A	2 (3)	4 (9)		c.615 + 5G>A	-	Saheki *et al*[23]
IVS16ins3kb	4 (7)	4 (9)		c. 1750+72_1751-4dup17ins NM_138459.3:2667	p.A584fsX585	Tabata *et al*[20]
Other	4 (7)	9 (21)	2 (50)			
IVS11+1G>A			1 (25)	c.1019_1177del	-	Kobayashi *et al*[1]
E601K	1 (2)	1 (2)		c.1801G>A	p.E601K	Yamaguchi *et al*[17]
R184X		1 (2)		c.550C>T	p.R184X	Lu *et al*[19]
R360X			1 (25)	c.1078C>T	p.R360X	Tabata *et al*[20]
R319X		1 (2)		c.955C>T	p.R319X	Song *et al*[29]
IVS6+1G>A		2 (5)		c.615+1G>A	-	Fu *et al*[24]
L85P		1 (2)		c.254T>C	p.L85P	Fu *et al*[24]
R585H		1 (2)		c.1754G>A	p.R585H	Song *et al*[28]
1092_5delT	1 (2)			c.1092_5delT	p.R319X	Fu *et al*[24]
Q259X		1 (2)		c.775C>T	p.Q259X	Wen *et al*[30]
985insT	1 (2)			c.985_986insT	p.A329fsX372	Present study
F96S		1 (2)		c.287T>C	p.F96S	Present study
E450G	1 (2)			c.1349A>G	p.E450G	Present study

Allele counts for the mutations are given together with their relative frequencies expressed as percentage value (in brackets). Novel mutations found in this study are indicated by bold letters. Variation in allele proportion (counts) between the three regions: $^1\chi^2 = 4.621$, $P = 0.032$, common mutation in south *vs* in border; $^2\chi^2 = 8.288$, $P = 0.041$, common mutation in south *vs* in north. GenBank reference sequences were NT_079595 and NM_014251.2.

acid p.E450G was 1.000, indicating a high chance of affecting protein function. The *P* value from MutationTaster is more than 0.999, suggesting that the mutation might affect the protein's features.

Distribution of SLC25A13 gene mutations

The distribution of *SLC25A13* mutations in carriers originating from different regions of China is given in Figure 1. Sixty (56%) mutant alleles originated from the southern region with Zhejiang, Jiangxi, Fujian, Guangdong, Hunan, Guizhou, and Taiwan accounting for 27, 15, 9, 1, 4, 3 and 1 mutant allele, respectively. Forty-three mutant alleles (40%) originated from the border region, with Jiangsu, Shanghai, Anhui, Hubei, Sichuan, and

Table 2 Conservation analysis of *SLC25A13* gene mutations F96S and E450G among different species

F96S																						
Human	L	C	A	P	D	A	L	F	M	V	A	F	Q	L	F	D	K	A	G	K	G	E	V
Canis	L	C	A	P	D	A	L	F	M	V	A	F	Q	L	F	D	K	A	G	K	G	E	V
Bos	L	C	A	P	D	A	L	F	M	V	A	F	Q	L	F	D	K	A	G	K	G	E	V
Equus	L	C	A	P	D	A	L	F	M	V	A	F	Q	L	F	D	K	A	G	K	G	E	V
Pan	L	C	A	P	D	A	L	F	M	V	A	F	Q	L	F	D	K	A	G	K	G	E	V
Mus	L	C	A	P	D	A	L	F	M	V	A	F	Q	L	F	D	K	A	G	K	G	E	V
Gallus	L	C	A	P	D	A	L	F	M	V	A	F	Q	L	F	D	K	A	G	K	G	E	V
Xenopus	L	C	A	P	D	A	L	F	M	V	A	F	Q	L	F	D	K	A	G	K	G	E	V
E450G																							
Human	G	G	S	Q	V	I	F	T	N	P	L	E	I	V	K	I	R	L	Q	V	A	G	E
Canis	G	G	S	Q	V	I	F	T	N	P	L	E	I	V	K	I	R	L	Q	V	A	G	E
Bos	G	G	S	Q	V	I	F	T	N	P	L	E	I	V	K	I	R	L	Q	V	A	G	E
Equus	G	G	S	Q	V	I	F	T	N	P	L	E	I	V	K	I	R	L	Q	V	A	G	E
Pan	G	G	S	Q	V	I	F	T	N	P	L	E	I	V	K	I	R	L	Q	V	A	G	E
Mus	G	G	S	Q	V	I	F	T	N	P	L	E	I	V	K	I	R	L	Q	V	A	G	E
Gallus	G	M	C	Q	V	V	F	T	N	P	L	E	I	V	K	I	R	L	Q	T	A	G	E

Chongqing accounting for 14, 6, 12, 4, 5 and 2, respectively. In the northern region, only four mutant alleles (4%) were found, including 1 from Henan, 1 from Liaoning and 2 from Shanxi provinces.

The *SLC25A13* mutation spectra among the three regions of China presented significant differences. The four common mutations exhibited maximal relative frequencies in the southern China (56/60, 93%), and four other mutations were detected, including two novel mutations. On the contrary, in the area bordering the Yangtze River, there was a wide range of mutation types. The four common mutations accounted for 79% (34/43) of the mutant alleles, and eight other mutation types were found, including one novel mutation. In northern China the mutation c.851_854del accounted for 50% (2/4) of the mutant alleles. The other two mutations were c.1019_1177del and c.1078C>T. The proportion of the four common mutations was higher in south region (93%) than that in the border region (79%, χ^2 = 4.621, P = 0.032) and that in the north (50%, χ^2 = 8.288, P = 0.041) (Table 1).

Among the four common mutations, c.851_854del was the most. Proportion accounts for 68% (41/60) in southern region, 51% (22/43) in the border region and 50% (2/4) in the northern region. The difference of the proportion between the southern and border part of China was marginally significant (χ^2 = 3.109, P = 0.078).

DISCUSSION

The mutation spectra for the *SLC25A13* gene differ within the Asian population[18-20]. A different carrier rate for the common mutations between different parts of China has been reported[19,30]. Here we demonstrate that the mutation spectrum of *SLC25A13* gene varies considerably among specific regions of China with common mutations having a higher proportion in the southern region than in the border and northern regions.

In the southern region of China, four common mutations accounted for 93% and c.851_854del is the predominant mutation accounting for 68%. This is consistent with the published data[19,30]. The c.851_854del mutation is a common ancestral mutation, and the frequency difference between various regions of China may be associated with ancient migration.

The mutations found in patients from the border region exhibited significant variety. The total mutant allele number in patients from the border was less than that in patients from the southern region (43 *vs* 60), but the mutation types were much greater than that in the southern area (12 *vs* 8). In total, seven private mutations were found in patients from the border, compared with three private mutations found in patients from the southern region. This divergence may reflect the ethnic diversity of this area. Previously, the data on *SLC25A13* mutations in this region were very limited. This study is the first paper providing an estimate for the border region and significantly increases the data on southern and northern China. Considering the high proportion of uncommon mutations, the previously reported *SLC25A13* mutation carrier rate in this region may be underestimated and sequencing may be a prefect method for testing.

The c.851_854del mutation was the only one of the four common mutations detected in the northern region. The explanation for this may be that other common mutations are rare in that part of China. Two mutant c.851_854del alleles were found among the 4 known mutant alleles, suggesting mutation 851del4 is the frequent mutation in this region.

Since variants c.287T>C and c.1349A>G have not undergone functional testing, so we analyzed the data without these and statistical significance was reached even when those two variants were removed from the analysis. The proportion of the four common mutations was also higher in southern region (95%) than the border (81%, χ^2 = 4.929, P = 0.048) and the northern region (50%, χ^2 = 10.343, P = 0.029). Thus the primary conclusion remains valid.

This paper is the first study conducted the *SLC25A13* mutation spectrum in neonatal intrahepatic cholestasis

from different parts of China. The previous study evaluated the population frequency for the common mutations and conducted that the carrier frequency in China is 1/79-1/65[17,19]. Conversely only 94% (59/63) of cases with suspected citrin deficiency in our study had the common mutations. This suggests that point mutation testing alone is not sufficient to exclude citrin deficiency even in cases from the southern region but may be a cost effective way of confirming the diagnosis as the first step.

There were limitations of this study. Firstly, only a small number of patients came from the north of the Yangtze River, and only limited cases were reported from that area, so the sampling bias needs to be considered. The current literature has not shown a significant difference between *SLC25A13* mutation types and the phenotype observed, so the smaller sample size is not likely to lead to referral bias in favor of null or missense mutations in this study. Secondly, for 19 of the 126 alleles, we could not find any mutations. One possible explanation could be that the patients with one detected mutant allele are carriers and this may be a risk factor for cholestasis or they may have an alternate cause for cholestasis. Alternatively, as previously described[31,32] they may have a second mutation not detected by Sanger sequencing or the targeted test for the IVS16ins3kb rearrangement such as intronic mutations or large rearrangements.

In conclusion, the mutation spectra of the *SLC25A13* gene are significantly different among patients with neonatal intrahepatic cholestasis from different parts of China. These differences should be considered when establishing a molecular diagnostic strategy or interpreting their results.

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COMMENTS

Background

SLC25A13 gene mutations lead to Citrin deficiency, which includes adult-onset type Ⅱ citrullinemia and neonatal intrahepatic cholestasis caused by citrin deficiency. The carrier frequency is high in Asian populations, and the mutation spectrum of *SLC25A13* gene in Chinese population (most came from southern China) was found to be different from that of other population groups in East Asia.

Research frontiers

The mutant alleles of *SLC25A13* gene was conducted in southern, border and northern regions of China, providing a basis for the improvement of diagnostic strategies and interpretation of genetic diagnosis.

Innovations and breakthroughs

The proportion of four common mutations was higher in the southern region (56/60, 93%) than that in the border region (34/43, 79%, χ^2 = 4.621, P = 0.032) and the northern region (2/4, 50%, χ^2 = 8.288, P = 0.041). Three novel mutations were found, which has expanded the *SLC25A13* mutation spectrum.

Applications

The mutation spectra of the *SLC25A13* gene are significantly different among patients with neonatal intrahepatic cholestasis from different parts of China. These differences should be considered when establishing a molecular diagnostic strategy or interpreting their results.

Terminology

An allele is a single copy of a gene. For autosomal genes, an individual inherits two alleles at each locus, with one from each parent. Genotypes are described as homozygous if the two alleles are the same and as heterozygous if the alleles are different. The mutant allele is the mutated form of a gene.

Peer review

This is a retrospective study aimed at investigating the regional distribution of *SLC25A13* mutations in Chinese patients with neonatal intrahepatic cholestasis. The topic is relevant, since biochemical diagnosis of citrin deficiency is not widely available and mutation analysis of the *SLC25A13* gene is crucial to diagnosis. The study was well-conducted and the manuscript is reasonably well written with good scientific value.

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BRIEF ARTICLE

Efficacy of capecitabine and oxaliplatin regimen for extrahepatic metastasis of hepatocellular carcinoma following local treatments

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Abstract

AIM: To investigate the efficacy and safety of capecitabine and oxaliplatin (CapeOx) for extrahepatic metastasis after local treatment of hepatocellular carcinoma (HCC).

METHODS: Thirty-two patients with extrahepatic metastasis of HCC after local treatment were prospectively enrolled. The CapeOx regimen consisted of capecitabine 1000 mg/m^2 taken orally twice daily on days 1-14, and oxaliplatin was administered at a total dose of 100 mg/m^2 on day 1. The treatment was repeated every 3 wk until disease progression or unacceptablle toxicity. Efficacy and safety were assessable for all enrolled patients. The primary objective of this study was to assess the overall response rate. The secondary objectives were to evaluate the overall survival (OS), the time to tumor progression (TTP) and the toxicity profile of the combined strategy. TTP and OS were assessed by the Kaplan-Meier method and differences between the curves were analyzed using the log-rank test. The statistical software SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL, United States) was used for statistical analysis. All *P* values were 2-tailed, with statistical significance defined by $P \leqslant 0.05$.

RESULTS: Thirty-two patients were assessable for efficacy and toxicity. The median follow-up duration was 15 mo (range, 12-20 mo). At the cut-off date of March 31, 2012, 27 patients died due to tumor progression and one patient died of myocardial infarction. Four patients were still alive (three patients with disease progression). OR was 21.9% (n = 7), the stabilization rate was 40.6% (n = 13), and the disease control rate was 62.5%. The responses lasted from 4 to 19 mo (median, 6 mo). Median TTP was 4.2 mo (95%CI: 2.5-7.4), and the median OS time was 9.2 mo (95%CI: 6.5-17.8). The 1-year survival rate was 43.6% (95%CI: 29.0-66.0). In a multivariate analysis, OS was significantly longer in patients with a Child-Pugh class A compared with class B patients (P = 0.014), with a median OS of 10.1 mo *vs* 5.4 mo, and there were trends towards longer OS (P = 0.065) in patients without portal vein tumor thrombosis. There were no significant effects of age, gender, performance status, cirrhosis, metastatic sites, and level of alpha fetoprotein (AFP) or hepatitis B virus-DNA on OS. Among the 22 patients with elevated AFP levels at baseline ($\geqslant$ 400 ng/mL), the level fell by more than 50% during treatment in 6 patients (27.3%). The most frequent treatment-related grade 3 to 4 toxicities included leucopenia/neutropenia, transient elevation of aminotransferases, hand-foot syndrome and fatigue.

CONCLUSION: CapeOx showed modest anti-tumor activity in metastatic HCC. However, the manageable

toxicity profile and the encouraging disease control rate deserve further study for these patients.

Key words: Hepatocellular carcinoma; Extrahepatic metastasis; Capecitabine; Oxaliplatin; Local treatments

Core tip: Distant metastases are still obstacles in improvement of outcome in hepatocellular carcinoma (HCC) patients after local treatment. Although, sorafenib is used as a standard systemic treatment for those patients, it is not suitable for patients with intermediate HCC who were not eligible to or failed in the locoregional therapy. This study reports the capecitabine and oxaliplatin regimen for extrahepatic metastasis after local treatment of HCC. The objective response rate was 21.9%, and 40.6% of patients had stable disease, and the median overall survival and the time to tumor progression were 4.2 and 9.2 mo, respectively. Furthermore, the result of this study showed that toxicity profile was tolerated well.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths worldwide, with the incidence on the rise[1]. The overall 5-year survival rate for all HCC patients has remained no more than 5%[2]. Surgical resection, local ablation, transarterial chemoembolization (TACE) and liver transplantation are the mainstay of treatment of localized HCC, but local recurrence and distant metastasis are still obstacles in the further improvement of outcome in HCC patients after local treatments. Sorafenib, a small molecule multikinase inhibitor, was the first systemic agent used to prolong survival of patients with advanced HCC, as demonstrated in two phase Ⅲ trials and it is now the reference standard for systemic treatment of these patients[3,4]. However, its efficacy and safety have not been demonstrated in patients with poor liver function (Child-Pugh class B)[5]. Moreover, patients with extrahepatic metastasis had a greater risk of death than those with intrahepatic disease treated by sorafenib[6]. Systemic treatment with oral targeted therapy may be lifelong and expensive. In addition, sorafenib is not covered in the scope of health insurance for advanced HCC in China. Therefore, systemic treatment options remain to be defined in patients with extrahepatic metastasis of HCC after local treatments.

Capecitabine is a rationally designed, orally administered, tumor-selective fluoropyrimidine that mimics continuous infusion of 5-fluorouracil (5-FU). Capecitabine was found to be safe in patients with cirrhosis and provided an 11% response rate (RR) including radiologically confirmed complete response (CR) in one patient[7]. Oxaliplatin has consistently shown preclinical and clinical anti-tumor activity against gastrointestinal cancers. In metastatic colorectal cancer, oxaliplatin in combination with 5-FU resulted in response rates of 20%-50% and median progression-free survival (PFS) of approximately 7.5-9.0 mo in randomized trials[8]. A phase Ⅲ study of 5-FU/oxaliplatin conducted in Asian patients suffering from inoperable or metastatic HCC showed the feasibility and demonstrated its superior efficacy compared with doxorubicin[9].

Response evaluation for intrahepatic lesions in patients with advanced HCC is difficult because of variability of both tumor growth pattern and results of previous local treatments including TACE, ablation or radiation therapy[10]. Therefore, this study selected advanced HCC patients with at least one measurable extrahepatic metastatic lesion. The regimen of capecitabine and oxaliplatin (CapeOx) for patients with extrahepatic metastatic HCC was based on (1) the synergy of these two drugs in patients with advanced or metastatic solid tumors[11]; (2) the regimen of oxaliplatin and 5-FU with a manageable toxicity profile in cirrhotic Child-Pugh class A-B or liver transplanted patients[12]; (3) the clinical activity and favorable toxicity profile of capecitabine alone and in combination with oxaliplatin in advanced or metastatic colorectal cancer[13,14]; (4) no dose adjustment required for capecitabine and oxaliplatin due to hepatic dysfunction[15]; and (5) the feasibility and efficacy of CapeOx alone or in combination with antiretroviral therapy in patients with human immunodeficiency virus- (and hepatitis C virus-co-) infection and HCC[16]. This study aims to evaluate the efficacy and safety of CapeOx regimen in patients with extrahepatic metastasis following local treatment.

MATERIALS AND METHODS

Patients

From March 2009 to March 2012, we enrolled 32 patients with extrahepatic metastasis. Eligibility criteria included the following: (1) initially received surgery, thermal ablation, TACE or TACE combined with radiotherapy; (2) at least one measurable extrahepatic lesion; (3) no previous systemic treatment; (4) World Health Organization (WHO) performance status (PS) 0-2; (5) Child-Pugh class of A or B; and (6) age between 18-70 years and adequate bone marrow, renal and hepatic function (absolute neutrophil count $\geqslant 1.5 \times 10^9$/L and platelet count $\geqslant 80 \times 10^9$/L; serum creatinine $\leqslant$ 1.5 mg/dL; aspartate aminotransferase and alanine aminotransferase $\leqslant 2.5 \times$ upper limits of normal; total bilirubin $\leqslant 1.5 \times$ upper limits of normal). Study entry required a complete medical history, physical examination, complete blood cell with a differential count, biochemistry panel, and a coagulation panel

and serum alpha-fetoprotein (AFP), chest or abdominal computed tomography (CT) scan or magnetic resonance imaging (MRI). Main exclusion criteria were Child-Pugh class C, previous systemic treatment, central nervous system metastases, severe cardiac and/or respiratory failure, concurrent malignancy, and baseline sensitive peripheral neuropathy; pregnant or lactating females. This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. The study was approved by the local ethics committee. Informed consent was obtained from all participants.

Before registration, complete blood cell and platelet counts were examined weekly, and physical examination, biology [serum alpha-fetoprotein (AFP), transaminases, alkaline phosphatases, bilirubin, lactate dehydrogenase, γ-glutamyltransferase, albumin, prothrombin time, and creatinine], and safety assessments were performed before each cycle of chemotherapy. Analysis of AFP level and tumor assessment by CT scan or MRI were undertaken every two cycles. Objective response (OR) was confirmed by a second evaluation 4 wk later. Objective and discordant responses were reviewed by an independent radiologist. Treatment was discontinued because of either disease progression and unacceptable toxicity, or patient's refusal. Other treatments were proposed in the event of disease progression.

Treatment protocol

CapeOx regimen was administered in a 3-wk cycle. In each cycle, oxaliplatin (ELOXATIN®, Sanofi-Aventis, Hangzhou, China) was administered at a total dose of 100 mg/m^2 as a 2-h *iv* infusion on day 1, and capecitabine (XELODA®, Shanghai Roche Shanghai, China) 1000 mg/m^2 was taken orally twice daily (total daily dose 2000 mg/m^2) on days 1-14. Hepatitis B surface antigen positive patients were treated with lamivudine (HEPTODIN®, GlaxoSmithKline, Suzhou, China) 100 mg/d before the first CapeOx cycle to prevent severe hepatitis during treatment. All patients with bone metastases received bisphosphonates treatment once a month. Depending on the severity of side effects, chemotherapy was paused or the dose was reduced. A 20% dose reduction was required based on predefined criteria. Briefly, capecitabine dose was reduced by 20% due to recurrence of grade 3 or 4 diarrhea or hand/foot syndrome. Oxaliplatin dose was reduced by 20% in case of grade 1 or 2 peripheral neuropathy, whereas in case of grade 3 or 4 neuropathy (defined as permanent functioning discomfort), oxaliplatin was discontinued and capecitabine was administered alone as initially scheduled. Patients were considered assessable for toxicity if they had received a minimum of one cycle of treatment.

Assessment of responses

Baseline evaluation included physical examination, assessment of medical history, evaluation of performance status, and blood counts. During treatment, patients were evaluated before each cycle of therapy with the above parameters. Response was assessed after every two cycles of chemotherapy by CT scan or MRI using the Response Evaluation Criteria in Solid Tumors 1.1 (RECIST 1.1) criteria[17]. CR was defined as the disappearance of all target and non-target lesions compared to baseline. Partial response (PR) was defined as at least a 30% decrease in the longest diameters of all target lesions, taking as a reference the baseline sum of the diameters with no new lesions appearing. Patients were considered to have progressive disease (PD) if any new lesions appeared, if the tumor size increased by at least 20% in the diameters of the target lesions, taking as reference the smallest sum on study, or if there was unequivocal progression of existing non-target lesions. A patient who did not meet the definition of CR, PR or PD was classified as having stable disease. The percentage of patients who had the best responses (other than PD) according to the RECIST 1.1 criteria, and had those responses maintained for at least 28 d after the first radiologic evaluation, was defined as the disease control rate (DCR). AFP and hepatitis B virus (HBV)-DNA levels were determined every 2 mo. Body weight, PS, and symptoms were recorded before each cycle. Toxic effects of chemotherapy were evaluated according to the National Cancer Institute-Common Terminology Criteria for Adverse Events version 3.0. This specific scale was used to assess oxaliplatin neurotoxicity[18].

Statistical analysis

The primary objective of this study was to assess the overall response rate. The secondary objectives were to evaluate the overall survival (OS), the time to tumor progression (TTP) and the toxicity profile of the combined strategy. TTP was the interval from the starting date of therapy to the date of progression; OS was defined as the time interval between the first cycle of chemotherapy and death due to any cause or the last clinical follow-up. TTP and OS were assessed by the Kaplan-Meier method and differences between the curves were analyzed using the log-rank test. For the statistical analysis, the statistical software SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL, United States) was used. All P values were 2-tailed, with statistical significance defined by $P \leqslant 0.05$.

RESULTS

Thirty-two patients (21 men and 11 women) were enrolled between March 2009 and March 2012. Median age of the patients was 59 years (range 19-70 years). Chronic HBV infection was the most common etiology of underlying liver disease (23 patients, 71.9%). Two patients (6.3%) had a history of alcohol abuse. Twenty-two (68.8%) patients belonged to Child-Pugh class A and 10 (31.2%) to Child-Pugh class B. Cirrhosis was present in 12 patients (37.5%), and 22 patients (68.8%) had serum AFP (≥ 400 ng/mL). Four patients received curative HCC resection and 19 patients were treated with TACE, 4 patients were treated by TACE combined with radiotherapy after diagnosis. Five patients underwent ablation of HCC. Patients' other baseline characteristics are summarized in Table 1.

Table 1 Patient and tumor characteristics at baseline (n = 32)

Characteristics	Patients, n (%)
Age (yr), median (range)	56 (19-70)
Gender	
Male	21 (65.6)
Female	11 (34.4)
ECOG performance status	
0	16 (50)
1	11 (34.4)
2	5 (15.6)
Underlying liver disease	
HBV	23 (71.9)
Alcohol	2 (6.3)
Other	7 (21.8)
Prior therapy	
Surgery	4 (12.5)
Ablation	5 (15.6)
TACE	19 (59.4)
TACE + radiotherapy	4 (12.5)
Child Pugh score	
A	22 (68.8)
B	10 (31.2)
Cirrhosis	
No	20 (62.5)
Yes	12 (37.5)
HBV-DNA	
< 1.0e3 cps/mL	16 (69.6)
⩾ 1.0e3 cps/mL	7 (30.4)
Portal vein thrombosis	
No	25 (78.1)
Yes	7 (21.9)
Median AFP (ng/mL)	
⩾ 400	22 (68.8)
< 400	10 (31.2)
Metastasis	
Lung	9 (28.1)
Bone	6 (18.8)
Adrenal gland	9 (28.1)
Lymph node	3 (9.4)
Peritoneum	3 (9.4)
Other	2 (6.2)

ECOG: Eastern Cooperation Oncology Group; HBV: Hepatitis B virus; TACE: Transarterial chemoembolization; AFP: Alpha fetoprotein.

In total, 142 cycles of CapeOx were administered, with a median of four cycles (range 1-9 cycles) per patient. Dose reductions including oxaliplatin in 9/32 patients (28.1%) were due to grade 1/2 toxicities, and capecitabine in 9/32 patients (28.1%) because of grade 3/4 toxicities. Oxaliplatin was discontinued in 2 patients with grade 3 neurotoxicity. Thirty-two patients were assessable for efficacy and toxicity. The median follow-up duration was 15 mo (range 12-20 mo). At the cut-off date of March 31, 2012, 27 patients died due to tumor progression and one patient died of myocardial infarction. Four patients were still alive (3 patients with disease progression). OR was 21.9% (n = 7), the stabilization rate was 40.6% (n = 13), and the DCR was 62.5%. The responses lasted 4-19 mo (median, 6 mo). Median TTP was 4.2 mo (95%CI: 2.5-7.4), and the median OS time was 9.2 mo (95%CI: 6.5-17.8; Figure 1A). The 1-year survival rate was 43.6% (95%CI: 29-66). In a multivariate analysis, OS was significantly longer in patients with a Child-Pugh

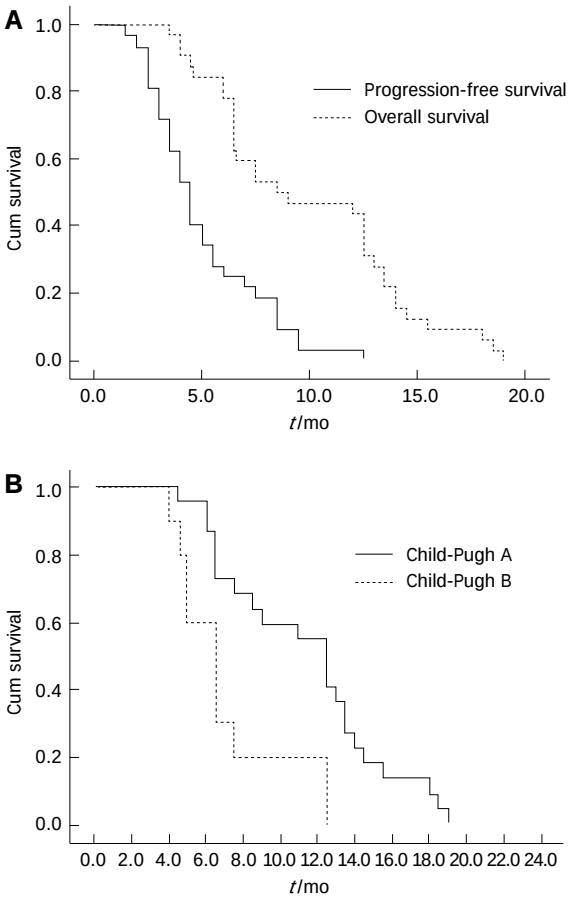

Figure 1 Kaplan-Meier estimation. A: Progression-free survival and overall survival (n = 32); B: Overall survival by Child-Pugh class group (n = 32).

class A compared with class B patients (P = 0.014), with a median OS of 10.1 mo *vs* 5.4 mo (Figure 1B), and there were trends towards longer OS (P = 0.065) in patients without portal vein tumor thrombosis. There were no significant effects of age, gender, PS, cirrhosis, metastatic sites, and level of AFP or HBV-DNA on OS (data not shown). Among the 22 patients with elevated AFP levels at baseline (⩾ 400 ng/mL), the level fell by more than 50% during therapy in 6 patients (27.3%). Moreover, 2 of the 5 patients whose initial PS was equal to 2, improved to 1 after two cycles of treatment. Three of 23 patients treated with lamivudine therapy switched to entecavir therapy because the level of HBV-DVA had exceeded the baseline level (⩾ 1.0e3 cps/mL) during treatment.

Safety

Toxicities are summarized in Table 2. Treatments were generally well tolerated in the majority of patients, and there were no treatment-related deaths. Thirty-two patients were assessable in toxicity. Grade 3-4 toxicity occurred in 11 patients (34.4%). Hematologic toxicity was the most common severe toxicity, including thrombocytopenia (6.3%; no bleeding events) and neutropenia (6.3%; fever in only one case). Grade 3 neurotoxicity was the most common severe non-hematologic toxicity, affecting 2 patients (6.39%), whereas grades 1 and 2 neurotoxicity

Table 2 Treatment-related toxicities in 32 patients

Adverse event	Grade 1	Grade 2	Grade 3	Grade 4
Neutropenia	2	2	1	1
Thrombocytopenia	4	3	1	1
Anemia	5	1	0	0
Nausea/vomiting	6	3	1	0
Mucositis	4	2	0	0
Stomatitis	3	2	1	0
Diarrhoea	0	1	1	0
Transaminases	7	3	1	0
Hyperbilirubinemia	2	0	0	0
Neurotoxicity	5	8	2	0
Hand-foot syndrome	4	2	1	0

Hepatitis B virus DNA level (real-time polymerase chain reaction, Abbott, Wiesbaden, Germany).

occurred in 6 (18.8%) and 3 (9.4%) patients, respectively.

Additional treatments

Nine patients had received additional treatments due to tumor progression. Six patients with bone metastasis received local palliative radiotherapy, and three patients received sorafenib therapy.

DISCUSSION

Sorafenib is currently considered standard of care systemic therapy for patients with advanced HCC. The use of sorafenib is based on phase Ⅱ and phase Ⅲ data in patients with metastatic HCC, with the treatment group showing close to a 3-mo survival advantage over the non-treated group in Child-Pugh class A[3,19]. In contrast, Child-Pugh class B patients did not seem to derive any benefit from sorafenib in phase Ⅲ trials[5,20]. Similarly, in a series of Asian patients, only patients with a score of B7 seemed to benefit from sorafenib, at the cost of higher rates of bleeding events[21]. National Institute for Health and Clinical Excellence does not recommend sorafenib for patients with advanced hepatocellular carcinoma, because it does not provide enough benefit to patients to justify its high cost[22]. In addition, the results of SOFIA study showed that only dose-adjusted, but not full-dose sorafenib was a cost-effective treatment compared to best supportive care in intermediate and advanced HCC. There was no cost-effective treatment for patients with intermediate HCC who were not eligible to or failed locoregional therapy even if they were treated with dose-adjusted sorafenib[23].

The survival rates of HCC patients have risen greatly concomitant with the progress in diagnostic and treatment methods. However, the survival prognosis for treatment-resistant progressive liver cancers is extremely poor[24]. Although surgical resection was used as treatment for pulmonary metastasis from HCC, the treatment might be only beneficial for patients with few than three lung lesions[25]. Chemotherapy used in combination with interferon is considered to be effective but lacks adequate scientific evidence[26].

From general point of view and in line with previous reports[12,27,28], CapeOx seems feasible and suitable for palliative care in patients with advanced HCC. With lack of renal toxicity of oxaliplatin[29], the low incidence of myelosuppression observed with capecitabine[30], the synergistic anti-tumor activity and safety of capecitabine and oxaliplatin combination in advanced HCC[31], and the absence of dose adjustment required for both agents in case of hepatic dysfunction, make the CapeOx regimen attractive in advanced HCC patients with cirrhosis or chronic HBV infection[15,32]. A multicenter, open-label, phase Ⅱ study of CapeOx reported a response rate of 6% and a disease control rate of 72%[33], however, patients who had not undertaken local therapies were eligible for this study. For patients with extrahepatic metastasis from HCC, systemic chemotherapy of carboplatin and 5-FU had demonstrated a statistically significant improvement in OS (10.7 mo *vs* 5.1 mo) in comparable patients with non-chemotherapy[34].

For these patients who had extrahepatic metastasis after local treatments and who had no significant alteration of their liver function, palliative chemotherapy can be delivered with tolerable toxicity[35]. Recently, research combining the use of CapeOx and cetuximab for advanced HCC reported an RR of 12.5%, TTP of 3.3 mo and overall survival of 4.4 mo[36]. Another phase Ⅱ trial of CapeOx with bevacizumab for advanced HCC in 2011 showed tumor response and disease control rates of 20% and 77.5%, respectively[31]. The median OS and PFS were 9.8 and 6.8 mo, respectively. In our study, although only the cytotoxic chemotherapy drugs oxaliplatin and capecitabine were used for patients with extrahepatic metastasis, the result was encouraging for both efficacy and toxicity. Partial response was seen in 21.9% patients, and 62.5% had their disease controlled. The study also showed a median TTP of 4.2 mo and a median OS of 9.2 mo in a patient population of 50% with Eastern Cooperation Oncology Group PS 1-2, and more than 31% of the patients with Child- Pugh class B disease status.

Obviously the underlying liver cirrhosis increases the risk of severe adverse events as many chemotherapeutic drugs are metabolized or eliminated *via* the liver. Moreover, severe complications might occur if a cytotoxicity-related side effect appears on a cirrhotic liver. Certain causes of the underlying cirrhosis, such as hepatitis B virus infection, may be reactivated after chemotherapy induced immunosuppression, producing an additive toxic effect[37].

In conclusion, palliative chemotherapy can be delivered to patients with extrahepatic metastasis from HCC following local treatments with tolerable toxicity. However, the efficacy was not satisfactory. More effective systemic chemotherapy regimens are needed for this subgroup of patients.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths worldwide. The overall 5-year survival rate for all HCC patients has remained no more than 5%. Sorafenib is the first agent to demonstrate a survival advantage over supportive care in HCC. Nevertheless, in a relatively fit group of sorafenib-treated patients (95% Childs-Pugh A), median survival was only 10.7 mo. The purpose of this study was to evaluate the safety and efficacy of the capecitabine-oxaliplatine combination (CapeOx) in patients with extrahepatic metastatic HCC following local treatments.

Research frontiers

Local recurrence and distant metastasis are still obstacles in further improvement of outcome in HCC patients after local treatments. Doxorubicin, until recently considered the standard chemotherapeutic for HCC, is associated with an objective response rate of approximately 10%. In this study, the authors aimed to valuate the efficacy and safety of CapeOx regimen in patients with extrahepatic metastasis from HCC following local treatments.

Innovations and breakthroughs

Sorafenib is currently considered standard of care systemic therapy for patients with advanced HCC, but results from recent several studies showed it not suitable in some patients with advanced HCC. In this study, CapeOx regimen showed modest anti-tumor activity in metastatic HCC and tolerated toxicities.

Applications

This study may represent a future strategy for therapeutic intervention in the treatment of patients with extrahepatic metastasis from HCC following local treatments even if with liver cirrhosis.

Terminology

Capecitabine is an orally administered, tumor-selective fluoropyrimidine that mimics continuous infusion of 5-fluorouracil. Oxaliplatin s a new platinum complex, diaminocyclohexane compound, which is thought to result from inhibition of DNA synthesis in cancer cells. The combination of oxaliplatin and capecitabine has also shown some promise in HCC because both drugs are tolerated in the setting of hepatic dysfunction.

Peer review

This study is an uncontrolled phase 2 evaluation of capecitabine and oxaliplatin for locally controlled HCC with extrahepatic metastases involving 32 patients, the majority of whom were hepatitis B virus infected and non-cirrhotic. The majority of patients' metastases were pulmonary or intra-abdominal with 6/32 being confined to bone. Twenty-eight percent of patients required dose reduction of capecitabine due to grade 3/4 toxicity but only 2 grade 3 oxaliplatin toxicities occurred. Ninety-seven percent of patients died or manifested tumor progression. Median time to tumor progression was 4.2 mo and median overall survival was 9.2 mo. This is an interesting prospective study on efficacy of CapeOx combination regimen for extrahepatic metastasis of HCC following local treatments, and gives a practical point of view in management of these patients.

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BRIEF ARTICLE

Surgical management of patients with bowel obstructions secondary to gastric cancer

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Abstract

AIM: To assess whole-body fluorodeoxyglucose (FDG) positron emission tomography/computed tomography (PET/CT) in the management of small bowel obstructions (SBOs) secondary to gastric cancer and its role in treatment strategies.

METHODS: The medical records of all of the patients who were admitted for an intestinal obstruction after curative resection for gastric cancer were retrospectively reviewed. PET/CT was performed before a clinical treatment strategy was established for each patient. The patients were divided into 2 groups: patients with no evidence of a tumor recurrence and patients with evidence of a tumor recurrence. Tumor recurrences included a local recurrence, peritoneal carcinomatosis or distant metastases. The primary endpoint was the 1-year survival rate, and other variables included patient demographics, the length of hospital stay, complications, and mortality.

RESULTS: The median time between a diagnosis of gastric cancer and the detection of a SBO was 1.4 years. Overall, 31 of 65 patients (47.7%) had evidence of a tumor recurrence on the PET/CT scan, which was the only factor that was associated with poor survival. Open and close surgery was the main type of surgical procedure reported for the patients with tumor recurrences. R0 resections were performed in 2 patients, including 1 who underwent combined adjacent organ resection. In the group with no evidence of a tumor recurrence on PET/CT, bowel resections were performed in 7 patients, adhesiolysis was performed in 7 patients, and a bypass was performed in 1 patient. The 1-year survival curves according to PET/CT evidence of a tumor recurrence *vs* no PET/CT evidence of a tumor recurrence were significantly different, and the 1-year survival rates were 8.8% *vs* 93.5%, respectively. There were no significant differences (P = 0.71) in the 1-year survival rates based on surgical *vs* nonsurgical management (0% with nonoperative treatment *vs* 20% after exploratory laparotomy).

CONCLUSION: ^{18}F-FDG PET/CT can be used to identify the causes of bowel obstructions in patients with a history of gastric cancer, and this method is useful for planning the surgical management of these patients.

Key words: Positron emission tomography/computed tomography; Small bowel obstructions; Gastric cancer; Clinical treatment strategy

Core tip: The management of patients who present with a small bowel obstruction (SBO) after treatment of primary carcinoma challenges the clinical judgement of

even the most experienced surgeons when the feared cause is metastatic disease. It is difficult to predict whether the quality and/or the quantity of life in this group of patients will be improved by surgery. This study evaluated the clinical role of ^{18}F-fluorodeoxyglucose (FDG) positron emission tomography/computed tomography (PET/CT) in identifying SBOs and its role in subsequent clinical treatment strategies. We found that ^{18}F-FDG PET/CT is an appropriate method to identity the causes of bowel obstructions secondary to gastric cancer, and this method is useful for the surgical management of these patients.

Wu WG, Dong P, Wu XS, Li ML, Ding QC, Zhang L, Yang JH, Weng H, Ding Q, Tan ZJ, Lu JH, Gu J, Liu YB. Surgical management of patients with bowel obstructions secondary to gastric cancer. *World J Gastroenterol* 2013; 19(28): 4559-4567 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i28/4559.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i28.4559

INTRODUCTION

An intestinal obstruction is a common problem in patients with an advanced malignancy. Approximately 3%-15% of all terminal cancer patients will develop an intestinal obstruction[1,2]. In advanced abdominal and pelvic malignancies, 5%-51% of patients with ovarian malignancies and 10%-28% of patients with gastrointestinal cancers will develop an intestinal obstruction[3-8]. An intestinal obstruction may be due to intra-abdominal adhesions, intra-abdominal hernias, local cancer recurrences, peritoneal carcinomatosis, or distant metastases from other tumors. Small bowel obstructions (SBOs) secondary to malignant disease are often a sign of end-stage disease and are associated with poor survival. The treatment of such patients presents a dilemma for the surgeon. Inappropriate surgery will not significantly improve morbidity and mortality outcomes and often has limited success in relieving symptoms. Nonoperative treatment is often ineffective at restoring bowel function, and when relief is obtained, early reobstruction frequently occurs[9,10]. The management of patients who present with a bowel obstruction after treatment of primary carcinoma challenges the clinical judgment of even the most experienced surgeons when the feared cause is metastatic or recurrent disease[11]. The management of these patients is difficult, and it is unclear which patients will benefit from surgery and which patients will have similar outcomes from medical management because many patients may have diffuse peritoneal metastatic disease and/or adhesions from previous surgery. It is difficult to predict whether the quality and/or the quantity of life in this group of patients will be improved by surgery because these patients have a poor prognosis at the time of presentation[12]. In addition, the management of these patients presents an additional difficulty because the intestinal obstruction may be due to more than one physiopathological process, such as an intraluminal obstruction from polypoid lesions that occlude the bowel lumen, an intramural obstruction from the infiltration of a tumor within the muscular coat of the bowel wall, and an extramural obstruction from mesenteric and omental masses and extrinsic compression from malignant adhesions.

Positron emission tomography (PET) with ^{18}F-fluorodeoxyglucose (FDG) detects the increased utilization of glucose by malignant cells to provide diagnostic information and is more accurate than conventional diagnostic methods in cases of primary and recurrent gastrointestinal tumors[12-15]. To date, the usefulness of integrated FDG PET/computed tomography (CT) in the treatment decisions for patients with bowel obstructions secondary to malignant disease has not been investigated. This study evaluated the clinical role of whole-body FDG PET/CT in the management of SBOs secondary to gastric cancer and its role in the formulation of subsequent clinical treatment strategies.

MATERIALS AND METHODS

Patients

This retrospective chart review was approved by the institutional review board and was performed at the Department of General Surgery, Xinhua Hospital, School of Medicine, Shanghai Jiaotong University. A retrospective review of our electronic database was conducted to find all patients with a history of curative resection for gastric cancer who were admitted for an intestinal obstruction from August 1, 2008 to January 1, 2010. Adult patients with discharge diagnoses of bowel obstructions and gastric cancer were enrolled. Patients whose cancer was first diagnosed with the bowel obstruction and patients without radiographic confirmation of an obstruction were excluded. Patients with an early postoperative bowel obstruction, which is generally defined as a mechanical obstruction that occurs within 1 mo of abdominal surgery, were excluded. No patient with a bowel obstruction before a cancer diagnosis was included in this study. The diagnosis of a bowel obstruction was based on a combination of clinical signs and symptoms and radiologic findings. The enrolled patients had at least one of the following symptoms along with radiographic confirmation of an obstruction: nausea and vomiting, colicky pain, abdominal bloating, obstipation, or an inability to tolerate PO intake. Radiographic confirmation of the obstruction was usually by either plain abdominal films or a CT scan. In addition, at least one of the following findings was required: dilated loops of bowel with a paucity of air in the colon, air/fluid levels, or a transition from a dilated bowel to a decompressed bowel[16]. PET/CT was performed for each patient before the clinical treatment strategy was established. Based on the PET/CT results, the patients were divided into 2 groups: patients with no evidence of a tumor recurrence and patients with evidence of a tumor recurrence, which included a local recurrence, peritoneal carcinomatosis or distant metastases.

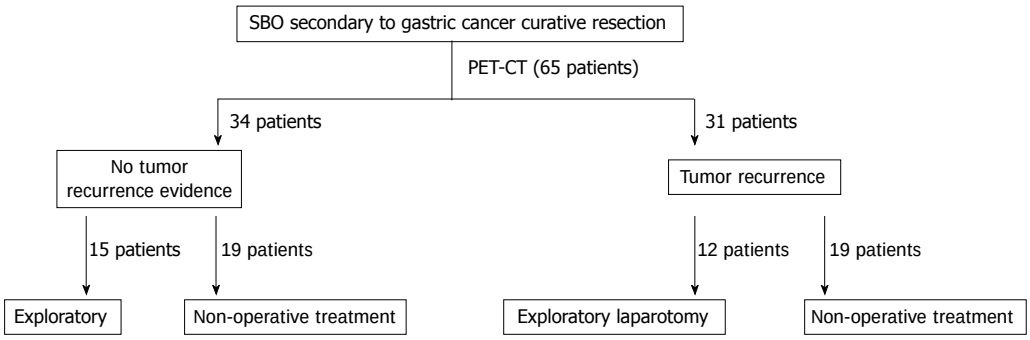

Figure 1 The treatment decision-making process. PET/CT: Positron emission tomography/computed tomography; SBO: Small-bowel obstruction.

PET/CT

PET/CT imaging was performed using a GE Discovery ST 8-slice scanner. The patients were scanned after 6 h of fasting. Blood glucose levels were checked immediately before the scan. An average of 296-370 MBq (*i.e.*, 8-10 mCi) FDG was injected intravenously, and whole-body images were obtained 1 h later. Low-dose CT images were used for attenuation correction. An oral contrast agent was administered to all of the patients for PET/CT imaging. A semiquantitative and visual analysis was made. The images were evaluated by 2 nuclear medicine specialists, and a consensus was required to prevent interobserver variability. The FDG uptake was defined as qualitatively positive when the focal FDG uptake was higher than the normal biodistribution of background FDG activity. In addition, to exclude the physiologic uptake, the FDG uptake in the bowel was considered positive only when wall thickening of the same bowel was simultaneously detected by CT. The PET/CT images were analyzed for the number and the sites with positive FDG uptake, and the standardized uptake value value of all of the positive FDG uptake values was measured.

Operative and non-operative management

The patients with an obstruction were divided into 2 treatment groups: patients who received conservative treatment and patients who underwent surgical management. The standard nonoperative management of small bowel obstructions consisted of fluid and electrolyte replacement, bowel rest, and tube decompression. A nonoperative course may be followed for 24-48 h. If the obstruction has not resolved within that time period, it is unlikely that the obstruction will ever resolve and laparotomy is usually advised. In the patients who underwent surgery for a bowel obstruction after curative resection of gastric cancer, the type of operation was determined by 3 expert gastrointestinal surgeons depending on the overall medical status of the patient, the wishes of the patient, and the abdominal examination. The primary endpoint of the analysis of surgical *vs* non-surgical treatment for the bowel obstructions in this study was the 1-year survival rate, and other recorded variables included patient demographics, the length of hospital stay, complications, and mortality. The modified Clavien system was used to grade any postoperative complications. In-hospital mortality was defined as the percentage of patients who died before hospital discharge. The length of hospital stay was defined as the number of days from the index procedure to hospital discharge. This study was approved by the Human Research Review Committee of our hospital.

Statistical analysis

Statistical analyses and graphics were generated using the SPSS 13.0 statistical package for Windows (SPSS, Inc., Chicago, IL, United States). If the P value was < 0.05, the results were considered statistically significant. Patency after palliation and the overall survival were estimated using the Kaplan-Meier actuarial method, and the curves were compared using the log-rank test. To identify the independent factors that influenced clinical success and the risk factors that were associated with the 1-year overall survival, univariate and multivariate analyses were performed. The results were expressed as the mean ± SD or as the percentages.

RESULTS

There were 72 cases of bowel obstructions in patients with a history of curative surgery for gastric cancer at our institution during the study period. Seven patients declined PET/CT imaging and were excluded from the analysis. The remaining 65 patients were all admitted for a SBO and were included in the analysis. The surgical decision-making process is shown in Figure 1. The average age at the time of the primary gastric cancer diagnosis was 62.5 ± 17.1 years. The mean age at admission for a SBO was 63.9 ± 15.6 years.

The median time between curative resection of gastric cancer and the detection of a SBO was 1.4 years. The clinicopathological data of the patients are listed in Table 1. Each patient underwent PET/CT before the final clinical treatment strategy was determined. PET/CT indicated that 31 patients (47.7%) had evidence of a tumor recurrence, including a local recurrence, peritoneal carcinomatosis, and distant metastases (Table 2; Figure 2A). The remaining 34 (52.3%) patients had no evidence of a tumor recurrence (Figure 2B). Both the univariate

Table 1 Factors on presentation associated with a malignant etiology of the small-bowel obstruction

Factors	Value
All	65 (100.0)
Sex	
Female	11 (16.39)
Male	54 (83.1)
Age (yr)	
< 70	45 (69.2)
⩾ 70	20 (30.8)
Site	
Lower	41 (63.1)
Middle	8 (12.3)
Upper	16 (24.6)
Diffuse	0 (0.0)
Comorbidities	
Yes	42 (64.6)
No	23 (35.4)
Surgery	
Subtotal gastrectomy	33 (50.8)
Total gastrectomy	19 (29.2)
Extended total gastrectomy	13 (20.0)
Types of digestive reconstruction	
Billroth Ⅰ	20 (30.8)
Billroth Ⅱ	15 (23.1)
Roux-en-Y	30 (46.1)
Grading	
Well differentiated	22 (33.8)
Moderately differentiated	22 (33.8)
Poorly differentiated	21 (32.4)
Undifferentiated	0 (0.0)
T stage	
T1	0 (0.0)
T2	21 (32.3)
T3	38 (58.5)
T4	6 (9.2)
No. metastatic nodes	
N0	8 (12.3)
N1	22 (33.8)
N2	23 (35.4)
N3	12 (18.5)
M stage	
M0	65 (100.0)
M1	0 (0.0)
Intra-abdominal chemotherapy	
Yes	35 (53.8)
No	30 (46.2)
Postchemotherapy	
Yes	55 (84.6)
No	10 (15.4)
Recurrence in PET/CT	
Yes	31 (47.7)
No	34 (52.3)
Re-surgery	
Yes	27 (41.5)
No	38 (58.5)

PET/CT: Positron emission tomography/computed tomography.

and multivariate analyses for factors that may have correlated with survival revealed that the only factor that was associated with poor survival was PET/CT evidence of a tumor recurrence (Table 3).

In patients who received surgical treatment, the type of operation was determined by 3 expert gastrointestinal surgeons based on abdominal examinations and the general condition of the patient. A total of 27 patients (12 in the group with PET/CT evidence of a tumor recurrence and 15 with no evidence of a tumor recurrence) underwent laparotomy. The types of surgical procedures that were performed are summarized in Table 4. Open and close surgery was the main type of surgical procedure reported for the patients with tumor recurrences. R0 resections were performed in 2 patients, including 1 who underwent combined adjacent organ resection. In the group with no evidence of tumor recurrences on PET/CT, bowel resections were performed in 7 patients, adhesiolysis was performed in 7 patients, and a bypass was performed in 1 patient. The overall incidence of postoperative complications was 44.4% (12 of 27 patients). There were 7 patients with Clavien grade I complications, including 3 with wound infections and 4 with pleural effusions. Another 3 patients were classified as having grade Ⅱ complications, including 1 with an anastomotic leakage and 2 with pneumonia. One patient had the grade Ⅲb complication of an abdominal abscess, and 1 patient had the grade V complication of multiple organ failure and died in the hospital 1 wk after surgery.

Table 2 Recurrence site, number and standardized uptake value of recurrence in positron emission tomography/computed tomography

Variables		*n*	SUV mean (range)
Recurrence	Yes	31	7.3 (2.6-28.3)
	No	34	/
Recurrence site	Locoregional recurrence (Remnant stomach or anastomosis site)	2	6.8 (4.1-16.2)
	Distant metastasis	29	7.9 (2.6-28.3)
	Lymph-node	16	7.5 (2.6-28.3)
	Liver	8	8.0 (2.8-15.5)
	lung	6	7.1 (2.6-14.2)
	Other site (bone, skin, *etc.*)	10	6.2 (3.2-12.5)
	Peritoneum	12	5.4 (2.7-11.2)

SUV: Standardized uptake value.

The 1-year survival curves according to the PET/CT findings are shown in Figure 3A. There was a significant difference in the survival between patients with and without evidence of recurrences on PET/CT, and the 1-year survival rates were 8.8%, and 93.5%, respectively (P = 0.00). The 1-year survival curves according to exploratory laparotomy and nonoperative treatment are shown in Figure 3B. There were no significant differences (P = 0.71) in the 1-year survival based on surgical *vs* nonsurgical management (0% with nonoperative treatment *vs* 20% after exploratory laparotomy). The 1-year survival curves according to evidence of a tumor recurrence on PET/CT are shown in Figure 3C.

Other variables in the analysis included 30-d readmission, the length of hospital stay, complications, and the mortality rates. These variables are listed in Table 5. In both the PET/CT-positive and -negative groups, exploratory laparotomy resulted in a shorter mean length of hospital stay than nonsurgical management (P < 0.05).

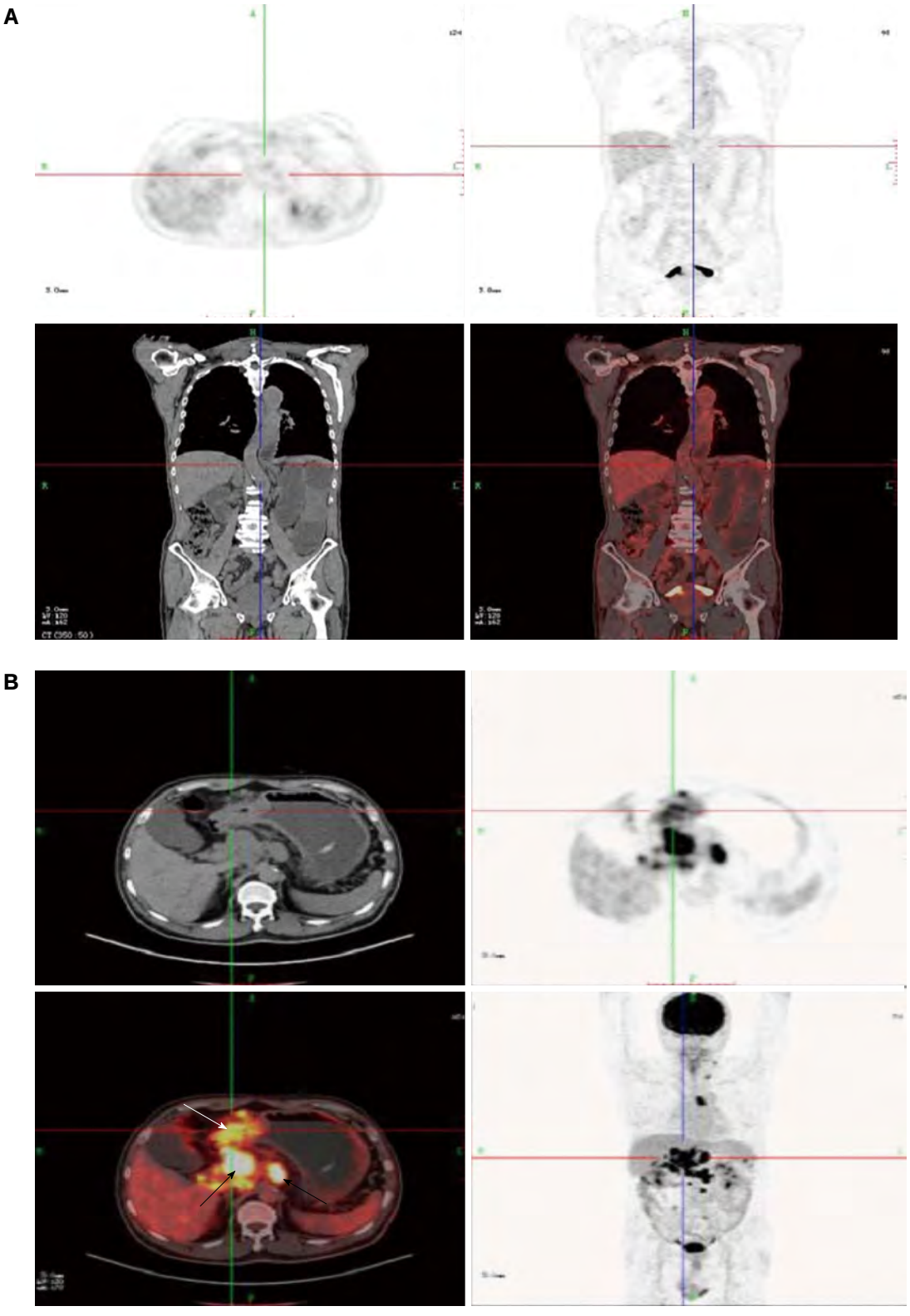

Figure 2 Patients who had had gastric cancer resection underwent positron emission tomography/computed tomography because of small-bowel obstruction. A: A 68-year-men who had had gastric cancer resection 2 years previously underwent positron emission tomography (PET)/computed tomography because of small-bowel obstruction. Whole body PET projection image and axial PET image showed no focal hypermetabolic activity; B: A 38-year-female who had had gastric cancer resection 1 years previously underwent positron emission tomography/computed tomography because of small-bowel obstruction. Whole body PET projection image and axial PET image showed the remnant stomach (white arrow) and lymph-node (black arrow) focal hypermetabolic activity.

Table 3 The clinicopathologic factors of the 65 patients

Factors	Survive (*n*)	Death (*n*)	Univariate analysis *P* value	Multivariate analysis *P* value
Sex			0.751	
Female	6	5		
Male	26	28		
Age (yr)			0.180	
< 70	25	20		
⩾ 70	7	13		
Site			0.336	
Lower	20	21		
Middle	1	7		
Upper	11	5		
Diffuse	0	0		
Comorbidities			0.798	
Yes	20	22		
No	12	11		
Surgery			0.683	
Subtotal gastrectomy	17	16		
Total gastrectomy	10	9		
Extended total gastrectomy	5	8		
Types of digestive reconstruction			0.446	
Billroth Ⅰ	12	8		
Billroth Ⅱ	6	9		
Roux-en-Y	14	16		
Grading			0.241	
Well differentiated	11	11		
Moderately differentiated	8	14		
Poorly differentiated	13	8		
Undifferentiated	0	0		
T stage			0.447	
T1	0	0		
T2	8	13		
T3	21	17		
T4	3	3		
No. metastatic nodes			0.105	
N0	1	7		
N1	11	11		
N2	14	19		
N3	6	6		
Intra-abdominal chemotherapy			0.459	
Yes	19	16		
No	13	17		
Postchemotherapy			0.511	
Yes	26	29		
No	6	4		
Recurrence in PET/CT			0.000	0.000
Yes	2	29		
No	31	3		
Re-surgery			0.804	
Yes	14	13		
No	18	20		

PET/CT: Positron emission tomography/computed tomography.

DISCUSSION

An intestinal obstruction is a common problem in patients with advanced cancers. Approximately 3%-15% of all terminally ill cancer patients will suffer from an intestinal obstruction and 10%-28% of patients with gastrointestinal cancers will develop an intestinal obstruction. Obstructions in patients with a history of gastric cancer may be secondary to a malignant process, either extrinsic or intrinsic to the bowel, or an underlying benign etiology, such as an intra-abdominal hernia or intraperitoneal adhesions. The current treatment options for patients with a bowel obstruction secondary to malignant disease include surgery to bypass/remove the obstruction, gastrointestinal decompression *via* a nasogastric tube, and medications (*e.g.*, octreotide)[17]. In inoperable cases, decompression *via* a nasogastric tube may be the only treatment available. Nasogastric tube decompression can provide symptomatic relief but may cause mucosal erosion, esophagitis, or aspiration pneumonia, which further diminish quality of life. However, surgical treatment is often contraindicated because of the poor physical status of the patient, and many patients with gynecological malignancies, especially ovarian cancer, are not candidates for surgery because of the presence of diffuse intraperitoneal carcinomatosis, multiple partial obstruction points, ascites, and/or a history of previous radiotherapy. A critical step in the management of patients with a bowel obstruction and a history of curative resection of gastric cancer is to determine whether a malignant process is present. Identifying the underlying etiology of the bowel obstruction, malignant or benign, will significantly impact management decisions. Additionally, distinguishing between a malignant obstruction and a benign obstruction is a key measure in deciding which patients should undergo early operation.

Table 4 Types of surgery performed for small-bowel obstruction

Procedure	*n*
Open and close	10
R0 resection	2
Bypass	1
Bowel resection	7
Adhesiolysis	7

It is well documented that the complete surgical removal of gastric tumors with lymph node dissection is the only curative treatment that is currently available; however, disease recurrence after radical surgery still occurs in approximately 22%-48% of patients, and its prognosis is poor[18-21]. Tumor marker evaluation, endoscopy, and imaging studies have previously been used to monitor patients for gastric cancer recurrences; however, there are several limitations to tumor markers and endoscopy. Tumor markers cannot be used to determine the site of recurrence, and endoscopy cannot detect extraluminal recurrences[22]. The most important limitation of CT in the detection of locally recurrent gastric cancer is the lack of specificity because the diagnostic ability of CT depends on the morphological changes of the involved organs and distorted anatomical features. In addition, CT uses size criteria. These factors result in difficulties in image interpretation, and CT cannot precisely identify the presence and the quality of tumors.

Whole-body ^{18}F-FDG PET detects increased glucose metabolism in malignant cells to produce diagnostic evidence and can be widely applied for staging, re-

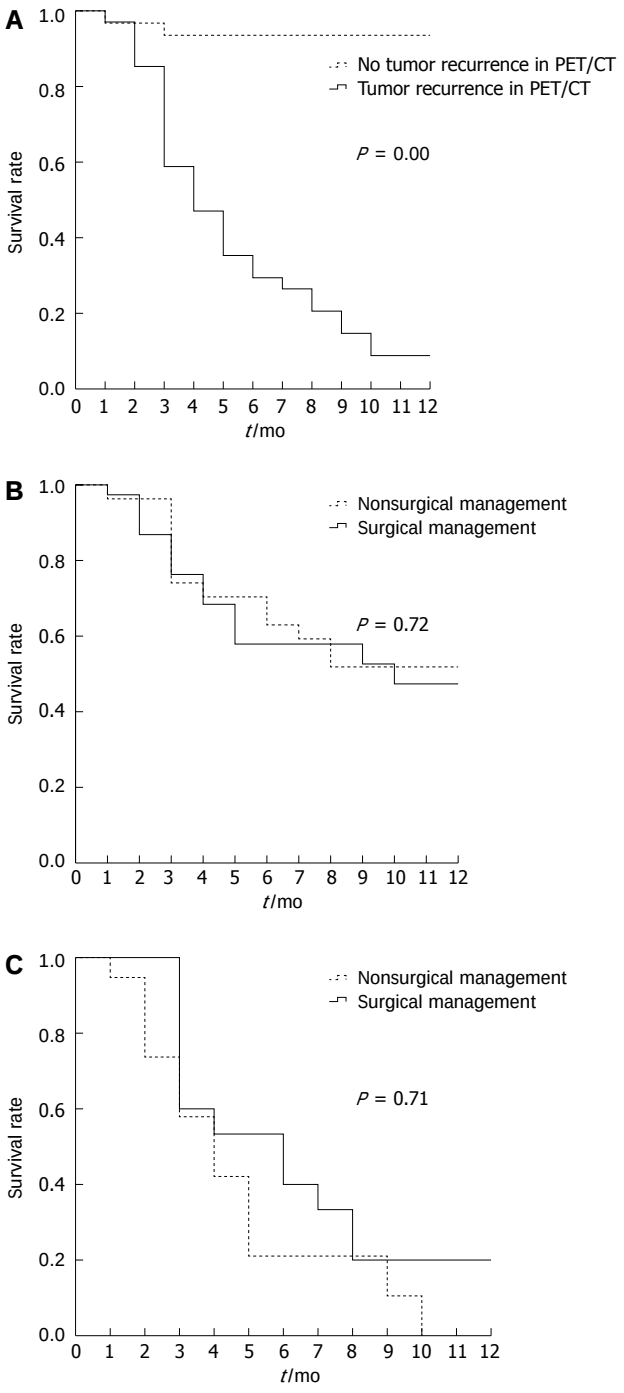

Figure 3 1-year survival curves. A: With respect to no tumor recurrence and tumor recurrence group in positron emission tomography/computed tomography. There was significant difference between two subgroups (P = 0.00). The 1-year survival rate in positron emission tomography/computed tomography (PET/CT) tumor recurrence group is 8.8%, while 93.5% in no tumor recurrence group; B: With respect to exploratory laparotomy and nonoperative treatment group. There was no difference in 1-year survival based on type of surgical *vs* nonsurgical management (P = 0.72); C: With respect to tumor recurrence group in positron emission tomography/computed tomography. The 1-year survival rates for patients in each subgroup were, respectively, 0.0% for tumor nonoperative treatment, and 20% for exploratory laparotomy group. There is also no significant difference between two subgroups (P = 0.71).

staging, and monitoring therapy-induced tumor changes and response to therapy in patients with various cancers. The usefulness of integrated ^{18}F-FDG PET/CT for the diagnosis of recurrences in patients with gastric cancer has been investigated in previous studies, which have indicated that ^{18}F-FDG PET/CT is an effective and helpful diagnostic method in the evaluation of recurrences. Other trials have studied the impact of ^{18}F-FDG PET/CT on the clinical decision-making process[23-25], FDG-PET results led to a radical change in the clinical management of 20% of the patients who were analyzed for resection of colorectal liver metastases. FDG-PET was considered a decisive technique for determining whether to perform surgery, and management was changed in 29% of the patients. This study has confirmed the critical role of whole-body ^{18}F-FDG PET/CT during the clinical course of patients with a bowel obstruction and a history of gastric cancer.

Table 5 Outcomes variables of the 65 patients

Variables	No recurrence in PET/CT		Recurrence in PET/CT	
	Surgical management	Non-surgical management	Surgical management	Non-surgical management
Mean length of stay (d)	10.5 ± 2.3	18.2 ± 8.7	8.2 ± 3.1	19.1 ± 9.6
30-d re-admission	0.00%	21.10%	25.00%	26.10%
In-hospital mortality	0.00%	0.00%	0.10%	0.00%
Overall complications	53.30%	0.00%	33.30%	0.00%

PET/CT: Positron emission tomography/computed tomography.

Several patients in our study with a SBO and a history of gastric cancer did not have end-stage disease that was associated with poor survival. Distinguishing between a malignant obstruction and a benign obstruction is a key measure in deciding which patients should undergo early operation. Patients with metastatic cancer who develop a bowel obstruction have a short median survival time (approximately 3 mo)[16], and decisions regarding the treatment of bowel obstructions must be carefully weighed in these patients. Surgery can offer good palliative benefits for these patients; however, surgery may result in complications that reduce quality of life and cause patients to spend an excessive amount of time in the hospital, which could have been avoided. Therefore, optimal palliation may result from the nonoperative and medical management of symptoms and lead to a potential decrease in the length of hospital stay. Attempting surgery in these patients may not be the best decision, and the finding in our study that there were no differences in the survival of patients with recurrent disease based on the type of management shifts the focus of care for these patients from a selection process to surgical *vs* nonsurgical management.

Multiple specialists are usually involved in the treatment of these patients, including gastroenterologists, interventional and diagnostic radiologists, radiation oncologists, and medical and surgical oncologists. These specialists may have divergent opinions regarding definitive individualized treatment. Our results suggest that patients who had evidence of a tumor recurrence on a

PET/CT scan face an end-of-life scenario and optimal symptom control is the goal for these patients. In addition, FDG PET/CT is a superior post-therapy surveillance modality for the diagnosis of recurrent gastric cancer compared with other imaging methods after initial surgery. In addition, FDG PET/CT has been specifically helpful in optimizing treatment plans and may play an important role in treatment stratification in the future[26]. Miller *et al*[11] compared operative therapy with nonoperative therapy in patients with small bowel obstructions secondary to malignant disease and found a rate of reobstruction that was 15% higher in the nonoperative group. Additionally, they reported shorter times to a reobstruction in patients who had received conservative nonoperative therapy, and they observed that a palpable abdominal mass was an important predictor of poor outcomes in their series. In this study, we concluded that patients with a history of gastric cancer who present with a SBO and who have no evidence of a tumor recurrence on PET/CT will receive benefits in both survival and quality of life after surgery to relieve the obstruction.

In conclusion, ^{18}F-FDG PET/CT can be used to identify the causes of bowel obstructions in patients with a history of curative resection of gastric cancer, and this method is useful for planning the surgical management of these patients. Surgical intervention in a patient who has an obstruction after curative resection of gastric cancer and who has no evidence of a tumor recurrence on a PET/CT examination benefits the quality of life of the patient. Patients with poor survival, including patients with PET/CT evidence of a local recurrence, peritoneal carcinomatosis or distant metastases, would not benefit from surgery.

COMMENTS

Background

The management of patients who present with a small bowel obstruction (SBO) after treatment of primary carcinoma challenges the clinical judgement of even the most experienced surgeons when the feared cause is metastatic disease. It is difficult to predict whether the quality and/or the quantity of life in this group of patients will be improved by surgery because these patients have a poor prognosis at the time of presentation. Positron emission tomography (PET) with ^{18}F-fluorodeoxyglucose (FDG) detects the increased utilization of glucose by malignant cells and is more accurate than conventional diagnostic methods for the diagnosis of primary and recurrent gastrointestinal tumors.

Research frontiers

The management of SBOs after treatment of primary carcinoma is difficult, and it is unclear which patients will benefit from surgery and which patients will have similar outcomes from medical management because many patients may have diffuse peritoneal metastatic disease and/or adhesions from previous surgery. It is difficult to predict whether the quality and/or the quantity of life in this group of patients will be improved by surgery because these patients have a poor prognosis at the time of presentation. In addition, the management of these patients presents an additional difficulty because the intestinal obstruction may be due to more than one physiopathological process, such as an intraluminal obstruction from polypoid lesions that occlude the bowel lumen, an intramural obstruction from the infiltration of a tumor within the muscular coat of the bowel wall, and an extramural obstruction from mesenteric and omental masses and extrinsic compression from malignant adhesions.

Innovations and breakthroughs

^{18}F-FDG PET/computed tomography (CT) can be used to identify the causes of bowel obstructions in patients with a history of curative resection of gastric cancer, and this method is useful for planning the surgical management of these patients. Surgical intervention in a patient who has an obstruction after curative resection of gastric cancer and who has no evidence of a tumor recurrence on a PET/CT examination benefits the quality of life of the patient. Patients with poor survival, including patients with PET/CT evidence of a local recurrence, peritoneal carcinomatosis or distant metastases, may not benefit from surgery.

Applications

^{18}F-FDG PET/CT can be used to identify the causes of bowel obstructions in patients with a history of gastric cancer, and this method is useful for planning the surgical management of these patients.

Terminology

PET with ^{18}F-FDG detects the increased utilization of glucose by malignant cells to provide diagnostic information and is more accurate than conventional diagnostic methods in cases of primary and recurrent gastrointestinal tumors.

Peer review

This article is interesting. The authors present their experience of using whole-body PET/CT in the surgical management of patients with bowel obstructions secondary to gastric cancer. PET/CT significantly improves survival because of its ability to identify the causes of bowel obstructions. Overall, the paper is well written and acceptable for publication in its current form.

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BRIEF ARTICLE

Fibroblast growth factor receptor 4 Gly388Arg polymorphism in Chinese gastric cancer patients

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Abstract

AIM: To investigate the contribution of the fibroblast growth factor receptor 4 (FGFR4) Gly388Arg polymorphism as a genetic risk factor for gastric cancer (GC) and to investigate any associations between this polymorphism and clinicopathological parameters and survival.

METHODS: Tumors and matched adjacent non-cancer tissues were collected from 304 GC patients, and 5 mL of venous blood was collected from 62 GC patients and 392 age- and sex-matched healthy controls without cancer history from the same ethnic population. DNA was extracted, and direct sequencing analyses were performed to genotype the *FGFR4* Gly388Arg polymorphism in all the samples. Differences in the genotype frequencies of the *FGFR4* Gly388Arg polymorphism between GC patients and healthy controls were estimated using the χ^2 test. Binary logistic regression was used for all analysis variables to estimate risk as the ORs with 95%CIs. The relationships between the *FGFR4* genotype and clinicopathological parameters were tested with the χ^2 test. The Kaplan-Meier product-limit method, the log-rank test, and the Cox regression model were applied to evaluate the effect of the *FGFR4* genotype on the overall survival of patients with GC.

RESULTS: In the present GC cohort, 118 patients (38.8%) were homozygous for the Gly388 allele, 124 patients (40.8%) were heterozygous, and 62 patients (20.4%) were homozygous for the Arg388 allele. The frequencies of the Gly/Gly, Gly/Arg, and Arg/Arg genotypes in the healthy controls were 33.6%, 48.0%, and 18.4%, respectively. The distributions of genotypes (χ^2 = 3.589, P = 0.166) and alleles (χ^2 = 0.342, P = 0.559) of the FGFR4 Gly388Arg polymorphism were not different between the GC patients and the healthy controls. Although we observed no correlation between the *FGFR4* Gly388Arg polymorphism and clinicopathological parameters or survival in the total cohort of GC patients, the presence of the Arg388 allele was associated with shorter survival time in patients with GC if the tumor was small (log rank χ^2 = 5.449, P = 0.020), well differentiated (log rank χ^2 = 12.798, P = 0.000), T1 or T2 stage (log rank χ^2 = 4.745, P = 0.029), without lymph node involvement (log rank χ^2 = 6.647, P= 0.010), and at an early clinical stage (log rank χ^2 = 4.615, P = 0.032).

CONCLUSION: Our results suggest that the *FGFR4* Gly388Arg polymorphism is not a risk factor for GC cancer initiation but that it is a useful prognostic marker for GC patients when the tumor is relatively small, well differentiated, or at an early clinical stage.

Key words: Fibroblast growth factor receptor 4; Gly388Arg; Genetic susceptibility; Single nucleotide polymorphism; Gastric cancer

Core tip: This study investigated the contribution of the fibroblast growth factor receptor 4 (FGFR4) Gly388Arg polymorphism as a genetic risk factor for gastric cancer (GC) and any associations between this polymorphism and clinicopathological parameters such as age, gender, clinical stage, tumor grade, human epidermal growth factor receptor 2 status and survival. The results suggested that the FGFR4 Gly388Arg polymorphism was not a risk factor for GC cancer initiation but that it was a useful prognostic marker for GC patients when the tumor was relatively small, well differentiated, or at an early clinical stage.

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INTRODUCTION

Gastric cancer (GC) is one of the most common cancers in the world, and it has a complex etiology. Disease development is related to environmental factors, such as diet and *Helicobacter pylori* (*H. pylori*) infection, as well as genetic predisposition. In the last decade, GC incidence rates have steadily declined owing to changing diets and the application of antibiotics to treat *H. pylori* infection[1]. However, the treatment of patients with advanced GC remains a significant challenge. Surgical resection and chemotherapy are only effective for a fraction of patients, and their prognoses remain very poor. Recently, a number of molecularly targeted agents modulating different signal transduction pathways have been researched in clinical trials for many cancer types[2]. Trastuzumab, a monoclonal antibody against human epidermal growth factor receptor 2 (HER2; also known as ERBB2) was demonstrated to be effective in advanced HER2-positive GC. The addition of trastuzumab to chemotherapy improved survival in patients with advanced GC or gastroesophageal junction cancer compared with chemotherapy alone in a Trastuzumab for Gastric Cancer trial[3]. However, only 7% to 34% of GC cases are HER2 positive[4-6], meaning that only a fraction of GC patients can benefit from trastuzumab. Accordingly, there is an urgent need to better understand the genesis of GC to establish a sound basis for future treatments.

Fibroblast growth factor receptor 4 (FGFR4) is a member of the receptor tyrosine kinase family. These receptors have highly conserved structures containing an extracellular ligand-binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain. The FGFR4 protein interacts with specific growth factors, especially acidic fibroblast growth factor, and is critically involved in cell growth, differentiation, migration, angiogenesis, and tumorigenesis. Several prior studies have investigated the role of the FGFR4 signaling pathway in GC. Notably, a soluble variant of FGFR4 was first detected in human gastrointestinal epithelial cells and cancer cells in a study by Takaishi *et al*[7]. Shin *et al*[8] and Ye *et al*[9]. also demonstrated the upregulation of FGFR4 mRNA and protein in GC, suggesting the possibility that FGFR4 signaling could play a role in gastric carcinogenesis.

Recently, a single nucleotide polymorphism (SNP) at codon 388 (cDNA 1162) from G to A, which results in a change of amino acid from glycine to arginine, was identified in the transmembrane domain of the *FGFR4* gene[10]. Significant scientific effort has been put into the investigation of this *FGFR4* Gly388Arg polymorphism in cancer progression, and the results showed that patients with the Arg/Arg or Gly/Arg genotype (compared to those with a Gly/Gly genotype) had a shorter survival time or a higher proportion of nodal involvement in many types of cancer, including breast, lung, colon, prostate, soft tissue sarcoma, melanoma, and head and neck squamous cell carcinoma[10-19]. However, several researchers have opposed the association between this polymorphism and poor outcomes or lymph node involvement[20-23]. Furthermore, it has been reported that the *FGFR4* Arg388 polymorphism may not be involved in tumor initiation in several different tumor types with the exception of prostate cancer[13,14,18,24]. Xu *et al*[25] conducted a meta-analysis of 2618 patients and 2305 controls and demonstrated that the *FGFR4* Gly388Arg polymorphism was associated with both progression and risk in prostate cancer .

Recently, Ye *et al*[26] reported that the FGFR4 Arg allele was an independent prognostic factor in Chinese patients with GC. To our knowledge, this is the only report on the association between the *FGFR4* Gly388Arg polymorphism and the progression of GC in Chinese patients, and it therefore needs further confirmation. Moreover, no study has been conducted on the correlation between this polymorphism and the risk of GC. In the present study, we expanded the sample sizes by enrolling 304 patients with GC and 392 healthy controls and genotyped every sample for the FGFR4 Gly388Arg polymorphism to confirm the findings of Ye *et al*[26] and investigate the association between the FGFR4 Gly388Arg polymorphism and the risk of GC.

MATERIALS AND METHODS

Patients and healthy controls

A total of 304 Chinese patients diagnosed with GC were recruited and underwent surgery between 2007 and 2009 at Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University. All GC cases were pathologically confirmed, and patient records were used to obtain clinical data. The study included a total of 223 males and 81

Table 1 General characteristics of gastric cancer patients and healthy controls *n* (%)

Characteristics	GC *n* = 304	Healthy controls *n* = 392	Pearson's χ^2 value	*P* value
Age (yr)			2.548	0.110
< 60	125 (41.12)	138 (35.20)		
⩾ 60	179 (58.88)	254 (64.80)		
Sex			0.115	0.735
Male	223 (73.36)	292 (74.49)		
Female	81 (26.64)	100 (25.51)		
Smoker			0.314	0.575
Yes	92 (30.26)	111 (28.32)		
No	212 (69.74)	281 (71.68)		
Hypertension			0.012	0.931
Yes	78 (25.66)	102 (26.02)		
No	226 (74.34)	290 (73.98)		
Diabetes			2.591	0.108
Yes	52 (17.11)	50 (12.76)		
No	252 (82.89)	342 (87.24)		

GC: Gastric cancer.

females. The age of the patients at the time of surgery ranged from 22 to 87 years, with a median age of 63.5 years. The clinical stage was determined according to the Union for International Cancer Control TNM staging system, and the tumor grade was based on the World Health Organization classification. The expression or amplification of HER2 in GC was detected using standard methods, including immunohistochemistry and fluorescence in situ hybridization. The HER2 status was determined according to the Hofmann HER2 scoring system[6]. Follow-up was performed regularly. The median follow-up time for patients still alive at analysis was 49 mo (range, 20-61 mo).

Tumors and matched adjacent non-cancer tissues were snap frozen in liquid nitrogen immediately after surgical removal and stored at -80 ℃. Each tumor sample was evaluated microscopically, and macro-dissection was performed to ensure that more than 70% of the sample was tumor tissue before DNA extraction. Five milliliters of venous blood was collected from 62 GC patients and 392 age- and sex-matched healthy controls without cancer history from the same ethnic population. Informed consent was obtained from all the patients and controls for the use of their specimens and clinicopathologic data for this study. The study was approved by the Institutional Human Ethics Committee.

Genotyping of the FGFR4 Gly388Arg polymorphism

DNA samples from tumor and normal adjacent tissue were prepared with the Puregene DNA extraction kit (Qiagen, Valencia, CA, United States). Genomic DNA was extracted and purified from the blood of GC patients and the healthy control group using the QIAamp DNA Blood Midi kit (Qiagen, Germany). Sanger sequencing was applied to genotype the *FGFR4* Gly388Arg polymorphism with the following PCR primers: 5'-GCGGCCAGTCTCACCACTGAC-3' and 5'-TGGAGTCAGGCTCTTCCGGCA-3'. Each primer was tagged with the M13 primer as a uniform sequencing primer for individual PCR products to facilitate the sequencing process. All PCR assays were carried out in a 25 μL volume containing 10 ng genomic DNA, 0.1 μmol/L of each primer, and 1 × AmpliTaq Gold Pre Mix (AB). The PCR conditions were as follows: 95 ℃ for 10 min; 30 cycles of 95 ℃ for 30 s, 58 ℃ for 30 s, and 72 ℃ for 30 s; and 5 min at 72℃. The PCR product was 355 bp and was sequenced in both directions using the BigDye Terminator kit 3.1 (Applied Biosystems; Foster City, CA, United States) and ABI Genetic Analyzer3730 × l (Applied Biosystems) according to the manufacturer's instructions. Sequence traces were analyzed for the polymorphism after assembly and quality calling with SeqScape2.5 sequence analysis software (Applied Biosystems).

Statistical analysis

Differences in the general characteristics and genotype frequencies of the *FGFR4* Gly388Arg polymorphism between GC patients and healthy controls were estimated using the χ^2 test. Hardy-Weinberg equilibrium analyses were performed to compare observed and expected genotype frequencies using the χ^2 test. Binary logistic regression was used for all analysis variables to estimate risk as the ORs with 95%CIs. The relationships between the *FGFR4* genotype and clinicopathological parameters were tested with the χ^2 test. The Kaplan-Meier product-limit method, the log-rank test, and the Cox regression model were applied to evaluate the effect of the *FGFR4* genotype on the overall survival of patients with GC. The covariates included in the models were age, gender, clinical stage, tumor grade, HER2 status and *FGFR4* genotype. All analyses were carried out with SPSS for Windows software (SPSS, Chicago, IL, version 16.0). A P value < 0.05 was considered to be statistically significant.

RESULTS

FGFR4 genotype in healthy controls and GC patients

Data from the 62 patients who provided both tumor tissue and blood samples showed that the Gly388Arg genotypes were identical between tumor tissue and blood from the same individual. This result confirms that there were no somatic mutations at this locus in any of these patients.

The clinical characteristics of all the subjects are shown in Table 1. There were no significant differences in age, gender, smoking, hypertension, or diabetes status between the GC patients and healthy controls. The frequencies of the *FGFR4* Gly388Arg polymorphism genotypes are summarized in Table 2. Among the 304 GC patients, 118 patients (38.8%) were homozygous for the Gly388 allele, 124 were heterozygous (40.8%), and 62 were homozygous (20.4%) for the Arg388 allele. In the healthy controls, the frequencies of the Gly/Gly, Gly/Arg, and Arg/Arg genotypes and the Arg allele were 33.6%, 48.0%, 18.4% and 42.3%, respectively. The fre-

Table 2 Distribution and regression analysis of the fibroblast growth factor receptor 4 Gly388Arg genotype in gastric cancer patients and healthy controls *n* (%)

Gly388Arg	GC *n* = 304	Healthy controls *n* = 392	OR (95%CI)	*P* value
Genotype				
GG	118 (38.8)	132 (33.6)	1	
AG	124 (40.8)	188 (48.0)	0.738 (0.527-1.033)	0.076
AA	62 (20.4)	72 (18.4)	0.963 (0.633-1.466)	0.862
AA + AG	186 (61.2)	260 (66.3)	0.800 (0.586-1.093)	0.161
Allele				
G	360 (59.2)	452 (57.7)	1	
A	248 (40.8)	332 (42.3)	0.938 (0.756-1.163)	0.559

FGFR4: Fibroblast growth factor receptor 4; GC: Gastric cancer.

Table 3 Association analysis of the fibroblast growth factor receptor 4 Gly388Arg polymorphism and clinicopathological parameters in gastric cancer patients

Variables	Total *n* = 304	Gly/Gly *n* = 118	Gly/Arg + Arg/Arg *n* = 186	Pearson's χ^2 value	*P* value
Age (yr)				0.013	0.909
< 60	125	49	76		
≤ 60	179	69	110		
Sex				1.398	0.237
Male	223	91	132		
Female	81	27	54		
Tumor size				0.198	0.656
≤ 3 cm	58	24	34		
> 3 cm	246	94	152		
Differentiation				2.122	0.145
G1 + G2	100	33	67		
G3 + G4	204	85	119		
Invasion depth				0.000	0.995
T1 + T2	49	19	30		
T3 + T4	255	99	156		
N stage				0.640	0.200
N0	82	27	55		
N1 + N2 + N3	222	91	131		
M stage				1.089	0.297
M0	274	109	165		
M1	30	9	21		
Clinical stage				0.980	0.322
Ⅰ + Ⅱ	103	36	67		
Ⅲ + Ⅳ	201	82	119		
HER2 status				0.391	0.532
Negative	180	63	117		
Positive	45	18	27		

FGFR4: Fibroblast growth factor receptor 4; GC: Gastric cancer; HER2: Human epidermal growth factor receptor 2.

quencies were consistent with the Hardy-Weinberg equilibrium in the healthy controls. The genotype (χ^2= 3.589, P = 0.166) and allele frequencies (χ^2 = 0.342, P = 0.559) of the *FGFR4* Gly388Arg polymorphism were not different between the GC patients and the healthy controls. Binary logistic regression analysis indicated that the ORs for the carriers of Gly/Arg, carriers of Arg/Arg, and carriers of the Gly/Arg or Arg/Arg genotypes were 0.738 (95%CI: 0.527-1.033), 0.963 (95%CI: 0.633-1.466) and 0.800 (95%CI: 0.586-1.093), respectively. No differences were observed between patients and healthy controls. The distribution of the Gly388 and Arg388 alleles was not different between patients and healthy controls (OR = 0.938, 95%CI: 0.756-1.163). These results suggest that the *FGFR4* Gly388Arg polymorphism is not an independent risk factor for GC in Chinese patients.

FGFR4 Gly388Arg polymorphism is not associated with any clinicopathological parameters in GC patients

Based on previous reports and due to the requirements for accurate statistical analysis, the 304 GC patients were divided into two groups: patients with the Gly/Gly genotype and patients with the Arg/Arg or Arg/Gly genotypes. As shown in Table 3, no correlation was observed between the *FGFR4* Gly388Arg polymorphism and any of the following clinicopathological parameters: age at diagnosis, gender, tumor size, clinical stage, differentiation and HER2 status.

Impact of the FGFR4 Gly388Arg polymorphism on GC survival

When analyzing the entire patient cohort using Kaplan-Meier survival analysis, no difference was observed in survival between patients with the Gly/Gly genotype and patients with the Gly/Arg or Arg/Arg genotypes (log rank χ^2 = 0.047, P = 0.829). When the patient population was stratified by clinicopathological parameters, such as age at diagnosis, gender, tumor size, differentiation, clinical stage, HER2 status and chemotherapy history (5-fluorouracil and cisplatinum), we found that the presence of the Arg388 allele was associated with a shorter survival time in GC patients if the tumor was small (less than or equal to 3 cm in size) (log rank χ^2 = 5.449, P = 0.020, Figure 1A), well differentiated (log rank χ^2 = 12.798, P = 0.000, Figure 1B), of T1 or T2 stage invasion depth (log rank χ^2 = 4.745, P = 0.029, Figure 1C), without lymph node involvement (log rank χ^2 = 6.647, P = 0.010, Figure 1D), and at an early clinical stage (log rank χ^2 = 4.615, P = 0.032, Figure 1E). No survival differences were observed in any of the other subgroups (Table 4). In addition, the Cox proportional hazard analysis of survival demonstrated that the *FGFR4* Gly388Arg polymorphism was not an independent prognostic factor for GC patients (data not shown).

DISCUSSION

In the present study, the overall frequencies of the Gly/Gly, Gly/Arg, Arg/Arg genotypes and Arg388 allele in healthy controls were 33.6%, 48.0%, 18.4% and 42.3%, respectively. As shown in Table 5, the frequencies of the Arg allele or Arg/Arg genotypes in our study were similar to those of the Chinese patient populations reported by Chen *et al*[27], Zhu *et al*[28], Ma *et al*[29]and Yang *et al*[30]. Interestingly, the Arg388 allele frequencies in the Chinese population are much higher than those in Caucasian cohorts. In a meta-analysis by Xu *et al*[31], the Arg

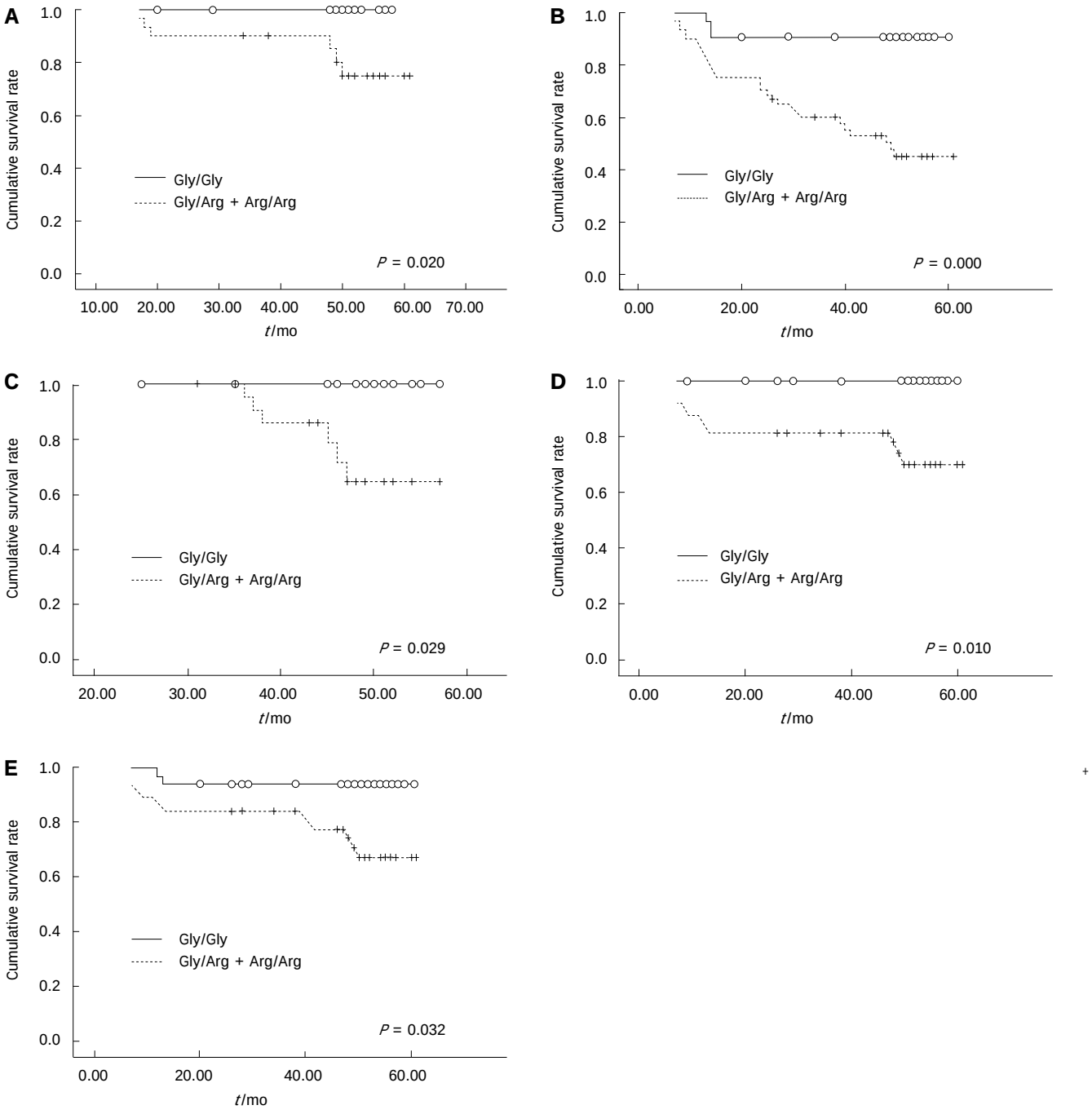

Figure 1 A considerable difference was found between patients with the Gly/Gly genotype and patients with Gly/Arg or Arg/Arg genotypes after stratified Kaplan-Meier survival analysis. A: Patients with tumor size ≤ 3 cm; B: Patients with well-differentiated gastric cancer (grades Ⅰ and Ⅱ); C: Patients classified as stage T1 or T2; D: Patients with no lymph node involvement; E: Patients at an early clinical stage (Ⅰ/Ⅱ).

allele was more highly represented among controls of Asian descent than controls of European and African-American descent. Our findings also support this result, which is contrary to a previous study that reported approximately 50% homo- or hetero-zygous carriers of the Arg allele in healthy controls independent of ethnic background[26].

For the first time, we report the distribution of the *FGFR4* Gly388Arg genotypes and alleles in both GC patients and matched healthy controls. No differences were found between GC patients and healthy controls. Our findings suggest that this polymorphism is not a risk factor for GC initiation in the Chinese population. This result is consistent with previous reports on several cancer types, including breast cancer and lung cancer, in different races[10,18,24]. However, it has been reported that the Arg388 allele is associated with an increased risk of prostate cancer[13,14]. Moreover, this polymorphism also plays a role in some types of non-cancer disease initiation. Zhu *et al*[28] and Ma *et al*[29] found that the *FGFR4* Gly388Arg polymorphism can act as a protective factor against coronary artery disease in the Chinese popula-

Table 4 Influence of fibroblast growth factor receptor 4 Gly388Arg polymorphism on gastric cancer survival

	Total *n* = 257	Gly/Gly *n* = 100	Gly/Arg + Arg/Arg *n* = 157	Log rank χ^2 value	*P* value
Age (yr)					
< 60	98	37	61	1.459	0.227
≥ 60	159	63	96	1.734	0.188
Sex					
Male	188	79	109	0.041	0.839
Female	69	21	48	0.018	0.894
Tumor size					
≤ 3 cm	55	24	31	5.449	0.020
> 3 cm	202	76	126	0.308	0.579
Differentiation					
G1 + G2	94	33	61	12.798	0.000
G3 + G4	163	67	96	2.637	0.104
Invasion depth					
T1 + T2	46	19	27	4.745	0.029
T3 + T4	211	81	130	0.037	0.848
N stage					
N0	73	24	49	6.647	0.010
N1 + N2 + N3	184	76	108	0.024	0.876
M stage					
M0	235	94	141	0.027	0.869
M1	22	6	16	0.139	0.710
Clinical stage					
Ⅰ + Ⅱ	91	33	58	4.615	0.032
Ⅲ + Ⅳ	166	67	99	0.048	0.827
Chemotherapy (5-fluorouracil and cisplatinum)					
Yes	156	55	101	0.019	0.891
No	51	21	30	0.442	0.506
HER2 status					
Negative	157	54	103	0.458	0.499
Positive	36	15	21	1.014	0.314

FGFR4: Fibroblast growth factor receptor 4; GC: Gastric cancer; HER2: Human epidermal growth factor receptor 2.

Table 5 Frequency of the codon 72 genotype

	Gly/Gly	Gly/Arg	Arg/Arg
This study (China)	132 (33.6)	188 (48.0)	72 (18.4)
Chen *et al*[27] (China)	133 (29.1)	229 (50.1)	95 (20.8)
Zhu *et al*[28] (China)	231 (33.4)	346 (50.0)	115 (16.6)
Ma *et al*[29] (China)	243 (33.2)	368 (50.3)	121 (16.5)
Yang *et al*[30] (China)	123 (32.0)	195 (50.6)	67 (17.4)
Ma *et al*[14] (Japan)	67 (37.4)	87 (48.6)	25 (14.0)
Morimoto *et al*[15] (Japan)	39 (38.2)	50 (49.0)	13 (12.7)
Ho *et al*[34] (Singapore)	30 (34.1)	38 (43.2)	20 (22.7)
Bange *et al*[10] (Italy)	55 (44.7)	60 (48.9)	8 (6.5)
Spinola et al[11] (Italy)	112 (50.9)	83 (37.7)	25 (11.4)
Ho *et al*[35] (United Kingdom)	150 (51.5)	117 (40.2)	24 (8.2)
Wang *et al*[36] (United States-European)	53 (54.6)	40 (41.2)	4 (4.1)
Wang *et al*[36] (United States-African)	76 (80.9)	18 (19.1)	0 (0.0)
FitzGerald *et al*[13] (United States-European)	631 (50.4)	496 (39.6)	124 (9.9)
FitzGerald *et al*[13] (United States-African)	60 (75.0)	18 (22.5)	2 (2.5)

tion. Based on a recent case-control study, the *FGFR4* Gly388Arg polymorphism is also considered to be a genetic risk factor that contributes to the aggravation of gallstone disease[27]. Therefore, these findings may reflect a tissue-specific effect of this polymorphism.

In our study, we found a significant difference following stratification by tumor size, differentiation, invasion depth, lymph node involvement or clinical stage ($P < 0.05$). Clinical stage depends on invasion depth and lymph node involvement. Therefore, our results demonstrate that the *FGFR4* Gly388Arg polymorphism is a prognostic factor in relatively small (less than 3 cm), well-differentiated (grades Ⅰ and Ⅱ) and early-stage GC (stages Ⅰ and Ⅱ) tumors but not in the total cohort of GC patients, which is in contrast to previous reports by Ye *et al*[26]. Because this study (and that of Ye *et al*[26]) focused on patients of Chinese origin, we suggest two possibilities to explain the conflicting results. The first possibility is the sample size. One major strength of our study is its large size. Our study included a higher proportion of patients with large tumors and late-stage disease than the study by Ye *et al*[26]. Thus, our results are more reliable given the isolation of the target patients from a pool of varied cases. Second, another possible explanation for the discord is that different DNA analysis methods were used in these two studies. Our study used a direct sequencing approach for genotype analysis, which was a more reliable method than the PCR-RFLP approach used by Ye *et al*[26]. Moreover, we detected the association between HER2 status and the FGFR4 Gly388Arg polymorphism in GC for the first time. No correlation was observed between HER2 status and the FGFR4 genotype ($P = 0.532$), which was consistent with previous reports in breast cancer[32].

The biochemical function of the *FGFR4* Gly388Arg polymorphism in GC is still unclear. No correlation was found between the *FGFR4* genotype and mRNA expression by Ye *et al*[26], and therefore, we do not attribute the polymorphism's effect to FGFR4 up-regulation. The FGFR4 Arg388 allele may be in linkage disequilibrium with other genetic changes that contribute to poor prognosis in GC. A previous study indicated that 39 head and neck cancer cell lines harboring the FGFR4 Arg388 allele exhibited an increased sensitivity to cisplatinum[33]. In breast cancer, no significant survival differences between *FGFR4* genotypes were found in patients without adjuvant systemic therapy. However, with adjuvant systemic therapy, breast cancer patients with the Gly/Gly genotype exhibited better disease-free survival and overall survival duration. Notably, this association seemed to be attributable to relatively poor therapy response in Arg388 carriers[32]. In our study, 156 patients underwent chemotherapy (5-fluorouracil and cisplatinum) following surgery. However, the median survival time of patients with the Gly/Arg and Arg/Arg genotypes did not differ from that of patients with the Gly/Gly genotype who underwent chemotherapy. Therefore, our data do not provide any evidence to support the theory that the FGFR4 Gly388Arg polymorphism is associated with sensitivity to chemotherapy.

In conclusion, our results demonstrate that the *FGFR4* Gly388Arg polymorphism plays no role in the initiation

of GC in Chinese patients, but it may be a factor in the survival of patients harboring relatively small, well-differentiated tumors in early GC stages.

COMMENTS

Background

Gastric cancer (GC) is one of the most common cancers in the world. Surgical resection and chemotherapy are only effective for a limited number of patients, and their prognoses remain very poor. Accordingly, there is an urgent need for a better understanding of the genesis of GC to establish a sound basis for future treatments.

Research frontiers

A germline polymorphism in the fibroblast growth factor receptor 4 (FGFR4) gene resulting in an amino acid change from glycine to arginine was identified several years ago. The presence of the FGFR4 Arg388 allele is associated with decreased disease-free survival in many cancers, including breast, lung, colon, prostate, soft tissue sarcoma, melanoma, and head and neck squamous cell carcinoma. Recently, Ye *et al* reported that the FGFR4 Arg388 allele was an independent prognostic factor in Chinese patients with GC. To our knowledge, this is the only report on the association between the FGFR4 Gly388Arg polymorphism and the progression of GC in Chinese patients, and this association needs further confirmation. Moreover, no study has been conducted on the correlation between this polymorphism and the risk of GC.

Innovations and breakthroughs

The correlation between the *FGFR4* Gly388Arg polymorphism and the risk of GC was investigated for the first time, and the authors suggested that this polymorphism was not a risk factor for GC cancer initiation. Another interesting finding was that the Arg388 allele frequency was much higher in this Chinese population than in Caucasian cohorts. The authors also investigated the associations between the *FGFR4* Gly388Arg polymorphism and clinicopathological parameters and survival in a larger sample series using more reliable methods. This result demonstrates that this polymorphism may contribute to the survival of patients with relatively small, well-differentiated tumors in the early stages of GC.

Applications

The FGFR4 Gly388Arg polymorphism is helpful for predicting the prognosis of early-stage GC patients with relatively small, well-differentiated tumors.

Terminology

Single nucleotide polymorphism (SNP): Genetic polymorphisms are natural variants in the genomic DNA sequence that are present in more than 1% of the population. One SNP represents the DNA variations in a single nucleotide. SNPs are widely used to better understand disease processes, thereby paving the way for genetic-based diagnostics and therapeutics.

Peer review

The results are interesting and may convey a useful prognostic marker for some GC patients.

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BRIEF ARTICLE

Helicobacter pylori and Crohn's disease: A retrospective single-center study from China

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Abstract

AIM: To investigate the association between *Helicobacter pylori* (*H. pylori*) infection and the prevalence of Crohn's disease (CD).

METHODS: Subjects were selected from patients admitted the gastrointestinal (GI) department at The First Affiliated Hospital School of Medicine (Zhejiang University) for abdominal pain, hematochezia, diarrhea and other GI symptoms between January 2008 and September 2012. CD was diagnosed by endoscopy and biopsy. *H. pylori* infection was detected by a ^{14}C-urea breath test and culturing of the biopsy sample. Demographic, anthropometric and serologic data were collected for each patient. *H. pylori* infection rate was compared between CD and control groups, followed by a subgroup analysis based on extent and severity of CD. Student's *t*, Mann-Whiney *U*, and χ^2 tests were used to analyze the data.

RESULTS: A total of 447 patients were analyzed, including 229 in the CD group and 248 in the control group. There were no significant differences in age, sex, and rates of hypertension or diabetes. However, the CD group showed significantly higher rates of smoking history (34.9% *vs* 18.1%), alcohol intake (17.4% *vs* 8.1%), white blood cell count (9.7 ± 2.9 × 10^9/L *vs* 4.3 ± 0.9 × 10^9/L), and C-reactive protein (36.3 ± 20.8 mg/L *vs* 5.5 ± 2.3 mg/L) but lower body mass index (24.5 ± 2.0 kg/m^2 *vs* 26.0 ± 2.2 kg/m^2) than the control group. The *H. pylori* infection rate in the CD group was 27.1%, significantly lower than that of 47.9% in the control group. Furthermore, the *H. pylori* infection rates in patients with colonic, small intestine, ileocolonic and extensive CD were 31.1%, 28.9%, 26.8% and 25.9% respectively, all of which were significantly lower than in the control group. Finally, the *H. pylori* infection rates in patients with remission, moderate and severe CD were 34.3%, 30.7% and 22.0% respectively, which were also significantly lower than in the control group.

CONCLUSION: Lower *H. pylori* infection in CD patients suggests a correlation between bacterial infection and CD, suggesting caution when considering *H. pylori* eradication in CD patients.

Key words: Crohn's disease; *Helicobacter pylori*; Urea breath test; Biopsy; Pathogenesis; Inflammatory bowel disease; Bacteria

Core tip: The association between *Helicobacter pylori* (*H. pylori*) infection and Crohn's disease (CD) prevalence is still unclear. In this retrospective study, we collected 229 CD patients and 248 control subjects to investigate the risk factors for prevalence, extent and severity of CD. Through extensive analysis, we found significantly lower *H. pylori* infection rates in CD patients having different disease extent and severity, providing evidence for bacteria involvement in CD pathogenesis and serving as a reminder for clinicians to remain cautious when considering *H. pylori* eradication in CD patients.

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INTRODUCTION

Crohn's disease (CD) is a chronic and relapsing inflammatory disease affecting any part of the intestine, most commonly involving the distal ileal, ileocecal and colonic sections[1]. CD is more common in northern Europe and America than in southern Europe and developing countries, and its prevalence has increased since the mid-1970s[2,3]. The pathogenesis of CD is unclear and increasing evidence supports the hypothesis of combinational involvement of genetic predisposition, immune response, and the environment, especially the gut bacteria and antigens[4]. Though several pathogens have been considered as infectious agents in CD, conclusive data is still lacking[5]. Therefore, identifying the correlation between a potential bacteria and CD pathogenesis is of clinical importance.

Helicobacter pylori (*H. pylori*) belongs to the family of curved or spiral flagellated, Gram-negative microaerophilic bacterium that is thought to have co-existed with humans for over 5000 years[6,7]. Since its discovery in 1984[8], *H. pylori* has been characterized as the causative agent for peptic ulceration and has been implicated in various autoimmune diseases[9]. *Helicobacter* are also excellent colonizers of the gastrointestinal surface for their microaerophilic metabolism, spiral shape, and peculiar motility. Considering the immune regulation, the capacity for colonization, and the nature of autoimmune-related damage in CD, it is theoretically plausible that *H. pylori* infection may take part in the pathogenesis of CD.

In immunodeficient rodents, *Helicobacter hepaticus* and *Helicobacter bilis* induce a persistent inflammation of the colon and cecum[10,11]. Nevertheless, the observations from human studies were confusing. *Helicobacter* was absent or only detected in the intestinal mucosa of a small subgroup of patients in both an English and an Australian study[12,13]. In contrast, Bohr *et al*[14] identified enterohepatic *Helicobacter* species in patients with inflammatory bowel disease (IBD). Furthermore, a meta-analysis suggested a protective role of *H. pylori* infection in CD pathogenesis but the heterogeneity among enrolled studies and the possibilities of publication bias limited the confidence of these results[15]. Therefore, we conducted a large-scale case control study to investigate the association between *H. pylori* infection and different severity and types of CD.

MATERIALS AND METHODS

Ethics statement

The protocol was approved by the institutional review board at Zhejiang University and conducted in accordance with the Declaration of Helsinki. We followed guidelines from the STROBE statement when designing the study and preparing the manuscript[16]. Written informed consent was collected from all patients.

Patients

Study subjects were selected from patients who were admitted for abdominal pain, hematochezia, diarrhea, and other GI symptoms between January 2008 and September 2012. Patients either underwent both a *H. pylori* test and endoscopy (gastroscopy, colonoscopy, or capsule) screen during admission or had recorded evidence of current *H. pylori* infection and CD. For the CD group, diagnosis was based on endoscopy manifestation and biopsy, as adopted by the Asia-Pacific consensus[17]. Exclusion criteria included previous acid inhibition or *H. pylori* eradication, 5-aminosalicylic administration and differential diagnosis with intestinal tuberculosis, ulcerative colitis (UC), Behçet's disease, or ischemic colitis. The control group was comprised of patients who underwent the initial screening but were subsequently excluded by negative results for CD and other known GI diseases.

CD patients were further categorized into subgroups according to the severity and extent of disease following the Chinese IBD guidelines[18]. For convenience, we adopted the Harvey-Bradshaw index (HBI)[19], a simplified version of the Crohn's disease activity index (CDAI), to evaluate CD severity. This index comprises general conditions, degree of abdominal pain, frequency of diarrhea, existence of abdominal mass, and complications such as arthritis, nodular erythema, gangrenous pyoderma, aphthous stomatitis, fistula, and abscess. Scores of 0-4 were appointed to each parameter according to disease severity. The HBI score was the summary of scores from each parameter, where $\leqslant 4$ was remission, 5-8 was moderate, and $\geqslant 9$ was severe. In addition, according to the extent revealed from radiology and endoscopy, CD was further divided into small intestinal CD, colonic CD, ileocolonic CD, and extensive CD, where the involved scale was over 100 cm.

Analysis of demographic, anthropometric and serologic data

Demographic and anthropometric data were retrieved

from the medical records of enrolled patients and included age, sex, smoking history, alcohol intake history (with positive designation made according to the Chinese guideline of alcoholic liver disease[20]), hypertension (defined as a patient on antihypertensive drug for blood pressure over 140/90 mmHg), body mass index (BMI; calculated as weight in kilograms divided by height in meters squared), and diabetes mellitus (DM; defined as fasting glucose $\geqslant$ 7.0 mmol/L or with past history of diagnosed DM). Patient blood samples were routinely gathered and tested for general condition and inflammation, including C-reactive protein (CRP) and complete blood cell counts. The main complications of CD were also recorded, including fistula and obstruction.

^{14}C-urea blood test and biopsy sample culture

H. pylori infection was detected by a ^{14}C-urea blood test (UBT) and biopsy sample culture. Serum *H. pylori*-IgG was not accepted as a diagnostic tool, as it does not reflect the current infection status. First described in 1989[21], ^{14}C-UBT is considered a rapid diagnostic procedure for *H. pylori* detection for its ability to convert urea to ammonia and carbon dioxide. UBT is also recommended in leading society guidelines as a preferred non-invasive choice for *H. pylori* detection[22]. According to the manufacturer's instruction (Headway, Shenzhen, China), patients were given a tablet of urea labeled with an uncommon isotope of radioactive carbon-14. In the following 30 min, the amount of isotope labeled carbon dioxide was measured in exhaled breath by scintillation. A positive result indicated the existence of *H. pylori*. For biopsy sample culture, samples from the gastric antrum obtained by gastroscopy were cultured for *H. pylori* as previously described[23].

Statistical analysis

Data were assessed for normality, and log-transformed as needed. Quantitative variants were expressed as mean ± SD, and analyzed by Student's *t* test or Mann-Whiney *U* test. For qualitative variants, percentages or frequencies were calculated and a χ^2 test was used for comparison. SPSS 17.0 software (Chicago, IL, United States) was used for all statistical analyses, and a P value < 0.05 was considered statistically significant.

RESULTS

Characteristics of study subjects

Following careful review of medical records, we identified 1563 patients that had been admitted to our ward for abdominal pain, hematochezia, diarrhea, and other GI symptoms over the past five years. Among these patients, 1231 were selected according to their having undergone both endoscopy and *H. pylori* test. The endoscopic diagnosis of CD was given to 921 patients by colonoscopy, 603 by gastroscopy, and 105 by capsule endoscopy. Furthermore, many of the patients had received more than one type of endoscopy. For the *H. pylori* infection test, 603 patients were investigated by biopsy sample culturing and 628 patients were investigated by ^{14}C-UBT. Among these 1231 subjects, 287 were diagnosed as CD by endoscopy and/or biopsy confirmation, with 18 of these patients having already received 5-aminosalicylic therapy, 11 being under proton pump inhibitor (PPI) therapy for reflux symptoms, and 21 having achieved *H. pylori* eradication. Among the remaining patients, 8 were further excluded due to positive serum *H. pylori*-IgG but negative ^{14}C-UBT or biopsy sample culture results. Finally, a total of 229 patients were enrolled into the CD group. Among them, 132 and 97 patients had ^{14}C-UBT and biopsy sample culture, respectively.

Among 1231 subjects, 251 were diagnosed with UC, 279 with different degrees of hemorrhoids, 41 with ischemic colitis, 7 with antibiotic-associated colitis, 5 with radiation enterocolitis, 11 with intestinal tuberculosis, 71 with sigmoiditis and proctitis, and 2 with Behçet's disease. The remaining 277 patients had GI symptoms but normal endoscopy and biopsy results. Nevertheless, there were still 14 patients under anti-acid therapy (10 with PPI and 4 with H_2 receptor antagonist) and 15 patients in which *H. pylori* had been eradicated. Finally, there were 248 patients enrolled into control group. Among them, 147 and 101 patients had ^{14}C-UBT and biopsy sample cultures, respectively.

Demographic, anthropometric and serologic data of enrolled patients

The average age and sex distribution of patients were balanced between two groups (Table 1). Differences in the rates of hypertension and DM between the two groups were not significant. However, BMI was significantly lower in the CD group than that in the control group, while the rate of smoking history was approximately twice that of the CD group ($P < 0.01$), reinforcing a correlation between smoking history and CD pathogenesis. In addition, percentage of alcohol intake was also significantly higher in the CD group. Finally, the two inflammation-associated markers, CRP and white blood cell (WBC) count, were significantly higher in the CD group, supporting the potential involvement of inflammation in CD.

Association between CD and H. pylori infection

Total *H. pylori* infection rate in the CD group was 27.1%, significantly lower than that of 47.9% in the control group. In the CD group, there were 45 patients with colonic CD, 28 with small intestine CD, 112 with ileocolonic CD, and 34 with extensive CD. In a subgroup analysis, all of the above-mentioned CD subgroups had significantly lower *H. pylori* infection rate than that in the control group, but the differences among these subgroups did not reach statistical significance (Table 2). We further divided the CD group into three subgroups according to severity determined by endoscopic appearance. Briefly, there were 32, 88 and 109 patients

Table 1 Demographic, anthropometric and serologic data of enrolled patients

Group	CD (n = 229)	Control (n = 248)	P value
Age (yr)	46.2 ± 10.2	46.8 ± 9.4	0.79
Sex (male/female)	133/96	141/107	0.07
Smoking history	34.90%	18.10%	< 0.01
Alcohol intake	17.40%	8.10%	< 0.01
BMI (kg/m^2)	24.5 ± 2.0	26.0 ± 2.2	< 0.01
Hypertension	14.80%	16.10%	0.09
Diabetes,	7.90%	6.90%	0.13
CRP (mg/L)	36.3 ± 20.8	5.5 ± 2.3	< 0.01
WBC (× 10^9/L)	9.7 ± 2.9	4.3 ± 0.9	< 0.01

BMI: Body mass index; CRP: C-reactive protein; WBC: White blood cell.

Table 2 *Helicobacter pylori* infection rate between different Crohn's disease types n (%)

Group	*H. pylori*-positive	*H. pylori*-negative	P value
CD	62 (27.1)	167 (72.9)	< 0.01
Colonic CD	14 (31.1)	31 (68.9)	< 0.01
Small intestine CD	11 (28.9)	27 (71.1)	< 0.01
Ileocolonic CD	30 (26.8)	82 (73.2)	< 0.01
Extensive CD	7 (25.9)	27 (74.1)	< 0.01
Control	119 (47.9)	129 (52.1)	

H. pylori: *Helicobacter pylori*; CD: Crohn's disease.

in the CD subgroups of remission, moderate CD, and severe CD, with corresponding *H. pylori*-positive rates of 34.3%, 30.7% and 22.0% respectively (Table 3). All three subgroups had significantly lower *H. pylori* infection rates than the control group. Nevertheless, though there was a decrease trend in *H. pylori* infection rate from the CD remission group to the severe CD group, there was no significant difference among these three groups.

DISCUSSION

Crohn's disease can affect the entire digestive system from mouth to anus, but it is most commonly seen in the final segment of the small bowel and the first part of the colon. The frequency of CD has significantly increased in the last century, becoming a heavy economic burden[24]. The etiology of CD is still not completely understood[25], although *NOD2/CARD15* was the first CD susceptibility gene to be discovered[26]. Oxidative stress, autophagy, endoplasmic reticulum stress and other molecular pathways have been correlated with CD prevalence[27,28]. Moreover, an infectious organism might be the initiating factor for CD pathogenesis. This hypothesis was supported by evidence from animal models showing that spontaneous colitis did not develop in a germ-free environment[29]. Nevertheless, none of suggested bacterial causes have been conclusively proven[5].

The role of *H. pylori* in IBD pathogenesis has been enticing. Generally, *H. pylori* had two main subgroups: gastric *Helicobacter* that preferentially colonize the stomach, and enterohepatic *Helicobacter* that infect the intestinal or hepatobiliary system[30]. Accumulating evidence from gene knockout rodents indicate that the presence of enterohepatic helicobacter worsens the severity or hastens the development of colitis[31,32]. A more causative role was suggested by the observation that *Helicobacter muridarum* can provoke CD in severe combined immunodeficiency mice upon receipt of T cells[33]. In addition, lower *H. pylori* infection rate in CD patients was not influenced by antibiotic use[34]. However, the results from human studies are confusing. The largest study examining the association between *H. pylori* infection and CD was conducted in the Netherlands by Wagtmans *et al*[35], which enrolled 386 CD patients and 277 controls. Though their results supported the positive association between *H. pylori* infection and CD prevalence, the credibility of these findings was weakened by the use of serum *H. pylori*-IgG as the diagnostic tool. A meta-analysis was conducted that supported the involvement of *H. pylori* infection in CD, but study heterogeneity and publication bias decreased its credibility[15]. In contrast, two independent studies found higher *H. pylori* infection in CD patients than in controls (12% *vs* 4% and 14.0% *vs* 1.4%, respectively)[14,36]. Furthermore, other studies investigating *Helicobacter* species in human colon also failed to find any correlation with CD[37,38].

To tackle this discordancy, we retrospectively investigated the association between *H. pylori* infection and CD in a large case control study of Chinese patients. The initial results showed a significantly lower *H. pylori* infection rate in the CD group, which is in accordance with the previous meta-analysis[15]. The ^{14}C-UBT and biopsy sample culture had higher sensitivity and specificity than the serum *H. pylori*-IgG test, increasing the credibility of these findings. The significantly lower BMI in CD patients may be due to malnutrition caused by diarrhea and other GI symptoms. Based on subgroup analysis (Tables 2 and 3), we found significantly lower *H. pylori* infection in each CD subgroup and a trend of decreased *H. pylori* infection paralleling with increased CD severity, increasing the correlation between *H. pylori* infection and CD. Theoretically, it is possible for a protective role of *H. pylori* infection in CD[39]. In detail, *H. pylori* is able to decrease immune-mediated intestinal injury by triggering Th1 dominated cell defense[40] and inhibit other bacterially-induced mucosal damage by inducing antibacterial peptide production[41].

Several limitations of this study should be acknowledged. First, *H. pylori* infection in colon biopsy was not detected, which may decrease disease occurrence rate. Furthermore, other members of the *Helicobacter* family are associated with the development of gut inflammation and some are found more commonly in people with IBD when compared to healthy controls. However, these members colonize the lower gut, rather than having a location limited to the stomach. Therefore, while these data are convincing, it would be helpful to detect them in another independent experiment. Second, it is better to use ^{13}C-UBT instead of ^{14}C-UBT, since the for-

Table 3 *Helicobacter pylori* infection between different severity of Crohn's disease and control *n* (%)

Group	*H. pylori*-positive	*H. pylori*-negative	*P* value
CD	11 (34.3)	21 (65.7)	< 0.01[1]
Colonic CD	27 (30.7)	61 (69.3)	< 0.01[1]
Small intestine CD	24 (22.0)	85 (88.0)	< 0.01[1]
Control	119	129	

[1]*vs* control group. *H. pylori*: *Helicobacter pylori*; CD: Crohn's disease.

mer has no radiation and is safer for patients[42]. We have plans to implement this technique in our laboratory in the future. Third, wireless capsule endoscopy was used to detect small intestine CD. Though the effect of capsule endoscopy in small intestine CD diagnosis has been recognized[43], the lack of biopsy results may decrease its credibility. It would be helpful to include double balloon small bowel endoscopy. Fourth, the trend of decreased *H. pylori* infection paralleling with increased CD severity should be repeated in a larger clinical trial for statistical significance. Fifth, it is better to report *H. pylori* infection rate in IBD, where *H. pylori* in UC should be reported. We found a 30.5% *H. pylori* infection rate in UC patients and are currently preparing these data for submission. Finally, the causative effect of *H. pylori* infection in CD cannot be established through case control studies and further prospective clinical trial data are necessary.

In conclusion, our results provide evidence for the involvement of *H. pylori* in CD prevalence. These findings should serve as an important reminder to clinicians when considering *H. pylori* eradication in CD patients.

COMMENTS

Background

Crohn's disease (CD) is a chronic and relapsing inflammatory disease affecting any part of the intestine, with distal ileal, ileocecal and colonic regions most commonly involved. CD is more common in Northern Europe and America than in Southern Europe and developing countries, with increased incidence since the mid-1970s and unknown etiologies.

Research frontiers

Increasing evidence supports the combinational involvement of genetic predisposition, immune response, and environment, especially gut bacteria and antigens. Though some pathogens have been considered as infectious agents in CD, conclusive data is still lacking. Considering the immune regulation and colonization capacity of *Helicobacter pylori* (*H. pylori*) and the nature of autoimmune-related damage in CD, it is plausible that *H. pylori* infection may take part in the etiology of CD. However, results in humans remain unclear and contradictory. While *Helicobacter* was absent or only detected in the intestinal mucosa of a few patients in English and Australian studies, another study identified enterohepatic *Helicobacter* species in patients with inflammatory bowel disease. Furthermore, a meta-analysis suggested a protective role of *H. pylori* infection in CD pathogenesis. However, the heterogeneity among enrolled studies and the possibilities of publication bias limited the confidence of those results.

Innovations and breakthroughs

The authors conducted a large-scale case control study to investigate the association between *H. pylori* infection and different severity and type of CD. These findings are the first example of a significant correlation between *H. pylori* infection rate in CD patients and different subtypes.

Applications

Lower *H. pylori* infection in CD patients provides evidence for bacterial involvement in the pathogenesis of CD and reminds clinicians remain cautious when considering *H. pylori* eradication in CD patients.

Peer review

Their results supported the potential involvement of *H. pylori* infection in CD pathogenesis and raised concern for the necessity of *H. pylori* eradication in CD patients. This manuscript provides some key information, and builds on the published literature. The authors should be able to enhance this by extensive revisions.

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BRIEF ARTICLE

Antinociceptive effect of berberine on visceral hypersensitivity in rats

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Abstract

AIM: To assess the protective effect of berberine administration and the role of nitric oxide (NO) in visceral hypersensitivity.

METHODS: Fifty male Sprague-Dawley rats were randomly assigned to five groups. An inflammatory bowel disease model was induced in rats by intracolonic instillation of 1 mL 4% acetic acid at 8 cm proximal to the anus for 30 s and restraint stress. After subsidence of inflammation on day 7 of the experiment, the rats were subjected to rectal distension, performed by a balloon (6-Fr, 2 mm external diameter, disposable silicon balloon-urethral catheter for pediatric use) which was rapidly inflated with increasing volumes of prewarmed (37 ℃) water (0.1, 0.2, 0.3, 0.4, 0.6, 0.8 and 1 mL) for 30 s at four-minute intervals, and then the abdominal withdrawal reflex (AWR) and the level of fecal output were measured, respectively. AWR scores either 0, 1, 2, 3 or 4 were obtained by blinded observers. Rats had been pretreated with berberine or aminoguanidine (NO synthetase inhibitor) or berberine + aminoguanidine before measurement.

RESULTS: The rats in the placebo group showed a hypersensitive response to rectal distension (2.69 ± 0.08 *vs* 1.52 ± 0.08, P = 0.000) and defecated more frequently than those in the control group (5.0 ± 0.16 *vs* 0.44 ± 0.16, P = 0.000). Comparing the berberine with placebo group, the AWR scores were reduced for all distension volumes and were significant at 0.2-1 mL (1.90 ± 0.08 *vs* 2.69 ± 0.08, P = 0.000), while the numbers of hard pellets, soft pellets, formless stools, and total fecal output in the placebo group were significantly larger than in the berberine group (5.0 ± 0.16 *vs* 2.56 ± 0.16, P = 0.000). Administration of aminoguanidine or berberine + aminoguanidine before VH score measurement reversed the antinociceptive effect of berberine (2.52 ± 0.08 *vs* 1.90 ± 0.08, P = 0.000; 2.50 ± 0.08 *vs* 1.90 ± 0.08, P = 0.000). The numbers of hard pellets, soft pellets, formless stool, and total of fecal output in aminoguanidine group were significantly larger than the corresponding values in control group, berberine group, and berberine + aminoguanidine group (4.81 ± 0.16 *vs* 0.44 ± 0.16, P = 0.000; 4.81 ± 0.16 *vs* 2.56 ± 0.16, P = 0.000; 4.81 ± 0.16 *vs* 3.75 ± 0.16, P = 0.000). The berberine and berberine + aminoguanidine groups showed reduced defecation, but aminoguanidine alone did not reduce defecation (2.56 ± 0.16 *vs* 4.81 ± 0.16, P = 0.000; 3.75 ± 0.16 *vs* 4.81 ± 0.16, P = 0.000).

CONCLUSION: Berberine had an antinociceptive effect on visceral hypersensitivity, and NO might play a role in this effect.

Key words: Berberine; Irritable bowel syndrome; Visceral hypersensitivity; Nitric oxide

Core tip: Berberine had an antinociceptive effect on visceral hypersensitivity. This effect was reduced by nitric oxide (NO) synthetase inhibitor, thus NO might play a role in the effect of berberine.

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INTRODUCTION

It is believed that chronic visceral hypersensitivity (VH), abnormal gastrointestinal motility and altered central processing may be major pathophysiological mechanisms of irritable bowel syndrome (IBS)[1]. Gut hypersensitivity may lead to alterations in gut motility by disturbing regulatory reflex pathways and secretory function[2]. These abnormalities typically reflect the symptom pattern of IBS, which is characterized by abdominal pain or discomfort, and is associated with alterations in defecation frequency, stool passage, and stool form[3,4].

According to the recent Rome Ⅲ Criteria[5], IBS can be diagnosed based on at least 3 mo, with onset at least 6 mo, of recurrent abdominal pain or discomfort associated with two or more of the followings: (1) improvement with defecation; (2) onset associated with a change in stool frequency; and (3) onset associated with a change in stool form (appearance). Additionally, IBS patients are further subdivided into IBS with diarrhea, IBS with constipation, mixed IBS with diarrhea and unspecified IBS.

VH is a consistent finding in a large proportion of patients with IBS and provides a physiological basis for the development of IBS symptoms[2,6]. However, the exact mechanism of its action is still unclear. Recently, it has been documented that increasing the nitric oxide (NO) level in the extracellular space of the target tissue is one of the major mechanisms involved and this has been clarified in recent molecular studies[7]. There is growing evidence from previous studies that NO plays an important role in pain transmission and the antinociceptive action on VH or peritoneal pain[2,6,8-10].

It has been discovered that some drugs can be used to attenuate VH in IBS patients[11]. However, many drug treatments have not been satisfactory, with intractable adverse effects. Therefore, it is necessary to seek an effective and low-cost treatment for IBS. Berberine (Coptis chinensis Franch, var. asperma Don, family Ranunculaceae) is a botanical alkaloid isolated from the root and bark of *Rhizoma coptidis*, an ancient Chinese herb that has been used to treat gastroenteritis for many years, which is preferred for its inexpensiveness and low incidence of adverse effects[12]. It has been demonstrated that berberine has multiple pharmacological activities including anti-inflammatory[13], antimicrobial[14], anticancer[15,16], antidiabetic[17], antiarrhythmic[18], and antiseptic[19] effects. According to former studies, berberine has a significant effect in the treatment of experimental colitis[20-22]. Further evidence has shown that, in relation to the NO pathway, berberine has a significant effect on ethanol-induced gastric ulcers[23], endothelial progenitor cell mobilization and function[24], hyperglycemia-induced cellular injury and endothelial dysfunction[25], the early phase of hepatocarcinogenesis[26], and a rat model of Alzheimer's disease[27]. To establish whether berberine has a beneficial effect in IBS patients through reversal of VH, we examined the effects of berberine in a validated rodent model in which acute inflammation of the colon was associated with VH. The aim of this study was to evaluate whether berberine treatment prevents progression of VH to colorectal distension (CRD), and the involvement of NO in these effects.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats, weighing 270-300 g, were obtained from the Animal Facility of Southeast Hospital, Zhangzhou, China. The rats were housed individually in an access-restricted room with controlled conditions (22 ± 1 ℃ and 65%-70% humidity) with free access to standard laboratory food and water. All the experimental protocols in this study were reviewed and approved by the Animal Studies Ethics Committee of Southeast Hospital.

Experiment model

The rats were lightly anesthetized with ether after an overnight fast and colitis was induced by intracolonic instillation of 1 mL 4% acetic acid at 8 cm proximal to the anus for 30 s. Then, 1 mL PBS was instilled to dilute the acetic acid and flush the colon. The control animals were handled identically except that 1 mL saline was instilled instead of 4% acetic acid. Rats were left to recover from colitis for 6 d, and were used for the experiments 7 d after induction of colitis.

Histological examination of inflammation

To examine the extent of colonic inflammation, histological samples were collected at the selected time points (2 and 7 d post-enema in two rats of each group). Sections with a thickness of 5 μm were cut and processed for hematoxylin and eosin staining. The coded slides were analyzed by a pathologist blinded with regard to the treatment group and the time points.

Rectal distension procedure

At 7 d post-enema, eight rats in each group were used for studying progression of VH to CRD. A 6-Fr (2 mm external diameter) disposable silicon balloon-urethral catheter for pediatric use was used. The maximal inflation volume for the balloon was 1 mL and the length of the maximally inflated balloon was 1.2 cm. After an overnight fast, the animals were lightly anesthetized with

ether, and the balloon was carefully inserted into the rectum until the premarked line on the catheter (2 cm distal from the end of the balloon) was positioned at the anus. The catheter was taped to the base of the tail to prevent displacement. After this procedure, the rats were placed in a transparent cubicle (20 cm × 8 cm × 8 cm) on a mirror-based, elevated platform while still sedated, and were allowed to recover and adjust for a minimum of 30 min before testing. The catheter was connected to a pressure transducer *via* a three-way connector. The signals from pressure transducer were processed and recorded on an IBM-compatible computer.

After the animals were fully awake and adjusted to the environment, ascending-limit phasic distension (0.1, 0.2, 0.3, 0.4, 0.6, 0.8 and 1.0 mL) was applied for 30 s every 4 min to induce CRD. The balloon was distended with prewarmed (37 ℃) water. We chose this protocol because hypersensitivity was reported to be best elicited by rapid phasic distension. The abdominal withdrawal reflex (AWR) was semiquantitatively scored as previously described[4]. The AWR score was assigned as follows: 0 = no behavioral response to distension; 1 = brief head movements followed by immobility; 2 = contraction of abdominal muscle without lifting of the abdomen; 3 = lifting of the abdomen; and 4 = body arching and lifting of pelvic structure.

After the experiments, the balloon was withdrawn and immersed in 37 ℃ water. The compliance of balloon was not infinite, therefore, we measured intraballoon pressure at each distension volume in 37 ℃ water, and digitally subtracted the value from that recorded during the CRD experiment to calculate the intrarectal pressure.

Restraint stress procedure

The rats were housed individually with no restrictions on food intake before testing. At 7 d post-enema, eight rats from each group were placed in restraint cages (5 cm × 5 cm × 20 cm), which could limit their body movement, but not restrict breathing. The rats were in the restraint cages for 3 h at room temperature. The feces excreted during restraint stress were divided into three types: hard pellet, soft pellet, and formless, and counted separately.

Experimental protocol

Ten healthy rats without treatment served as controls. In the placebo group, IBS was induced as described above and eight rats were treated once with physiological saline 1 d after enema. In the berberine group, IBS was induced as described above and eight rats were treated once daily with berberine (50 mg/kg) 1 d after enema. In the aminoguanidine group, eight rats were treated once daily with aminoguanidine (100 mg/kg) *via* intraperitoneal injection 1 d after enema. In the berberine + aminoguanidine group, eight rats were treated once daily with berberine (50 mg/kg) 1 d post-enema, and then were treated once daily with aminoguanidine (100 mg/kg) *via* intraperitoneal injection.

Statistical analysis

Data were expressed as mean ± SD. Significant differences between the three groups (AWR score) at each distension volume were statistically analyzed using ANOVA. The relationship between the intraballoon volume and intrarectal pressure was determined by linear regression analysis, and the estimated slope coefficients and intercepts were compared between groups using ANOVA. The level of fecal output was compared using ANOVA and further analyzed using Bonferroni's or Tamhane's *T2* test. Differences with $P < 0.05$ were considered to be significant. Multiple comparisons between the groups were corrected by SPSS version 13.0 software.

RESULTS

Histology of colonic tissue

Figure 1 shows the histology of the distal colon at days 2 and 7 after acetic acid instillation in the control group, placebo group, berberine group, aminoguanidine group, and berberine + aminoguanidine group. Mucosal hemorrhage with an inflammatory infiltrate in the lamina propria and the edematous submucosa were observed in the IBS model group, berberine group, aminoguanidine group and berberine + aminoguanidine group 2 d after acetic acid instillation. At day 7 after induction of colitis, all signs of inflammation had disappeared. No remarkable inflammatory features were detected at 7 d after acetic acid instillation in each group.

Detection of VH

The AWR scores were recorded in at least eight rats in each group after CRD was induced. The changes in AWR score paralleled the balloon volume in CRD, which confirmed that the AWR score reflected the intensity of distension. Comparing the berberine with placebo group, the AWR scores were reduced for all distension volumes and were significant at 0.2-1.0 mL (1.90 ± 0.08 *vs* 2.69 ± 0.08, $P = 0.000$). These data indicated that progression of VH to CRD was attenuated by berberine (Figure 2A). As shown in Figure 2A, rats in the placebo group showed a hypersensitive response to the ascending-limit phasic rectal distension, and berberine effectively reduced VH (2.69 ± 0.08 *vs* 1.90 ± 0.08, $P = 0.000$). However, VH was not effectively reduced in the aminoguanidine and berberine + aminoguanidine groups. The pain threshold (minimal volume to induce AWR 2) was measured in rats that underwent CRD. As shown in Figure 2, berberine significantly increased the nociceptive threshold in rats. Administration of aminoguanidine or berberine + aminoguanidine before VH score measurement reversed the antinociceptive effect of berberine (2.52 ± 0.08 *vs* 1.90 ± 0.08, $P = 0.000$; 2.50 ± 0.08 *vs* 1.90 ± 0.08, $P = 0.000$). In order to examine whether VH in rats was related to changes in rectal compliance, we compared the intraballoon volume-intrarectal pressure relationship in the five groups. A distension volume of 0.2-1.0 mL and

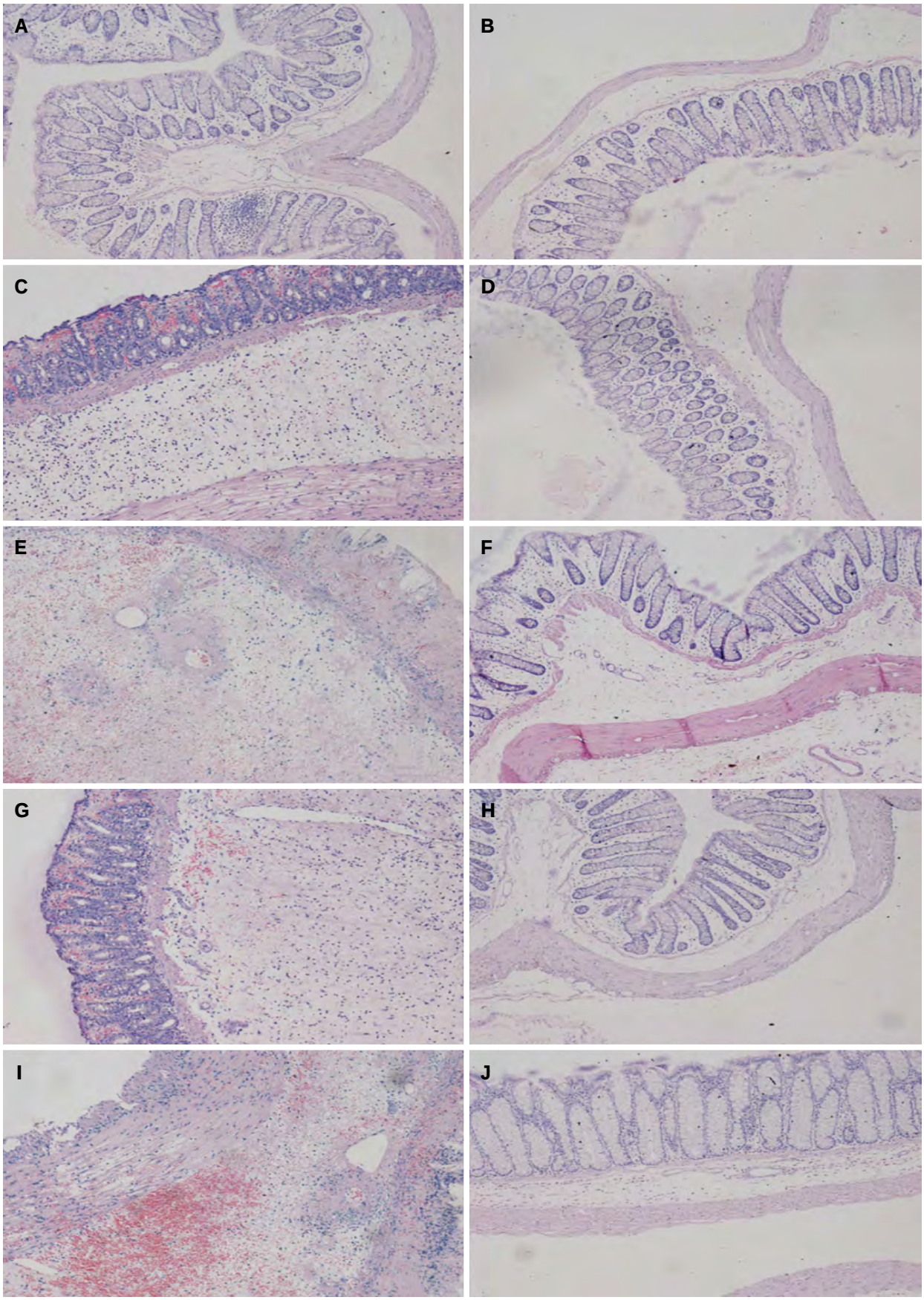

Figure 1 Photomicrographs (hematoxylin and eosin stain, × 100) of distal colon at 2 and 7 d, respectively. A, B: Control group; C, D: Placebo group; E, F: Berberine group; G, H: Aminoguanidine group; I, J: Berberine + aminoguanidine group. At 2 d, histological inflammatory features including mucosal hemorrhage, submucosal edema, and inflammatory infiltration in the lamina propria and the submucosa were observed in the placebo, berberine, aminoguanidine, and berberine + aminoguanidine groups (A, C, E, G, I). At 7 d, there was no marked inflammatory feature compared with the control group (B, D, F, H, J).

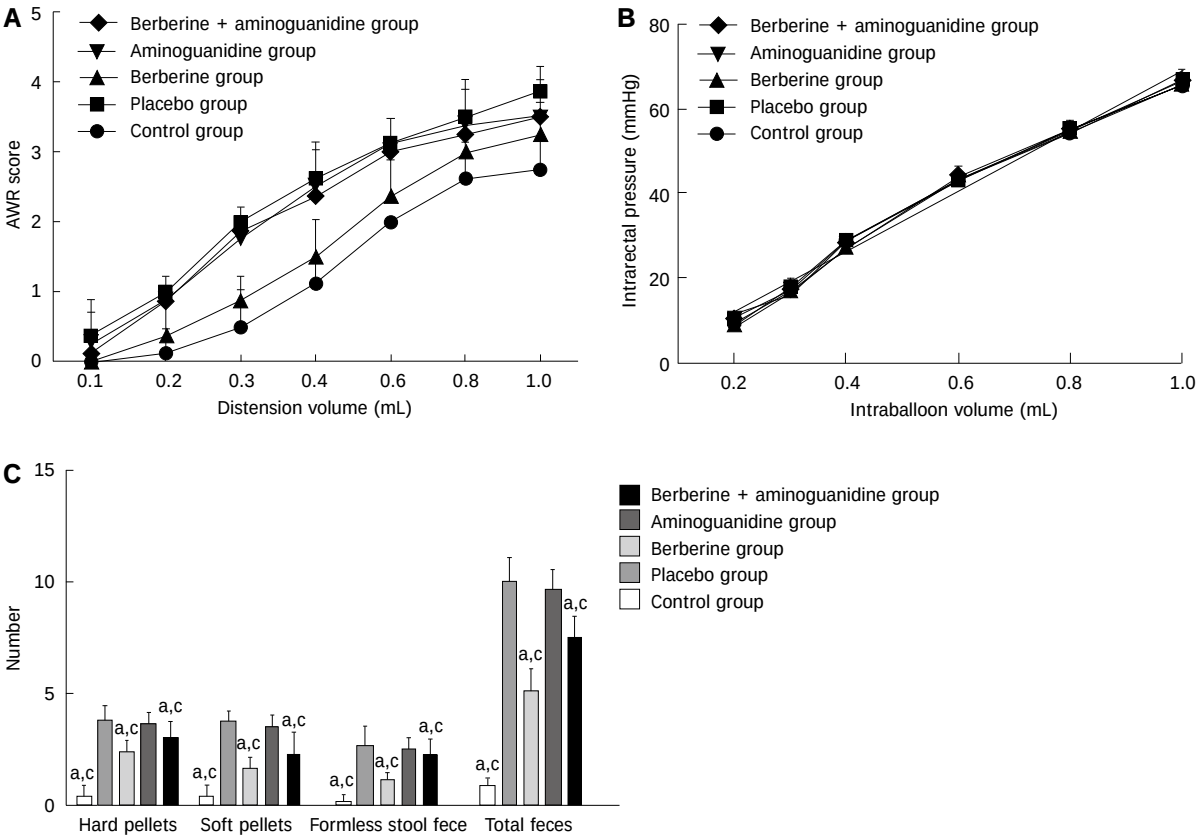

Figure 2 Summarized plots. A: Colorectal distension-induced abdominal withdrawal reflex (AWR) in each group. Comparing the berberine and placebo groups, the AWR scores were reduced at all distension volumes and were significant at 0.2-1.0 mL. The AWR score in the berberine group was significantly lower than in the aminoguanidine and berberine + aminoguanidine groups; B: The relationship between intraballoon volume and intrarectal pressure in each group. The distension volume from 0.2 to 1.0 mL and the corresponding intrarectal pressure were plotted for regression analysis. Intrarectal pressure was linearly increased as the balloon was inflated. The fitted functions of the five groups were not significantly different; C: Restraint-stress-induced defecation in each group. Defecation in the placebo and aminoguanidine groups was significantly more frequent than in the control, berberine and berberine + aminoguanidine groups ($^{a}P < 0.05$ *vs* placebo group; $^{c}P < 0.05$ *vs* aminoguanidine group). The berberine and berberine + aminoguanidine groups showed reduced defecation, but aminoguanidine group alone did not effectively reduce defecation.

the corresponding intrarectal pressure were plotted for regression analysis. Intrarectal pressure increased linearly as the balloon was inflated ($r = 0.9758$, $P < 0.001$, in the control group; $r = 0.9842$, $P < 0.001$, in the placebo group; $r = 0.9822$, $P < 0.001$, in the berberine group; $r = 0.9773$, $P < 0.001$ in the aminoguanidine group; and $r = 0.9827$, $P < 0.001$ in the berberine + aminoguanidine group). The fitted functions of the five groups were not significantly different (Figure 2B).

Restraint-stress-induced defecation

As demonstrated in Figure 2C, the numbers of hard pellets, soft pellets, formless stools, and total fecal output in the placebo were significantly larger than in the berberine groups (5.0 ± 0.16 *vs* 2.56 ± 0.16, $P = 0.000$). As shown in Figure 2C, the number of hard pellets, soft pellets, formless stool,and total of fecal output in aminoguanidine group were significantly larger than the corresponding values in control group, berberine group, and berberine + aminoguanidine group (4.81 ± 0.16 *vs* 0.44 ± 0.16, $P = 0.000$; 4.81 ± 0.16 *vs* 2.56 ± 0.16, $P = 0.000$; 4.81 ± 0.16 *vs* 3.75 ± 0.16, $P = 0.000$). The berberine and berberine + aminoguanidine groups showed reduced defecation, but aminoguanidine alone did not reduce defecation (2.56 ± 0.16 *vs* 4.81 ± 0.16, $P = 0.000$; 3.75 ± 0.16 *vs* 4.81 ± 0.16, $P = 0.000$).

DISCUSSION

This study was performed in order to clarify the effects of berberine administration on VH in a rat model of IBS. Berberine effectively attenuated the heightened visceral nociceptive response, that is, an increase in AWR score to CRD, in rats recovering from experimental colitis. In the placebo group, 7 d after instillation of acetic acid when there was no sign of inflammation in the colon, these rats still had VH and a high frequency of defecation in response to restraint stress. Stool form in the placebo group was softer and more shapeless than in the control group. These findings are in accordance with the clinical symptoms in IBS patients, but there is a great dilemma in using this model to investigate the effect of drugs on IBS.

To avoid the ambiguity that any sign of improvement can be interpreted as a drug effect on colitis, histopathological parameters of inflammation in each group, 2 and 7 d after acetic acid instillation, were evaluated and there

was no difference. Therefore, we concluded that berberine administration, at least at the dose used in our study, had neither a positive nor negative effect on the histopathological parameters of inflammation in the colon, and did not impair establishment of the postinflammatory model. Considering all these factors, we elucidated the effects of berberine administration at a dose that showed a beneficial effect on the inflammatory response. This indicated that berberine significantly reduced VH and stool frequency and increased stool consistency. We investigated the role of NO in the protective effects of berberine using an experimental model with the NO synthetase (NOS) inhibitor aminoguanidine. We demonstrated that aminoguanidine significantly reduced the effect of berberine on VH, which suggests this effect of berberine is at least partly mediated through NOS.

Berberine has been used in the treatment of gastroenteritis and infectious diarrhea in Chinese traditional medicine for thousands of years. Some recent studies have indicated that it has various pharmacological effects, including anti-inflammatory[20] and antimicrobial[28] effects; each of which may contribute to the antidiarrheal effect. The fact that berberine has low bioavailability and shows poor absorption through the colon wall (< 5%)[29] support the thesis that it may exert its antidiarrheal effect on the intestinal epithelial cells before absorption. However, until now the effect of berberine on HV has not been confirmed.

NO is a key neurotransmitter in both short- and long-acting inhibitory motor neurons[7] and plays a critical part in mediating gastrointestinal motility. Some studies have revealed that NOS neuronal activity considerably changes after inflammation and is responsible for some acute postinflammatory consequences in bowel-like ileus[30,31]. Apart from its major role in the peripheral nervous system, such as in the enteric inhibitory nerves of the myenteric plexus, NO is believed to be an intracellular messenger or neurotransmitter in the central nervous system (CNS). It has been verified that due to its free diffusibility, NO acts as a retrograde transmitter in the CNS, mediating some nervous paradigms, for instance, long-term potentiation, and is the key neurotransmitter in descending inhibitory neurons modulating nociception at the spinal level[32]. Furthermore, it proves that NO is involved in the modulation of visceral perception, for example, intraperitoneal injection of acetic acid in rats increases nitrergic neurons in specific regions of the brain, and NOS immunoreactivity has been confirmed in lumbosacral afferents and preganglionic neurons innervating the pelvic viscera[33]. Therefore, some hypotheses can be performed based on our results, which interpret the protective effects of berberine through NO on VH in IBS at the myenteric plexus level, CNS, and smooth muscles. Our results could be interpreted at the myenteric plexus level.

Our findings were similar to a recent study that identified no significant difference in NO-containing neurons of the colonic myenteric plexus between IBS rats and controls[34]. Thus, we hypothesize that basal NO synthesis is not significantly decreased in IBS, and it might be that the positive effects of berberine decrease VH through increasing NO levels. Therefore, we speculate that the positive effects of berberine on VH in the postinflammatory rat model are at least partly exerted through NO synthesis potentiation.

We also examined the effect of aminoguanidine (NOS inhibitor) on stool form (hard pellets, soft pellets, and shapeless) in IBS rats under restraint stress. Aminoguanidine had no noticeable effect on stool form, but it did diminish the protective effects of berberine on stool form. NO plays a major role in mediating gastrointestinal motility, which is critical for stool formation. As a result of the short time between acute drug administration and defecation measurement, aminoguanidine did not produce any change in the stool consistency pattern of rats. The effects of aminoguanidine on stool frequency were more sophisticated and hard to explain. The protective effects of berberine on stool frequency were diminished by aminoguanidine, but the specific mechanism was not established. Further study will be needed to explore the specific mechanism. However, it should be noted that this study investigated only the antinociceptive effect of berberine on VH. One of the limitations of this study is the lack of measurement of NOS inhibitors.

In conclusion, we indicated that berberine administration prevented progression of VH to CRD. It is possible that NO released by berberine may affect colonic hypersensitivity. All of our data suggest that berberine may be of interest in the treatment of visceral hyperalgesia, particularly in IBS.

COMMENTS

Background

It is believed that chronic visceral hypersensitivity (VH), abnormal gastrointestinal motility, and altered central processing may be major pathophysiological mechanisms of irritable bowel syndrome (IBS). Berberine is an ancient Chinese herb that has been used to treat gastroenteritis for many years, which is preferred for its low cost and low incidence of adverse effects. Recently, berberine has been shown to have a considerable effect in the treatment of experimental colitis. However, the mechanism remains unknown.

Research frontiers

Berberine was used to treat rats with VH induced by 4% acetic acid. Berberine administration significantly increased the nociceptive threshold in rats, whereas the administration of aminoguanidine or berberine + aminoguanidine before VH measurement reversed the antinociceptive effect of berberine. The mechanism underlying the effect of berberine on VH of rats appears to be partly mediated by nitric oxide (NO).

Innovations and breakthroughs

Recently, it has been demonstrated that berberine has multiple pharmacological activities. In this study, authors indicated that berberine may be of interest in the treatment of VH, particularly in IBS.

Applications

The present study demonstrated that berberine administration prevented progression of VH to colorectal distension in a rat model of IBS. The effect of berberine is mediated by NO pathways, thus providing evidence for the treatment of VH in IBS.

Terminology

IBS is a common gastrointestinal disorder characterized by chronic visceral pain and bloating in association with altered gut movements. Berberine is an ancient Chinese herb that might play a role in the treatment of IBS.

Peer review

This paper describes positive effects of berberine on VH in rats with IBS. It is reported that berberine administration significantly increased the nociceptive threshold in rats, whereas administration of aminoguanidine or berberine + aminoguanidine before VH measurement reversed the antinociceptive effect of berberine. The results presented are crucial for clinicians and for the fundamental scientific community as well.

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BRIEF ARTICLE

Increased expression of matrix metalloproteinase-9 associated with gastric ulcer recurrence

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Abstract

AIM: To compare matrix metalloproteinase (MMP)-9 and tissue inhibitor of metalloproteinase (TIMP)-1 in gastric ulcer (GU) and chronic superficial gastritis (CSG).

METHODS: This study enrolled 63 patients with GU and 25 patients with CSG. During upper gastroduodenal endoscopy, we took samples of gastric mucosa from the antrum and ulcer site from patients with GU, and samples of antral mucosa from patients with CSG. Mucosal biopsy tissues were cultured for 24 h, and the culture supernatant was measured for levels of MMP-9 and TIMP-1. After receiving eradication therapy for *Helicobacter pylori* (*H. pylori*) and 8 wk proton-pump inhibitor therapy for GU, follow-up endoscopy examination was performed after 6 mo and whenever severe symptoms occurred.

RESULTS: Levels of MMP-9 and TIMP-1 at the ulcer site or in the antrum were significantly higher in GU than CSG patients. MMP-9 levels at the ulcer site were significantly higher than in the antrum in GU patients, and had a significantly positive correlation with TIMP-1. MMP-9 levels were significantly higher in *H. pylori*-positive than *H. pylori*-negative GU and CSG patients. Levels of MMP-9 or TIMP-1 at the ulcer site were associated with the histological severity of activity and inflammation. About 57 GU patients were followed up, and seven had GU recurrence. *H. pylori*-infection and MMP-9 levels were risk factors for the recurrence of GU adjusted for age and sex by multiple logistic regression analysis.

CONCLUSION: MMP-9 may perform an important function in gastric ulcer formation and recurrence.

Key words: Gastric ulcer; Matrix metalloproteinase-9; Tissue inhibitor of metalloproteinase-1; *Helicobacter pylori*

Core tip: Gastric ulcer is a multifaceted process including acid secretion, reactive oxygen species generation, prostaglandin inhibition, and extracellular matrix degradation. Gastric mucosal damage is directly associated with extracellular matrix degradation in which matrix metalloproteinases (MMPs) play a crucial role. In this study, the authors compared MMP-9 and tissue inhibitor of metalloproteinase-1 levels in patients with gastric ulcer or chronic superficial gastritis.

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INTRODUCTION

Gastric ulcer (GU) is a multifaceted process including acid secretion, reactive oxygen species generation, prostaglandin inhibition, and extracellular matrix (ECM) degradation[1]. Gastric mucosal damage is directly associated with extracellular matrix degradation in which matrix metalloproteinases (MMPs) play a crucial role[2]. MMPs are endopeptidases that perform important functions in ECM remodeling, cell proliferation, and inflammatory processes. Recent studies have indicated that gastric ulceration is associated with cleaving and remodeling of the ECM by MMPs[3,4]. In several animal studies of GU, attention has focused on the role of MMP-1, MMP-2, MMP-3, MMP-9 and MMP-13[4-6]. In particular, MMP-9 is important in the early phase of chronic GU[7]. However, these data are mostly derived from animal studies, and human clinical data remains rare, especially in assessing MMPs expression in GU formation and recurrence. Here, we compared MMP-9 and tissue inhibitor of metalloproteinase (TIMP)-1 in patients with GU or chronic superficial gastritis (CSG), and how they correlated with GU recurrence.

MATERIALS AND METHODS

Patient selection

We examined 63 consecutive patients with GU and 25 with CSG who were diagnosed during upper gastroduodenal endoscopic examination at Liaocheng People's Hospital between January and December 2010. The patients were enrolled in the study if they met the following criteria: (1) age 18-75 years; (2) no nonsteroidal anti-inflammatory drugs (NSAIDs), antibiotics, or bismuth compounds in the 2 wk prior to the study; and (3) acute phase GU. Patients were excluded as follows: (1) a history of gastric or duodenal surgery; (2) allergy to the study drugs; (3) required long-term treatment with NSAIDs, corticosteroids, aspirin, or anticoagulant agents; (4) pregnant women; and (5) active cancer, acute serious medical illness, or terminal illness. The study protocol was approved by the Ethics Committee of our institution. All patients gave written informed consent before participating in the study.

Endoscopic examination

During endoscopic examination, three antral specimens were taken from all patients, including one for rapid urease test (Triwizard, Fujian, China), one for histological examination, and one for *in vitro* culture for measurement of levels of MMP-9 and TIMP-1. Two additional specimens were taken from the margin of the ulcer in GU patients; one for histological examination and one for *in vitro* cultures for MMP-9 and TIMP-1.

Helicobacter pylori infection detection

Helicobacter pylori (*H. pylori*) infection was confirmed by positive results for at least two of three diagnostic tests, namely rapid urease test, ^{13}C-urea breath test, or identification of the organism on tissue sections by Giemsa stain. Absence of infection was defined by a negative result in all three tests. Cases satisfying at least two test results were defined as positive for infection.

Tissue cultures and MMP-9, TIMP-1 assay

Mucosal biopsy tissues were weighed and then cultured in a 5% CO_2 incubator for 24 h in a culture bottle (Xiangya Gene Technology, Changsha, China) containing 5 mL RPMI 1640 medium with 5% heat-inactivated fetal calf serum, 15 mmol/L HEPES buffer, 100 U/mL penicillin-G, 100 mg/mL streptomycin and 10 mg/mL phytohemagglutinin-P. At the end of the culture period, the supernatant was drawn off and stored at -70 ℃ until measured by enzyme-linked immunosorbent assay for MMP-9 and TIMP-1 (Boster, Wuhan, China). A modified version of the Lowry method was used to assay total protein in biopsy homogenates (Boster). The amount of MMP-9 and TIMP-1 was expressed relative to protein content in the biopsy tissue homogenate (per milligram of biopsy protein).

Histology

Tissue sections stained with hematoxylin-eosin were used to assess activity, inflammation, glandular atrophy, and intestinal metaplasia. Grading was done on a four-item scale of 0, 1, 2 and 3, corresponding to none, mild, moderate and severe, respectively, in accordance with the updated Sydney system[8].

Follow up

H. pylori-positive GU patients received eradication treatment with triple therapy using lansoprazole (30 mg, *bid*), amoxicillin (1000 mg, *bid*), and clarithromycin (500 mg, *bid*) for 1 wk, and subsequently received lansoprazole (30 mg, *qd*) for 8 wk, whereas *H. pylori*-negative GU patients received lansoprazole (30 mg, *qd*) for 8 wk. After that, the presence of the ulcer scar was confirmed by endoscopy, and six patients who still had active ulcer were excluded from follow-up. An additional ^{13}C urea breath test or rapid urease test was conducted to assess the final *H. pylori* status after 6 mo for all GU patients. Follow-up endoscopy examination was performed at the end of the 6 mo and whenever severe symptoms occurred. Ulcer recurrence was defined as a lesion of white coat with a distinct depressed area and a diameter of $\geqslant$ 5 mm.

Statistical analysis

All data were expressed as mean ± SD. Frequency variables were compared using the χ^2 test. Quantitative variables were analyzed using Student's *t* test. Correlation was analyzed by Pearson's correlation or Spearman's rank correlation. Logistic analysis was used for risk factors for GU recurrence. SPSS version 17.0 (Chicago, IL, United States) was used, and $P < 0.05$ was regarded as significant.

RESULTS

A total of 88 patients were enrolled. The 63 GU patients

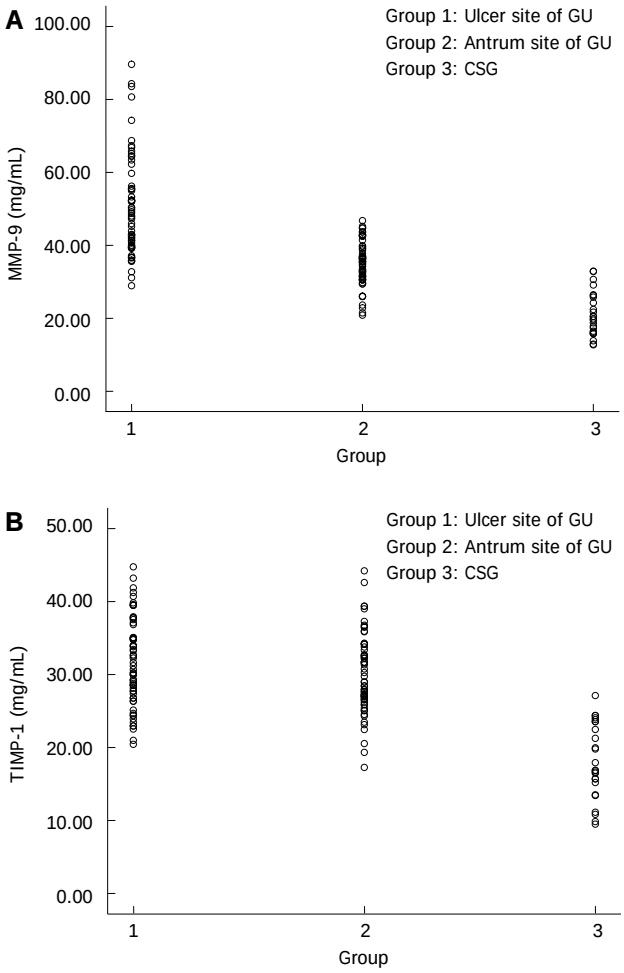

Figure 1 Production of matrix metalloproteinase-9 or tissue inhibitor of metalloproteinase-1 by gastric mucosa in patients with gastric ulcer (*n* = 63) or chronic superficial gastritis (*n* = 25). A: Tissue culture was performed for 24 h. Matrix metalloproteinase (MMP)-9 concentration in tissue culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA); B: Tissue inhibitor of metalloproteinase (TIMP)-1 concentration in tissue culture supernatants was measured by ELISA. GU: Gastric ulcer; CSG: Chronic superficial gastritis.

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

Characteristics	GU group (*n* = 63)	CSG group (*n* = 25)
Sex		
Male	31 (49.2)	15 (60.0)
Female	32 (50.8)	10 (40.0)
Age, yr (mean ± SD)	47.8 ± 12.9	51.3 ± 8.5
H. pylori infection		
Positive	54 (85.7)	10 (40.0)
Negative	9 (14.3)	15 (60.0)
Position of ulcer		
Corpus	18 (28.6)	-
Antrum	45 (71.4)	-

GU: Gastric ulcer; CSG: Chronic superficial gastritis; *H. pylori*: *Helicobacter pylori*.

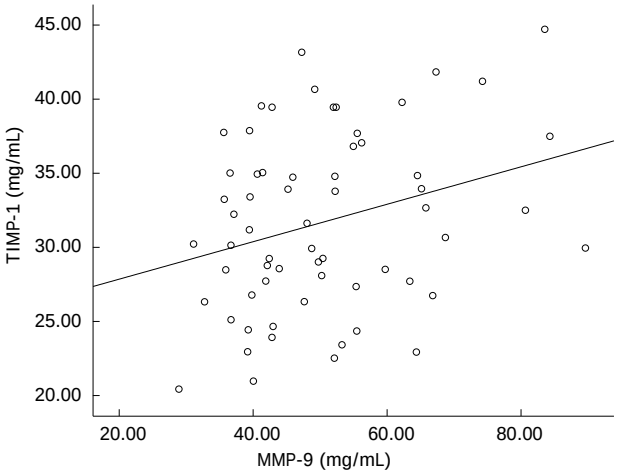

Figure 2 Correlation between matrix metalloproteinase-9 or tissue inhibitor of metalloproteinase-1 production by gastric mucosa in gastric ulcer patients (*n* = 63). Linear regression analysis showed a significant correlation between the two mediators ($r = 0.29$, $P = 0.021$). A significant positive correlation was observed between levels of matrix metalloproteinase (MMP)-9 and tissue inhibitor of metalloproteinase (TIMP)-1 at the ulcer site.

included 31 males and 32 females, with an average age of 47.8 years (range, 24-71 years). The 25 CSG patients included 15 males and 10 females, with an average age of 51.3 years (range, 29-68 years). Fifty-four GU patients were positive and nine were negative for *H. pylori*, and 10 CSG patients were positive and 15 were negative for *H. pylori* (Table 1). There were no significant difference between the GU and CSG patients in age and sex, except in *H. pylori* infection ($\chi^2 = 18.86$, $P < 0.01$).

In all patients, MMP-9 levels (Figure 1A) were significantly higher at the margin of the ulcer (50.50 ± 13.72 mg/mL, $t = 13.96$, $P < 0.01$) or in the antrum (35.08 ± 6.07 mg/mL, $t = 9.78$, $P < 0.01$) of the GU patients than the CSG patients (21.06 ± 6.04 mg/mL). In the GU patients, MMP-9 levels were significantly higher ($t = 8.16$, $P < 0.01$) at the margin of the ulcer (50.50 ± 13.72 mg/mL) than in the antrum (35.08 ± 6.07 mg/mL).

With regard to TIMP-1 levels (Figure 1B), a significant difference was seen between at the margin of the ulcer (18.17 ± 5.14 mg/mL *vs* 31.71 ± 5.97 mg/mL, $t = 9.96$, $P < 0.01$) or in the antrum (18.17 ± 5.14 mg/mL *vs* 30.07 ± 5.42 mg/mL, $t = 9.42$, $P < 0.01$) of the GU and CSG patients. There was no significant difference between the antrum and the margin of the ulcer (30.07 ± 5.42 mg/mL *vs* 31.71 ± 5.97 mg/mL, $t = 1.62$, $P = 0.108$) of the GU patients. A significant positive correlation was observed between levels of MMP-9 and TIMP-1 (50.50 ± 13.72 mg/mL *vs* 31.71 ± 5.97 mg/mL, $r = 0.29$, $P = 0.021$) at the margin of the ulcer in the GU patients (Figure 2).

For the GU patients, ulcers were classified according to their anatomical location, that is, 18 patients had corpus or fundus ulcers, and 45 had antral or prepyloric ulcers. Both MMP-9 (47.45 ± 11.92 mg/mL *vs* 51.72 ± 14.32 mg/mL, $t = -1.12$, $P = 0.267$) and TIMP-1 (30.85 ± 5.93 mg/mL *vs* 32.05 ± 6.02 mg/mL, $t = -0.72$, $P = 0.476$) levels were not significantly different at the margin of the ulcer between corpus or fundus ulcers and antral or prepyloric ulcers.

In the GU patients, levels of MMP-9 (Figure 3A) at

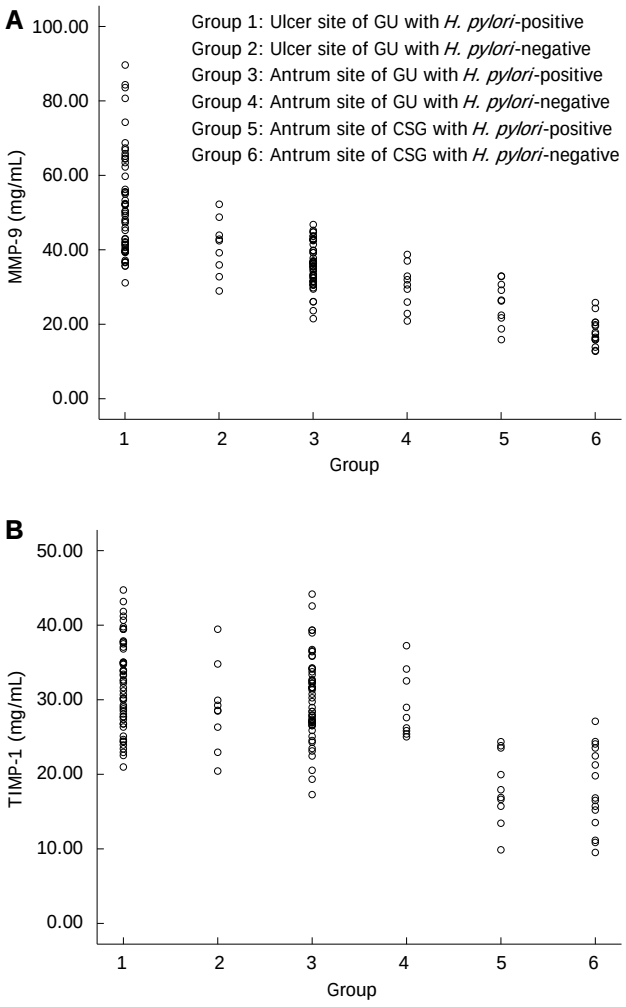

Figure 3 Production of matrix metalloproteinase-9 or tissue inhibitor of metalloproteinase-1 by gastric mucosa with negative or positive H. pylori-infection in patients with gastric ulcer (n = 63) or chronic superficial gastritis (n = 25). A: Tissue culture was performed for 24 h. Matrix metalloproteinase (MMP)-9 concentration in tissue culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA); B: Tissue culture was performed for 24 h. Tissue inhibitor of metalloproteinase (TIMP)-1 concentration in tissue culture supernatants was measured by ELISA. GU: Gastric ulcer; CSG: Chronic superficial gastritis; *H. pylori*: *Helicobacter pylori*.

the margin of the ulcer or in the antrum in the *H. pylori*-positive patients were significantly higher than in the *H. pylori*-negative patients (52.12 ± 13.90 mg/mL *vs* 40.77 ± 7.43 mg/mL, t = 2.38, P = 0.020; 35.92 ± 5.72 mg/mL *vs* 30.03 ± 6.01 mg/mL, t = 2.84, P = 0.006, respectively). In the CSG patients, levels of MMP-9 in the *H. pylori*-positive patients were significantly higher than in the *H. pylori*-negative patients (25.70 ± 5.89 mg/mL *vs* 17.96 ± 3.82 mg/mL, t = 4.00, P = 0.001).

In the GU patients, levels of TIMP-1(Figure 3B) at the margin of the ulcer or in the antrum in the *H. pylori*-positive patients did not differ significantly from those in the *H. pylori*-negative patients (32.18 ± 5.94 mg/mL *vs* 28.91 ± 5.71 mg/mL, t = 1.53, P = 0.130; 30.21 ± 5.60 mg/mL *vs* 29.22 ± 4.40 mg/mL, t = 0.50, P = 0.617, respectively). In the CSG patients, levels of TIMP-1 in the *H. pylori*-positive patients also did not differ significantly from those in the *H. pylori*-negative patients (18.22 ± 4.76 mg/mL *vs* 18.13 ± 5.55 mg/mL, t = 0.04, P = 0.967). Levels of MMP-9 (35.92 ± 5.72 mg/mL) and TIMP-1 (30.21 ± 5.60 mg/mL) in the antrum in the *H. pylori*-positive GU patients were significantly higher (t = 5.17, P < 0.01; t = 6.35, P < 0.01, respectively) than in the *H. pylori*-positive CSG patients (25.70 ± 5.89 mg/mL and 18.22 ± 4.76 mg/mL, respectively).

Table 2 Association between levels of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 and the histological degree at the margin of the gastric ulcer

Variables		Activity	Inflammation	Atrophy	Metaplasia
MMP-9	r	0.280	0.310	0.180	-0.030
	P	0.026[1]	0.014[1]	0.163	0.842
TIMP-1	r	0.270	0.280	0.120	0.050
	P	0.030[1]	0.025[1]	0.371	0.687

[1]By Spearman correlation analysis. MMP: Matrix metalloproteinase; TIMP: Tissue inhibitor of metalloproteinase.

For the GU patients, we compared levels of MMP-9 and TIMP-1 *in vitro* with the severity of histological gastritis (activity, inflammation, atrophy, and metaplasia) at the margin of the ulcer. A significant association was identified between levels of MMP-9 or TIMP-1 and the histological degree of activity and inflammation, but not with the degree of glandular atrophy or intestinal metaplasia (Table 2).

Of the 63 GU patients, six were excluded because they still had active ulcer after 8 wk PPI treatment. Among 57 follow-up patients, seven (12.3%) had recurrence at the time of or before endoscopy examination at the end of 6 mo. There were nine patients with *H. pylori* infection. A multivariate logistic regression analysis adjusted for age and sex demonstrated that *H. pylori* infection (OR = 17.705, 95%CI: 2.091-149.929, P = 0.008) and MMP-9 levels (OR = 1.078, 95%CI: 1.007-1.154, P = 0.031) were GU recurrence risk factors.

DISCUSSION

We found that MMP-9 production was increased in the gastric mucosa at the margin of the ulcer in GU patients. This increase had a significant positive correlation with production of TIMP-1, an MMP-9 inhibitor. Several studies have investigated the association between MMPs and GU. Indomethacin-induced ulcerated gastric tissues exhibited about 12-fold higher pro-MMP-9 activity as compared to control tissues. Similarly, ethanol induced about 22-fold higher pro-MMP-9 activities in rat gastric tissues[5]. One study showed that significant up-regulation of MMP-9 expression in indomethacin-induced GU in mice was correlated with increased activity of activator protein-1, and oxidative stress was preceded by chronic inflammation that enhanced expression of MMP-9[9].

MMPs have recently been shown to be up-regulated in gastric epithelial cells infected with *H. pylori*, and might contribute to the pathogenesis of peptic ulcer. Our study

showed that MMP-9 levels were associated with *H. pylori* infection. Significantly elevated serum levels of MMP-9 and reduced serum levels of TIMP-1 have been demonstrated in patients with *H. pylori* gastritis as compared to *H. pylori*-negative controls[10]. *H. pylori*-infected GUs had even higher MMP-9 and TIMP-1 expression in epithelial cells than in NSAID-related GU[11]. One study showed that there were no significant differences in serum levels of MMP-9 between *H. pylori*-positive and *H. pylori*-negative children[12].

We showed that levels of MMP-9 correlated with the histological degree of activity and inflammation at the margin of the ulcer. In BALB/c mice, NSAIDs caused dose-dependent induction in MMP-9 activity and expression in ulcerated gastric tissues, along with significant infiltration of inflammatory cells and disruption of the gastric mucosal layer[13]. GU is associated with infiltration of the gastric mucosa by neutrophils, lymphocytes, monocytes, and plasma cells. Inflammatory cells secrete an array of pro-inflammatory cytokines and growth factors (epidermal growth factor, platelet-derived growth factor, transforming growth factor-β, vascular endothelial growth factor, angiopoietins). MMPs can be induced by the activity of pro-inflammatory cytokines such as tumor necrosis factor-α, interleukin (IL)-1, IL-6 and IL-8[1,9,14]. Oxidative stress is preceded by chronic inflammation that enhances the expression of MMP-9. By decreased synthesis and secretion of MMP-9, as well as infiltration of inflammatory cells and oxidative damage in gastric tissues, we may block or heal acute GU[3,13].

The C/C genotype of MMP-9-1562 C/T gene polymorphism might be associated with *H. pylori* infection[15]. *H. pylori* infection increases the secretion of MMPs in the gastric mucosa, leading to severe mucosal damage. Genetic variations in the *MMP-9* gene may be part of a complex genetic risk profile to develop GU in chronic *H. pylori* infection[16]. MMP-9 levels decrease consistently and significantly after successful *H. pylori* eradication, whereas the elevated levels remain unchanged when treatment fails[17].

We found that patients with high levels of MMP-9 and *H. pylori* infection were the risk factors for GU recurrence. *H. pylori* infection associated with GU recurrence has been verified[18]. Some studies found that severity of GU was strongly correlated with increased secretion of proMMP-9 in ethanol-induced acute gastric ulceration in rats[19,20]. Higher levels of MMP-9 in chronic wound fluid correlate with a clinically worse wound[21]. Measurements of MMP-9 and TIMP-1 may help to identify diabetic foot ulcers at risk of poor healing[22]. These findings suggest that MMP-9 may be indicative of inflammation and poor wound healing, and that we can reduce GU recurrence by inhibition of MMP-9 activity.

In conclusion, we observed increased expression of MMP-9 and TIMP-1 in GU patients and found a significantly positive correlation between MMP-9 and TIMP-1 production at the margin of the ulcer. Increased production of MMP-9 was significantly correlated with increased GU recurrence. These results suggest that MMP-9 may play an important role in the occurrence of GU. A clearer understanding of the significance and implications of these findings may provide insights into ulcer healing. Further study is needed to clarify the roles of MMP-9 and elucidate any potential clinical implications in the healing of GU.

COMMENTS

Background

Gastric ulcer (GU) is a multifaceted process including acid secretion, reactive oxygen species generation, prostaglandin inhibition, and extracellular matrix (ECM) degradation. Gastric mucosal damage is directly associated with ECM degradation in which matrix metalloproteinases (MMPs) play a crucial role. In several animal studies of GU, attention has focused on the role of MMP-1, MMP-2, MMP-3, MMP-9 and MMP-13. However, these data are mostly derived from animal studies, and human clinical data remains rare, especially in assessing MMPs expression in GU formation and recurrence.

Research frontiers

In this study, the authors compared levels of MMP-9 and tissue inhibitor of metalloproteinase (TIMP)-1 in GU patients, and how they correlated with GU recurrence.

Innovations and breakthroughs

This study enrolled 63 patients with GU and 25 patients with superficial gastritis (CSG). Samples of gastric mucosa from the antrum and the ulcer site were harvested from GU patients and samples of antral mucosa were taken from CSG patients during upper gastroduodenal endoscopy. Levels of MMP-9 and TIMP-1 at the ulcer site or in the antrum were significantly higher in GU than CSG patients. MMP-9 levels at the ulcer site were significantly higher than in the antrum in GU patients, and had a significantly positive correlation with TIMP-1. MMP-9 levels were significantly higher in *Helicobacter-pylori*-positive than -negative GU and CSG patients. Levels of MMP-9 or TIMP-1 at the ulcer site were associated with the histological severity of activity and inflammation.

Applications

The authors found that the MMP-9 may perform an important function in gastric ulcer formation and recurrence.

Peer review

This study compared MMP-9 and TIMP-1 levels in GU and CSG patients. The authors measured the levels of MMP-9 and TIMP-1 from the tissues. The levels of MMP-9 and TIMP-1 at the ulcer site or in the antrum were significantly higher in GU than CSG patients. MMP-9 levels at the ulcer site were significantly higher than in the antrum in GU patients, and had a significantly positive correlation with TIMP-1. They concluded that MMP-9 may perform an important function in gastric ulcer formation and recurrence.

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META-ANALYSIS

Impact of being overweight on the surgical outcomes of patients with gastric cancer: A meta-analysis

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Abstract

AIM: To investigate the effect of being overweight on the surgical results of patients with gastric cancer.

METHODS: Comprehensive electronic searches of the PubMed, Web of Science, and Cochrane Library databases were conducted. Studies were identified that included patients with surgical complications from gastric cancer who were classified as normal weight [body mass index (BMI) < 25 kg/m^2] or overweight (BMI $\geqslant$ 25 kg/m^2). The operative time, retrieved lymph nodes, blood loss, and long-term survival were analyzed. A subgroup analysis was conducted based on whether patients received laparoscopic or open gastrectomy procedures. All statistical tests were performed using ReviewerManager 5.1.2 software.

RESULTS: This meta-analysis included 23 studies with 20678 patients (15781 with BMI < 25 kg/m^2; 4897 with BMI $\geqslant$ 25 kg/m^2). Overweight patients had significantly increased operation times [MD: -29.14; 95%CI: -38.14-(-20.21); P < 0.00001], blood loss [MD: -194.58; 95%CI: -314.21-(-74.95); P = 0.001], complications (RR: 0.75; 95%CI: 0.66-0.85; P < 0.00001), anastomosis leakages (RR: 0.59; 95%CI: 0.42-0.82; P = 0.002), and pancreatic fistulas (RR: 0.486; 95%CI: 0.34-0.63; P < 0.00001), whereas lymph node retrieval was decreased significantly in the overweight group (MD: 1.69; 95%CI: 0.75-2.62; P < 0.0001). In addition, overweight patients had poorer long-term survival (RR: 1.14; 95%CI: 1.07-1.20; P < 0.0001). No significant difference was detected for the mortality and length of hospital stay.

CONCLUSION: This meta-analysis demonstrates that a high BMI not only increases the surgical difficulty and complications but also impairs the long-term survival of patients with gastric cancer.

Key words: Overweight; Body mass index; Gastric cancer; Gastrectomy

Core tip: Surgical and postoperative complications are believed to be greater for overweight patients with gastric cancer, but this is controversial due to conflicting results from previous studies. This meta-analysis identified 23 studies with a total of 20678 patients, and the results indicate that overweight patients had significantly increased operation times, blood loss, complications, anastomosis leakages, and pancreatic fistulas, whereas lymph node retrieval was decreased significantly in the overweight group. In addition, overweight patients had poorer long-term survival. Therefore, being overweight not only increased the surgical difficulty and complications but also impaired the long-term survival of patients with gastric cancer.

Wu XS, Wu WG, Li ML, Yang JH, Ding QC, Zhang L, Mu JS, Gu J, Dong *P*, Lu JH, Liu YB. Impact of being overweight on the surgical outcomes of patients with gastric cancer: A meta-analysis. *World J Gastroenterol* 2013; 19(28): 4596-4606 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i27/4596.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i27.4596

INTRODUCTION

The increasing global prevalence of overweight and obese individuals is problematic[1,2] for Western countries[3] and is also a concern for Eastern countries such as China[4] and South Korea[5]. Consequently, abdominal surgeries are increasingly more difficult because increasing numbers of surgeries are performed on overweight and obese individuals. In particular, gastric cancer studies[6,7] reported that excess body weight is associated with unfavorable surgical results, including longer operating times, decreased lymph node retrieval, increased postoperative complications, and decreased survival rates. Radical gastrectomy with D2 node dissection is the recommended surgical approach for patients with resectable (curable) gastric cancer[8]. However, the results of postoperative morbidity, mortality, and long-term survival after D2 node dissection differed significantly between different studies from Asia and Europe[8-14]. This discrepancy may be due to the variable prevalence of overweight patients in Western and Eastern countries. Excess visceral fat in overweight patients theoretically complicates manipulation of the omentum and impedes lymph node dissection during radical gastrectomy due to decreased visualization of the branches of the arteria celiaca, which could increase surgical and postoperative complications and mortality. However, a number of studies[15-18] reported conflicting results about the effect of being overweight on both the short-term and long-term surgical outcomes for patients with gastric cancer. To more comprehensively understand this issue, we conducted a meta-analysis.

MATERIALS AND METHODS

Search strategy

Two authors (Wu XS and Wu WG) independently conducted comprehensive electronic searches of the PubMed, Web of Science, and Cochrane Library databases for all dates prior to January 2013. The search strategy was unrestricted for English-language journals and used combinations of MeSH and text words for overweight, body mass index (BMI), gastric cancer, and gastrectomy, *e.g.*, the string "Body Mass Index" (Mesh) or "overweight" (MeSH Terms) or overweight (Text Word) and "gastrectomy" (MeSH Terms) or gastrectomy (Text Word) or "stomach neoplasms" (MeSH Terms) or gastric cancer (Text Word). In addition, reference lists of all retrieved articles were manually searched for additional studies that were missed by the electronic search.

Inclusion and exclusion criteria

The inclusion criteria for the meta-analysis were studies that examined the influence of body weight on gastric cancer surgical outcomes (morbidity, anastomotic leakage, pancreatic fistula, postoperative mortality, operative time, lymph node retrieval, blood loss, postoperative hospital stay, and long-term survival). In the studies we chose, there were patients with normal-weight and overweight presurgical BMIs based on World Health Organization definitions (overweight BMI $\geqslant$ 25 kg/m^2; healthy-weight BMI < 25 kg/m^2)[19,20]. Reviews, case reports, and series reports were excluded. When data were presented in more than one publication, publications with smaller data sets were excluded. Disagreements regarding a study's eligibility were resolved based on a consensus of reviews from two additional authors (Li ML and Yang JH).

Outcome measures analyzed

Three outcome variables, including the operation time, number of retrieved lymph nodes, and blood loss, were analyzed as indices of the surgical difficulty. We estimated the influence of a high BMI on surgical safety, morbidity, anastomotic leakage, pancreatic fistula, postoperative mortality, and postoperative hospital stay. The long-term survival of overweight and healthy-weight patients was also compared as an index of successful clinical resolution.

Data extraction and risk of bias assessment

Data were extracted from each study by two independent reviewers (Ding QC and Zhang L), who also rated the overall quality of each outcome according to the recommendation of the Cochrane Handbook for Systematic Reviews of Interventions[21]. The criteria to assess nonrandomized studies were taken from the Grading of Recommendations Assessment, Development, and Evaluation Working Group[22]. By combining the aforementioned recommendations, the following aspects of each included study were evaluated: the application of an internal control, adequate control of confounding factors, adequate reporting of outcomes, and the absence of a variable definition. Agreement for ratings was achieved *via* author consensus, as needed.

Statistical analysis

The statistical analysis was performed using Reviewer-Manager (Version 5.1.2, 2011, The Nordic Cochrane Centre, Cochrane Collaboration, www.cochrane-handbook.org). Statistical methods were based on the *Cochrane Handbook for Systematic Reviews of Interventions*[21]. Heterogeneity was checked using χ^2 tests, and $P < 0.1$ was the cutoff for statistical significance. A random effects model was applied for the meta-analysis using a more conservative perspective. Data from different trials reporting the same or similar outcomes were combined. The results were expressed using the RR for binary variables and the MD for continuous variables. Methods for relevant data extraction were based on Tierney *et al*[23]. The cutoff for

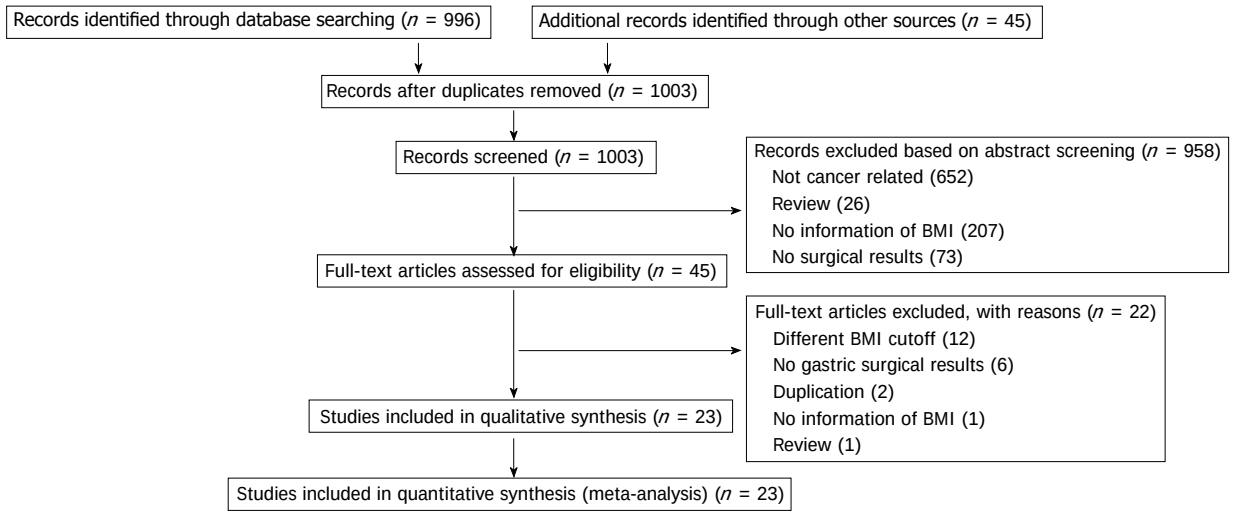


Figure 1 PRISMA flow chart showing study selection process. BMI: Body mass index.

statistical significance was $P < 0.05$, and the 95%CI was presented for each effect measure. Subgroup analysis was conducted based on whether patients received a laparoscopic gastrectomy or a total gastrectomy. Whenever possible, all analyses were based on the intention-to-treat principle. Publication bias exploration using a funnel plot and Egger's regression method[24] was performed if at least 10 trials were included in an outcome variable. Publication bias was considered to exist for $P < 0.05$.

RESULTS

Description of the included trials

We retrieved 996 records from the PubMed search and 45 records from the manual search. Twenty-three trials[15-18,25-43], which included multiple study types, procedures, percentages of patients with early gastric cancer, therapeutic modalities, and BMI cutoffs, met the eligibility criteria and were included in the meta-analysis (Table 1). Excluded reports largely had irrelevant topics. Twelve studies were excluded because they did not define overweight patients using the 25 kg/m^2 criteria. Figure 1 shows the flow chart for the selection of articles based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses[44]. This meta-analysis identified patients with healthy-weights (BMI < 25 kg/m^2) (n = 15781) and patients who were overweight (BMI ⩾ 25 kg/m^2) (n = 4897). For five included studies[27,28,30,33,35] that classified patients using more than one BMI cutoff point, binary variables were successfully combined, but it was not possible to pool these studies' continuous variables. Only four studies[16,17,32,35] were considered to have a low risk of bias, and all others were considered to have high risk of bias. Most of them were considered high risk because of the selective reporting or absence of variables definition.

Surgical results for all patients

Overweight patients had significantly longer operation times [MD: -29.14; 95%CI: -38.14-(-20.21); $P < 0.00001$, Figure 2A], greater blood loss [MD: -194.58; 95%CI: -314.21-(-74.95); $P = 0.001$], reduced lymph node retrieval (MD: 1.69; 95%CI: 0.75-2.62; $P < 0.0001$) (Table 2), and more postoperative complications (RR: 0.75; 95%CI: 0.66-0.85; $P < 0.00001$, Figure 2B). Specifically, anastomotic leakage (RR: 0.59; 95%CI: 0.42-0.82; $P = 0.002$, Figure 2C) and pancreatic fistula (RR: 0.486; 95%CI: 0.34-0.63; $P < 0.00001$, Figure 2D) were significantly greater in the overweight cohort. There was no significant difference between the two cohorts for the postoperative mortality or postoperative hospital stay. Patients in the normal-weight cohort had higher cancer-specific survivorship (RR: 1.14; 95%CI: 1.07-1.20; $P < 0.0001$, Figure 2E).

There was significant heterogeneity in the operation time, morbidity, anastomotic leakage, blood loss, long-term survival, and postoperative hospital stay results. No heterogeneity was detected for any of the other assessed outcomes. No publication bias was detected for the morbidity outcomes ($P = 0.05$), anastomotic leakage ($P = 0.291$), or mortality ($P = 0.272$).

Surgical results for patients receiving open gastrectomy

Overweight patients who received open gastrectomy had longer operation times [MD: -25.24; 95%CI: -33.53-(-16.95); $P < 0.00001$], greater intraoperative blood loss [MD: -212.93; 95%CI: -301.04-(-124.82); $P < 0.00001$], increased postoperative complications (RR: 0.78; 95%CI: 0.66-0.94; $P = 0.007$), more anastomotic leakage (RR: 0.58; 95%CI: 0.38-0.89; $P = 0.01$), and increased pancreatic fistulas (RR: 0.46; 95%CI: 0.38-0.67; $P < 0.0001$) compared with patients with healthy weights (Table 2). There were no significant differences between the cohorts for mortality, postoperative hospital stay, or number of retrieved lymph nodes. Non-overweight patients had better overall survival results than overweight ones (RR: 1.14; 95%CI: 1.07-1.20; $P < 0.0001$).

There was significant heterogeneity in the morbidity, anastomotic leakage, operative time, number of retrieved

Table 1 Basic data of included studies

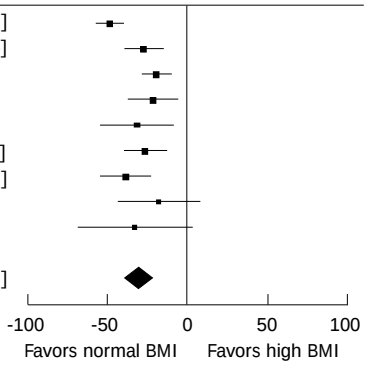
Study	Country	Inclusion period	Sample size	Study type	Follow up period	Percentage of T1 (normal weight *vs* overweight)	Percentage of N0 (normal weight *vs* overweight)	Percentage of stage 1/2 (normal weight *vs* overweight)	Percentage of differentiated (normal weight *vs* overweight)	Type of gastrectomy	Node dissection	Laparoscopic	Chemotherapy/ radiotherapy	BMI cutoff point	Risk of internal control	Risk of confounding factors	Risk of slective report	Risk of variables definition
Gretschel *et al*[27]	Germany	1992-2001	199	Retrospective	-	15.8% *vs* 31.6%	26.8% *vs* 45.3%	Not stated	Not stated	Total	D2	Not stated	Not stated	25, 30	Low	High	High	High
Yamada *et al*[17]	Japan	1999-2005	248	Retrospective	8-118 mo	53.2% *vs* 78.3%	Not stated	89.9% *vs* 93.3%	Not stated	Distal	D2	141 LADG	Not stated	25	Low	Low	Low	Low
Oh *et al*[29]	South Korea	2009-2009	61	Prospective	-	Not stated	Not stated	Not stated	Not stated	Total	D2	Not stated	Not stated	25	Low	High	Low	Low
Nobuoka *et al*[40]	Japan	1992-2008	644	Retrospective	-	Not stated	Not stated	53.8% *vs* 56.6%	Not stated	Total	D2	Not stated	Chemotherapy for advanced cancer and recurrence	25	Low	High	Low	Low
Tsujinaka *et al*[32]	Japan	1995-2001	523	Prospective	-	0.0% *vs* 0.0%	Not stated	Not stated	Not stated	Not stated	D2; D3	No	Not stated	25	Low	Low	Low	Low
Ohno *et al*[37]	Japan	2004-2009	197	Retrospective	-	73.9% *vs* 77.5%	Not stated	87.2% *vs* 95.0%	57.3% *vs* 57.5%	LADG; ODG	D1a; D1b; D2	120 LADG	Not stated	25	Low	Low	Low	High
Pacelli *et al*[33]	Italy	2000-2006	145	Retrospective	-	Not stated	Not stated	Not stated	Not stated	Distal; total	D2 + D3	No	No preoperative chemotherapy	18.5, 25, 30	Low	High	Low	Low
Yoshikawa *et al*[39]	Japan	2007-2009	66	Retrospective	-	Not stated	Not stated	92.4% *vs* 100.0%	Not stated	LADG; LATG[c]	D1ab + D2	56 LADG; 10 LATG	Not stated	25	Low	Low	Low	High
Murphy *et al*[35]	United Kingdom	1997-2002	50	Prospective	-	Not stated	Not stated	29.6% *vs* 65.2%	Not stated	Not stated	D2	No	No preoperative chemotherapy	20, 25, 30	Low	Low	Low	Low
Yasuda *et al*[34]	Japan	1994-2002	99	Retrospective	48 mo	100.0% *vs* 100.0%	93.8% *vs* 95.2%	Not stated	80.7% *vs* 87.5%	LADG	D1	LADG	Not stated	25	Low	Low	Low	High
Lee *et al*[38]	South Korea	-2005	1485	Retrospective	At least 3 mo	Not stated	Not stated	89.1% *vs* 89.0%	Not stated	LADG	D1a; D1b	LADG	Not stated	25	Low	High	Low	High
Kulig *et al*[18]	Poland	1986-1998	1992	Retrospective	104 mo	13.8% *vs* 10.6%	17.5% *vs* 15.0%	Not stated	Not stated	Distal; proximal; total	D1; D2; D2+	No	Not stated	25	Low	High	Low	High
Kim *et al*[30]	South Korea	2005-2010	1100	Prospective	-	Not stated	Not stated	Not stated	Not stated	LADG	D2	LADG	Not stated	25, 30	Low	High	Low	High
Inagawa *et al*[28]	Japan	1990-1997	293	Retrospective	10-104 mo	Not stated	Not stated	88.7% *vs* 94.0%	Not stated	Distal	D2	-	Not stated	20, 25	Low	Low	Low	High
Kawamura *et al*[36]	Japan	2003-2008	473	Retrospective	-	Not stated	Not stated	100.0% *vs* 100.0%	Not stated	Distal	Regional	249 LADG	Not stated	25	Low	Low	Low	High
Ojima *et al*[31]	Japan	1992-2002	689	Retrospective	At least 60 mo	55.3% *vs* 51.7%	66.1% *vs* 65.5%	Not stated	53.4% *vs* 56.9%	Distal; proximal; total	D1; D2; D2+	-	No preoperative chemotherapy	25	Low	High	Low	Low
Tokunaga *et al*[15]	Japan	1970-2004	7925	Retrospective	At least 60 mo	51.0% *vs* 60.0%	61.0% *vs* 68.0%	75.0% *vs* 83.0%	45.0% *vs* 40.0%	Distal; proximal; total	Not stated	-	Not stated	25	Low	High	High	Low
Oh *et al*[16]	South Korea	2000-2003	410	Retrospective	50 mo	15.7% *vs* 20.9%	41.8% *vs* 40.4%	47.6% *vs* 46.5%	32.5% *vs* 35.4%	Total	D2	-	Not stated	25	Low	Low	Low	Low
Tanaka *et al*[26]	Japan	2001-2007	191	Retrospective	-	Not stated	Not stated	Not stated	Not stated	Total	D1; D2	-	Not stated	25	Low	High	Low	High
Kunisaki *et al*[25]	Japan	2002-2008	152	Retrospective	-	Not stated	Not stated	Not stated	Not stated	LADG	D1a; D1b; D2	LADG	Not stated	25	Low	High	High	High
Lee *et al*[42]	South Korea	2006-2010	243	Retrospective	-	Not stated	Not stated	100.0% *vs* 100.0%	Not stated	Distal	Not stated	Not stated	Not stated	25	Low	Low	High	High
Bickenbach *et al*[43]	United States	1985-2007	1853	Retrospective	35 mo	24.6% *vs* 29.8%	44.5% *vs* 48.3%	58.6% *vs* 65.9%	Not stated	Not stated	D1; D2; D2+	Not stated	Not stated	25	Low	High	Low	Low
Deguchi *et al*[41]	Japan	1999-2008	1640	Retrospective	-	Not stated	Not stated	Not stated	Not stated	Proximal; total	D0; D1; D2; D2+	Not stated	Not stated	25	Low	High	Low	Low

LADG: Laparoscopic-assited distal gastrectomy; ODG: Opem distal gastrectomy; LATG: Laparoscopic-assited total gastrectomy.

A

Study or subgroup	Normal BMI Mean	SD	Total	High BMI Mean	SD	Total	Weight	Mean difference IV, random, 95%CI
Bickenbach *et al*[43]	272	84	728	320	102	1125	15.3%	-48.00 [-56.53, -39.47]
Kawamura *et al*[36]	236	60.7	349	262.4	58.2	124	13.4%	-26.40 [-38.46, -14.34]
Lee *et al*[38]	223.7	75.6	1053	242.5	84.6	432	15.0%	-18.80 [-27.99, -9.61]
Nobuoka *et al*[40]	245.1	69.3	545	265.9	74.1	99	11.5%	-20.80 [-36.51, -5.09]
Oh *et al*[5]	254.11	50.13	37	285.03	37.99	24	8.5%	-30.92 [-53.10, -8.74]
Oh *et al*[16]	173.7	47.6	311	199.2	61.8	99	12.8%	-25.50 [-38.77, -12.23]
Ojima *et al*[31]	277	85	573	315	75	116	11.7%	-38.00 [-53.32, -22.68]
Tanaka *et al*[26]	269	83	144	286	77	47	7.1%	-17.00 [-42.85, 8.85]
Yasuda *et al*[34]	239	48	83	271	69	16	4.7%	-32.00 [-67.35, 3.35]
Total (95%CI)			3823			2082	100.0%	-29.14 [-38.08, -20.21]

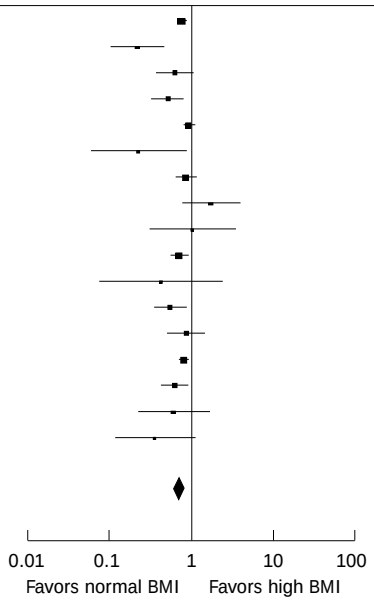
Heterogeneity: Tau^2 = 116.74; χ^2 = 26.79, df = 8 (P = 0.0008); I^2 = 70%
Test for overall effect: Z = 6.39 (P < 0.00001)



B

Study or subgroup	Normal BMI Events	Total	High BMI Events	Total	Weight	Risk ratio MH, random, 95%CI
Bickenbach *et al*[43]	279	728	551	1125	14.4%	0.78 [0.70, 0.87]
Inagawa *et al*[28]	12	239	12	54	2.5%	0.23 [0.11, 0.48]
Kawamura *et al*[36]	37	349	20	124	4.6%	0.66 [0.40, 1.09]
Kim *et al*[30]	45	791	33	309	5.7%	0.53 [0.35, 0.82]
Kulig *et al*[18]	533	1500	182	492	13.7%	0.96 [0.84, 1.10]
Kunisaki *et al*[25]	4	123	4	29	0.9%	0.24 [0.06, 0.89]
Lee *et al*[38]	147	1053	68	432	9.6%	0.89 [0.68, 1.16]
Lee *et al*[42]	23	157	7	86	2.2%	1.80 [0.81, 4.02]
Murphy *et al*[35]	5	27	4	23	1.1%	1.06 [0.32, 3.50]
Nobuoka *et al*[40]	192	545	48	99	10.5%	0.73 [0.58, 0.92]
Oh *et al*[5]	2	37	3	24	0.5%	0.43 [0.08, 2.40]
Oh *et al*[16]	43	311	24	99	5.5%	0.57 [0.37, 0.89]
Pacelli *et al*[33]	21	76	21	69	4.5%	0.91 [0.55, 1.51]
Tokunaga *et al*[15]	1276	6799	250	1126	14.1%	0.85 [0.75, 0.95]
Tsujinaka *et al*[32]	101	446	27	77	7.3%	0.65 [0.46, 0.92]
Yasuda *et al*[34]	13	83	4	16	1.5%	0.63 [0.23, 1.68]
Yoshikawa *et al*[39]	6	53	4	13	1.2%	0.37 [0.12, 1.12]
Total (95%CI)		13317		4197	100.0%	0.75 [0.66, 0.85]
Total events	2739		1262			

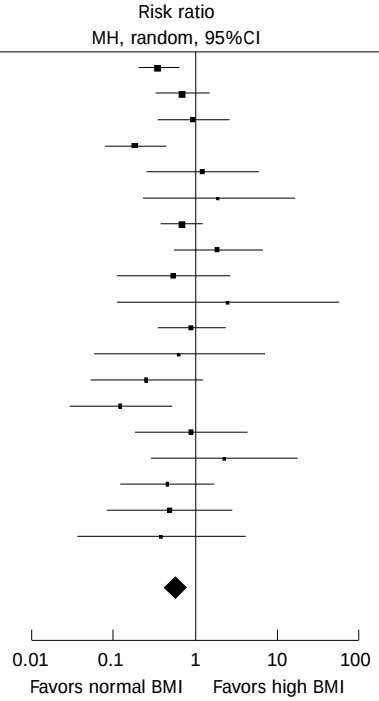
Heterogeneity: Tau^2 = 0.03; χ^2 = 38.26, df = 16 (P = 0.001); I^2 = 58%
Test for overall effect: Z = 4.48 (P < 0.00001)



C

Study or subgroup	Normal BMI Events	Total	High BMI Events	Total	Weight	Risk ratio MH, random, 95%CI
Bickenbach *et al*[43]	15	728	65	1125	12.3%	0.36 [0.21, 0.62]
Deguchi *et al*[41]	26	1320	9	320	9.7%	0.70 [0.33, 1.48]
Gretschel *et al*[27]	6	82	9	117	7.2%	0.95 [0.35, 2.57]
Inagawa *et al*[28]	9	239	11	54	8.8%	0.18 [0.08, 0.42]
Kawamura *et al*[36]	7	349	2	124	3.8%	1.24 [0.26, 5.91]
Kim *et al*[30]	5	791	1	309	2.2%	1.95 [0.23, 16.65]
Kulig *et al*[18]	36	1500	17	492	12.1%	0.69 [0.39, 1.23]
Lee *et al*[38]	14	1053	3	432	5.3%	1.91 [0.55, 6.63]
Lee *et al*[42]	3	157	3	86	3.7%	0.55 [0.11, 2.66]
Murphy *et al*[35]	1	27	0	23	1.1%	2.57 [0.11, 60.24]
Nobuoka *et al*[40]	25	545	5	99	7.7%	0.91 [0.36, 2.32]
Oh *et al*[16]	2	311	1	99	1.8%	0.64 [0.06, 6.95]
Ohno *et al*[37]	3	157	3	40	3.8%	0.25 [0.05, 1.22]
Ojima *et al*[31]	3	573	5	116	4.4%	0.12 [0.03, 0.50]
Pacelli *et al*[33]	3	76	3	69	3.8%	0.91 [0.19, 4.35]
Tanaka *et al*[26]	7	144	1	47	2.4%	2.28 [0.29, 18.09]
Tsujinaka *et al*[32]	8	446	3	77	5.0%	0.46 [0.12, 1.70]
Yamada *et al*[17]	3	106	2	35	3.1%	0.50 [0.09, 2.84]
Yasuda *et al*[34]	2	83	1	16	1.9%	0.39 [0.04, 4.00]
Total (95%CI)		8687		3680	100.0%	0.59 [0.42, 0.82]
Total events	178		144			

Heterogeneity: Tau^2 = 0.17; χ^2 = 27.27, df = 18 (P = 0.07); I^2 = 34%
Test for overall effect: Z = 3.07 (P = 0.002)



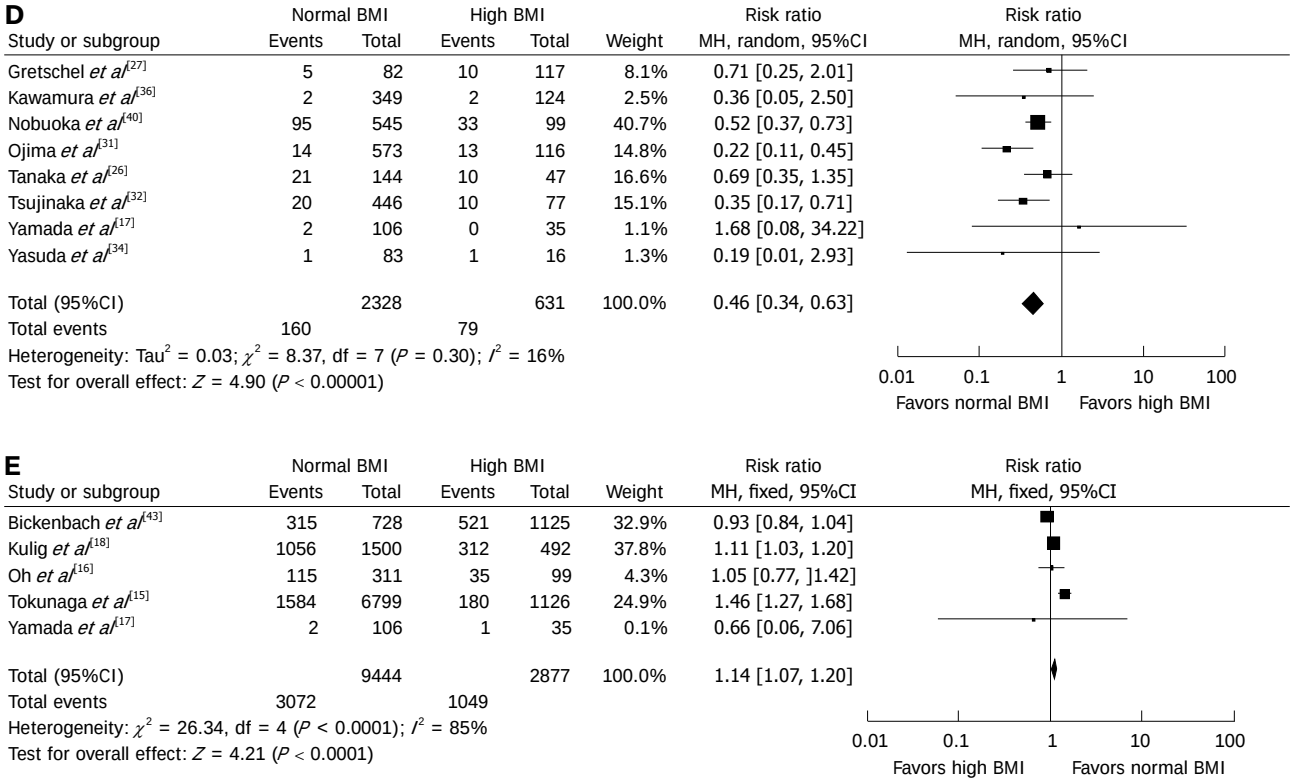


Figure 2 Forest plot. A: For operative time showing overweight in association with longer duration of operative time than non-overweight; B: For morbidity showing overweight in association with more postoperative complication than non-overweight; C: For anastomotic leak indicating that overweight correlates with higher rate of anastomotic leak; D: For pancreatic fistula showing overweight in association with more pancreatic fistula than non-overweight; E: For long-term survival favoring normal weight with better survival results. BMI: Body mass index.

lymph nodes, blood loss, and postoperative hospital stay. No heterogeneity was found in any of the other assessed outcomes. There was no evidence of publication bias ($P > 0.05$ for all 3 of the following outcomes: morbidity, anastomotic leakage, and mortality).

Surgical results for patients receiving laparoscopic gastrectomy

Overweight patients receiving laparoscopic gastrectomies had increased complications (RR: 0.48; 95%CI: 0.29-0.79; $P = 0.004$), longer operation times [MD: -15.06; 95%CI: -17.41-(-12.70); $P < 0.00001$], more blood loss [MD: -47.83; 95%CI: -68.12-(-27.53); $P < 0.00001$], and fewer retrieved lymph nodes (MD: 2.11; 95%CI: 1.35-2.88; $P < 0.00001$) than healthy-weight patients (Table 2). There were no significant differences in any of the other outcomes. Morbidity was a heterogeneous outcome with very low quality, whereas the other outcomes were rated as low quality. Egger's regression method was not applied in this subgroup analysis because none of the outcome variables included at least 10 trials.

Surgical results for patients receiving total gastrectomy

Overweight patients receiving total gastrectomies had increased complications (RR: 0.68; 95%CI: 0.56-0.84; $P = 0.0003$), more pancreatic fistulas (RR: 0.56; 95%CI: 0.42-0.74; $P < 0.0001$), longer operation times [MD: -23.94; 95%CI: -32.62-(-15.25); $P < 0.00001$], more blood loss [MD: -293.84; 95%CI: -401.80-(-185.87); $P < 0.00001$], and fewer retrieved lymph nodes (MD: 3.99; 95%CI: 1.14-6.83; $P = 0.006$) than healthy-weight patients. There were no significant differences in any of the other outcomes.

Surgical results for patients receiving subtotal gastrectomy

Overweight patients receiving subtotal gastrectomies had increased complications (RR: 0.61; 95%CI: 0.40-0.94; $P = 0.02$), longer operation times [MD: -22.02; 95%CI: -29.18-(-14.86); $P < 0.00001$], and more blood loss [MD: -58.36; 95%CI: -93.56-(-23.45); $P = 0.001$] than healthy-weight patients. There were no significant differences in any of the other outcomes, including pancreatic fistulas and the number of retrieved lymph nodes.

DISCUSSION

Theoretically, comorbidity risk factors[45] and surgical complications could cause prolonged surgical times, increased blood loss, more postoperative complications, and greater intraoperative mortality. However, the effects of comorbidity risk factors are uncertain because published papers[25-43] assessing the relationship between being overweight and poor surgical outcomes have reported conflicting results, especially for the outcome variables, such as morbidity, mortality, and long-term survival.

Table 2 Summary statistics of pooled data comparing normal body mass index *vs* high body mass index for overall patients, patients receiving open gastrectomy and laparoscopic gastrectomy

Outcome variables	Studies	Pooled patients	Pooled RR or MD or HR	95%CI	Test for overall effect		Test for heterogeneity	
					Z	*P* value	I^2	*P* value
Overall patients								
Operative time	9	5905	-29.14	-38.08, -20.21	6.39	< 0.00001	70%	0.0008
Retrieved lymph nodes	6	4612	1.69	0.75, 2.62	3.55	0.0004	9%	0.36
Blood loss	5	2096	-194.58	-314.21, -74.95	3.19	0.001	86%	< 0.00001
Morbidity	17	17514	0.75	0.66, 0.85	4.48	< 0.00001	58%	0.001
Anastomotic leak	19	12367	0.59	0.42, 0.82	3.07	0.002	34%	0.07
Pancreatic fistula	8	2959	0.46	0.34, 0.63	4.90	< 0.00001	16%	0.3
Mortality	13	16590	0.86	0.58, 1.29	0.71	0.48	0%	0.76
Postoperative hospital stay	6	4552	-5.83	-13.44, 1.78	1.5	0.19	98%	< 0.00001
Cancer-specific survival	5	12321	1.14	1.07, 1.20	4.21	< 0.0001	85%	< 0.0001
Patients receiving open gastrectomy								
Operative time	7	2179	-25.24	-33.53, -16.95	5.97	< 0.00001	53%	0.05
Retrieved lymph nodes	6	2838	3.81	-0.34, 7.96	1.8	0.07	91%	< 0.00001
Blood loss	5	1708	-212.93	-301.04, -124.82	4.74	< 0.00001	74%	0.004
Morbidity	11	12510	0.78	0.66, 0.94	2.68	0.007	64%	0.002
Anastomotic leak	14	7320	0.58	0.38, 0.89	2.51	0.01	37%	0.08
Pancreatic fistula	6	2470	0.46	0.38, 0.67	4.03	< 0.0001	37%	0.16
Mortality	10	12763	1.17	0.69, 2.01	0.58	0.56	0%	0.83
Postoperative hospital stay	4	1339	-2.04	-6.00, 1.91	1.01	0.31	80%	0.002
Cancer-specific survival	4	12180	1.14	1.07, 1.20	4.23	< 0.0001	89%	< 0.00001
Patients receiving laparoscopic gastrectomy								
Operative time	4	1845	-15.06	-17.41, -12.70	12.52	< 0.00001	0%	0.52
Retrieved lymph nodes	3	1746	2.11	1.35, 2.88	5.39	< 0.00001	0%	0.61
Blood loss	3	360	-47.83	-68.12,-27.53	4.62	< 0.00001	47%	0.15
Morbidity	6	3151	0.48	0.29, 0.79	2.91	0.004	67%	0.009
Anastomotic leak	6	3194	0.83	0.42, 1.65	0.53	0.6	32%	0.2
Pancreatic fistula	3	489	0.3	0.08, 1.20	1.7	0.09	10%	0.33
Mortality	3	1833	0.4	0.12, 1.30	1.53	0.13	0%	0.41
Postoperative hospital stay	3	1833	0.17	-0.80, 1.15	0.35	0.73	34%	0.22
Cancer-specific survival	1	141	1.65	0.13, 20.70	0.39	0.7	Not applicable	Not applicable

We evaluated the operation time, intraoperative blood loss, and number of retrieved lymph nodes as indices of the surgical difficulty. Both the operation time and blood loss for overweight patients with gastric cancer were significantly higher than for the normal-weight cohort, regardless of whether open gastrectomy or laparoscopic gastrectomy was performed. Being overweight was also correlated with significantly fewer retrieved lymph nodes. Two reasons may contribute to the lower number of retrieved lymph nodes[6]. First, the excess fat tissue in the abdomen could limit the node dissection for overweight patients. Second, pathologists would have difficulty obtaining lymph nodes from a large amount of adipose tissue.

The relationship between high BMI and surgical safety for patients with gastric cancer is controversial. In the 17 trials providing data about morbidity, ten studies[18,29,33-36,38,39,42,43] did not indicate that being overweight affected the overall postoperative complication rate, whereas the remaining 7[15,16,25,28,30,32,40] did. Our meta-analysis strongly suggests that overweight patients have more complications. More specifically, the rates of pancreatic fistula and anastomotic leakage were significantly higher in the overweight patients, which also was true in the subgroup analysis of patients receiving open gastrectomy. According to these results, it is clear that overweight patients have high risks of postoperative complications. However, it is still uncertain whether a high BMI has a direct influence on the postoperative morbidity. High BMIs directly affect the operation times for cholecystectomies, colectomies, and unilateral mastectomies but have no direct relationship with complications[46]. Increased operation times and blood loss secondary to high BMI are also responsible for high postoperative complication rates[28,31], which is likely because prolonged operative times prolong the duration of anesthesia and increase the risk of thromboembolic, cardiac, and respiratory complications. Our study found strong evidence (RR < 0.5) for an association between being overweight and high rates of pancreatic fistula, as suggested in earlier reports[13,32]. This effect on the occurrence of a pancreatic fistula could be because removal of overweight patients' pancreatic capsules is difficult; they have poor differentiation between the pancreas and excess pancreatic fat deposition[47,48]. This could also hamper peripancreatic node dissection and increase the potential for iatrogenic injury to pancreatic tissue. More interestingly, according to one included study[26], minimal damage to the pancreatic tissue, which would never cause a pancreatic fistula in patients with low visceral fat area (VFA), could result in pancreatic fistula in high-VFA patients. Visceral fat maybe play an important role in the pathogenesis from pancreatic injury to pancreatic fistula. Therefore, being overweight could have a direct influence on the postoperative complication rate, as is the case for pancreatic fistulas. Although overweight patients suffered

more complications, no difference was detected for mortality, which might be attributed to the advancement of perioperative management. Changes in perioperative management have dramatically decreased the death rate from serious postoperative complications such as pancreatic fistula and anastomotic leakage. Thus, it is safe to perform radical gastrectomy in overweight patients.

Relevant studies reported conflicting results on the relationship between being overweight and long-term survival[6,7,28,31,40,49,50]. Theoretically, excess visceral fat and being overweight could negatively affect survivorship by increasing the rates of coexisting disease and postoperative complications. In addition, according to Adachi *et al*[6], incomplete lymph node dissection in overweight patients could result in retention of metastatic nodes that are responsible for the worse survivorship. Increased long-term survival in normal-weight patients was found in the current review and is consistent with the hypothesis that excess accumulation of visceral fat could impair patient survival and promote tumor recurrence. Unfortunately, among the 23 analyzed studies, only five were included in the analysis of survivorship; thus, the survivorship results, with fewer data points, are less convincing. However, during the data extraction, we noticed that the percentage of patients with early gastric cancer was greater for the overweight cohort. Compared with advanced gastric cancer patients, patients with early gastric cancer have a significantly higher long-term survival rate[51]. Although the overweight cohort had more patients with early gastric cancer, who might have a more promising prognosis, the overall long-term survival was still significantly lower in this cohort. This is indirect evidence that being overweight can impair the long-term survival of gastric patients. In addition, we do not think that the decreased long-term survival was caused by the increasing comorbidity related to being overweight, such as diabetes and cardiovascular disease because we used cancer-specific survival as the indicator of long-term survival.

High BMIs increase the difficulty and decrease the safety of laparoscopic gastrectomy procedures, as is the case with open gastrectomy. These findings are consistent with some previous studies[25,30,36]. However, other studies[37-39] did not show significant differences in the morbidity between overweight and normal-weight cohorts for laparoscopic gastrectomy. Unlike open gastrectomy, laparoscopic gastrectomy can achieve excellent visibility even for overweight patients because the pneumoperitoneum creates sufficient extra space in the abdominal cavity. Although the laparoscopic procedure has these advantages, the results of our study still suggest that being overweight negatively affects the difficulty and safety of laparoscopic gastrectomy.

Moreover, being overweight increases the difficulty and impairs the safety of both total and subtotal gastrectomies. However, a subtotal gastrectomy seems to be safer than a total gastrectomy for overweight patients because subtotal gastrectomy did not increase the rate of pancreatic fistula occurrence in the overweight group, which is a severe complication after gastric surgery. In addition, after subtotal gastrectomy, the numbers of retrieved lymph nodes did not differ significantly between the two cohorts, while there was a difference in the number of lymph nodes retrieved after total gastrectomy.

Because of the relationship between being overweight and impaired surgical safety, surgeons should be more careful when performing radical gastrectomy in the future. In addition, for suitable cases, performing a subtotal gastrectomy might be safer than performing a total gastrectomy.

This meta-analysis has some limitations. First, most of the studies in this meta-analysis were rated as low or very low quality due to their retrospective study designs. All included studies are nonrandomized in nature and have a risk of bias. Although randomized trials are the gold standard for study design, random allocation of patients with different BMIs is hardly feasible. To overcome this limitation in the future, more rigorously designed studies with a good balance of other confounding factors, such as age and tumor-node-metastasis stage, are needed. Second, although BMI $\geqslant$ 25 kg/m^2 was used as a criterion for classifying patients as overweight, it may be not the best index because the distribution of fat tissue could differ greatly between individuals, even those with the same BMI[26,52]. Therefore, individuals with the same BMI could have different surgical outcomes due to their different fat distributions. Some studies[26,39,53] have indicated that the VFA is a better index than BMI. Third, the procedure type and extent of node dissection differed among the studies in our meta-analysis. Moreover, gastric cancer was more prevalent in Eastern countries than Western ones. As a result, surgeons from Eastern countries could have more experience in performing the surgeries and dealing with the postoperative complications. Additionally, the higher incidence of gastric cancer has led to earlier diagnosis in Asian countries. Therefore, the proportions of early gastric cancer cases differed between studies from the East and West in this review. All these factors could account for the heterogeneity of some results and jeopardize the reliability of the conclusions. The limitations in the previously published data could potentially affect the analysis of both groups. Publication bias was a possible source of bias during the meta-analysis because positive results are more likely to be published. Several methods have been proposed for detecting bias and, in this review, we detected publication bias by a funnel plot and Egger's regression method, which is reliable when the number of included trials is not less than 10. It turned out that our results did not show significant publication bias ($P > 0.05$) for the parameters in this review.

In conclusion, this meta-analysis indicates that overweight patients with gastric cancer have increased surgical complications and worse short-term operative outcomes than patients with healthy weights, and these results were consistent for patients who underwent either a laparoscopic gastrectomy or an open gastrectomy. Although no

evidence was detected to indicate that being overweight had higher postoperative morbidity, being overweight decreased the long-term survival.

COMMENTS

Background

The increasing global prevalence of overweight and obese individuals is problematic for Western countries and is also a concern for Eastern countries such as China and South Korea. Surgical results and postoperative complications are believed to be greater for overweight patients with gastric cancer, but this is controversial due to conflicting results from previous studies.

Research frontiers

The postoperative morbidity, mortality, and long-term survival after D2 node dissection differed between different studies from Asia and Europe. It is possible that this discrepancy is due to the differing prevalence of overweight patients in Western and Eastern countries. However, different studies have conflicting results for the effect of being overweight on both the short-term and long-term surgical outcomes for gastric cancer patients.

Innovations and breakthroughs

To the knowledge, this is the first meta-analysis studying the effect of being overweight on the surgical results of gastric cancer patients. The authors found that overweight patients with gastric cancer have increased surgical complications and worse short-term operative outcomes than patients with healthy weights, and these results were consistent for patients who underwent either a laparoscopic gastrectomy or an open gastrectomy.

Applications

This meta-analysis emphasizes the influence of being overweight on gastric cancer surgical results. Surgeons should pay particular attention when they perform radical gastric cancer surgery.

Peer review

Overall, this manuscript provides a detailed and comprehensive review of the influence of elevated patient body mass index on outcomes following gastrectomy as a treatment for cancer. This article includes information about the complications of gastric surgery and has potential clinical implications. Finally, this meta-analysis demonstrates that being overweight is significantly correlated with surgical difficulty, a high rate of postoperative complications, and poor survival in patients with gastric cancer.

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META-ANALYSIS

Prediction of the severity of acute pancreatitis on admission by urinary trypsinogen activation peptide: A meta-analysis

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Abstract

AIM: To undertake a meta-analysis on the value of urinary trypsinogen activation peptide (uTAP) in predicting severity of acute pancreatitis on admission.

METHODS: Major databases including Medline, Embase, Science Citation Index Expanded and the Cochrane Central Register of Controlled Trials in the Cochrane Library were searched to identify all relevant studies from January 1990 to January 2013. Pooled sensitivity, specificity and the diagnostic odds ratios (DORs) with 95%CI were calculated for each study and were compared to other systems/biomarkers if mentioned within the same study. Summary receiver-operating curves were conducted and the area under the curve (AUC) was evaluated.

RESULTS: In total, six studies of uTAP with a cut-off value of 35 nmol/L were included in this meta-analysis. Overall, the pooled sensitivity and specificity of uTAP for predicting severity of acute pancreatitis, at time of admission, was 71% and 75%, respectively (AUC = 0.83, DOR = 8.67, 95%CI: 3.70-20.33). When uTAP was compared with plasma C-reactive protein, the pooled sensitivity, specificity, AUC and DOR were 0.64 *vs* 0.67, 0.77 *vs* 0.75, 0.82 *vs* 0.79 and 6.27 *vs* 6.32, respectively. Similarly, the pooled sensitivity, specificity, AUC and DOR of uTAP *vs* Acute Physiology and Chronic Health Evaluation Ⅱ within the first 48 h of admission were found to be 0.64 *vs* 0.69, 0.77 *vs* 0.61, 0.82 *vs* 0.73 and 6.27 *vs* 4.61, respectively.

CONCLUSION: uTAP has the potential to act as a stratification marker on admission for differentiating disease severity of acute pancreatitis.

Key words: Acute pancreatitis; Urinary trypsinogen activation peptide; C-reactive protein; Acute Physiology and Chronic Health Evaluation Ⅱ score; Meta-analysis

Core tip: Currently, the assessment of acute pancreatitis severity on admission remains a challenge to clinicians. A single, rapid biochemical marker is the preferred choice than clinical and computed tomography scoring systems. In this study, the value of urinary

trypsinogen activation peptide (uTAP), on admission, in predicting severity of acute pancreatitis was assessed. It was found that the ability of uTAP to predict severity of acute pancreatitis on admission was comparable to C-reactive protein (at 48 h) and was potentially better than the Acute Physiology and Chronic Health Evaluation Ⅱ score (at 48 h), the most frequently used biochemical marker and clinical scoring system in acute pancreatitis, respectively.

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INTRODUCTION

Acute pancreatitis causes up to 210000 admissions in the United States annually and remains a diagnostic, prognostic and therapeutic dilemma for surgeons and physicians[1]. Although mild acute pancreatitis is associated with virtually no mortality, severe acute pancreatitis continues to be at the other end of the spectrum with mortality reaching up to 30%, mainly due to pancreatic necrosis and organ failure[2].

As severe acute pancreatitis may progress very quickly and is normally associated with a complicated clinical course and higher mortality, it is vital to identify these patients as early as possible to initiate appropriate supportive management, especially within the first 24 h after symptoms onset[3]. Therefore, in the last few decades many biomarkers[4], radiological[5] and clinical scoring systems[6,7] have been developed and validated to fulfil this role. These, however, have not been entirely successful. The Glasgow[8], Acute Physiology and Chronic Health Evaluation Ⅱ (APACHE Ⅱ)[9], and Ranson[10] scoring systems and plasma C-reactive protein (CRP)[11] are still the most widely used parameters and form part of many guidelines, however, their use does come with its own limitations.

There is enough evidence to establish trypsinogen activation as one of the earliest steps in the pathophysiology of the disease[12,13], and consequently, trypsinogen activation peptide (TAP) has been shown to be an excellent marker for severity stratification in different experimental acute pancreatitis models[14]. In human acute pancreatitis, TAP is rapidly excreted in urine and in urinary[15] and peritoneal fluid[16]. TAP concentrations correlate well with disease severity. Therefore, it is reasonable to hypothesize that pancreas-specific activation peptides would be elevated (in the urine) from the onset of disease and could potentially serve as early biomarkers. Urinary TAP (uTAP) is the most studied peptide for predicting severity of acute pancreatitis[17], but its diagnostic value in severe acute pancreatitis has not been systematically assessed. In this study, a meta-analysis was carried out to evaluate existing evidence of uTAP in predicting the severity of acute pancreatitis.

MATERIALS AND METHODS

Study selection

A comprehensive literature search of Medline, Embase, Science Citation Index Expanded and the Cochrane Central Register of Controlled Trials in The Cochrane Library was carried out to identify studies evaluating the prognostic efficacy of uTAP from January 1990 (the first human study)[15] to February 2013. The following medical subject headings (MeSH) and keywords were used: "trypsinogen activation peptide" or "activation peptide" and "acute pancreatitis" or "severe acute pancreatitis" or "post endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis". Equivalent free-text search terms were used in the search strategy. All abstract supplements from published literature or from relevant international meetings were searched manually. Relevant papers were also identified from the reference lists of previous papers. Only studies which were published in English as full-text articles were included. Final inclusion of articles was determined by consensus; when this failed, a third author adjudicated. Severe acute pancreatitis was defined as the development of organ failure and/or local complications[15,18].

Inclusion and exclusion criteria

Two authors independently identified and screened the search findings for potentially eligible studies.

Inclusion criteria: (1) English language studies published as full text articles in peer-reviewed journals; (2) Human studies; (3) Studies with available data; and (4) When similar studies were reported by the same institution, the best quality study was included.

Exclusion criteria: (1) Abstracts, letters, editorials, expert opinions, reviews and case reports; (2) Where only concentration or *P* value was reported; (3) Studies assessing the efficacy of serum/plasma TAP in predicting the severity of acute pancreatitis; and (4) Studies assessing the efficacy of uTAP in diagnosing acute pancreatitis.

Data extraction and quality assessment

Data were extracted by two independent observers using standardized forms. The recorded data included study design, demographics (age, gender, etiology and country of origin), severity of disease, duration from symptoms onset to admission, time point for the collection of samples and cut-off values. Diagnostic parameters including true positivity (TP), false positivity (FP), false negativity (FN) and true negativity (TN) were extracted directly or by calculating the sensitivity and specificity of uTAP for predicting the severity of acute pancreatitis. TP, FP, FN and

Table 1 Characteristics of included prospective studies for urinary trypsinogen activation peptide as a predictor of severity of acute pancreatitis

Ref.	Year	Country	Sampling time after admission	Cut-off value (nmol/L)	Total (*n*)	Male/ female (*n*)	Mild/ severe (*n*)	Mean age: male/ female (yr)	Etiology
Neoptolemos *et al*[32]	2000	Multicenter	On admission	35	172	87/85	137/35	52 (29-84)	Biliary 74, alcoholic 62, other 36
Liu *et al*[34]	2002	China	On admission	35	41	NA	29/12	NA	NA
Khan *et al*[33]	2002	United States	On admission	35	58	33/25	39/19	69 ± 19	Biliary 26, alcoholic 18, HTC 3, postoperative (including ERCP) 9, idiopathic 2
Lempinen *et al*[35]	2003	Finland	On admission	35	127	NA	98/29	NA	Biliary 24, alcoholic 74, other 29
Johnson *et al*[37]	2004	Multicenter	On admission	35	190	104/86	164/26	54 (42-70)	Biliary 70, alcohol 65, other 55
Huang *et al*[38]	2010	China	On admission	35	187	112/75	149/38	60.4 ± 6.7; 59.5 ± 8.1	Biliary 139, alcoholic 19, other 29

ERCP: Endoscopic retrograde cholangiopancreatography; HTC: Hypercholesterolemic; NA: Not available.

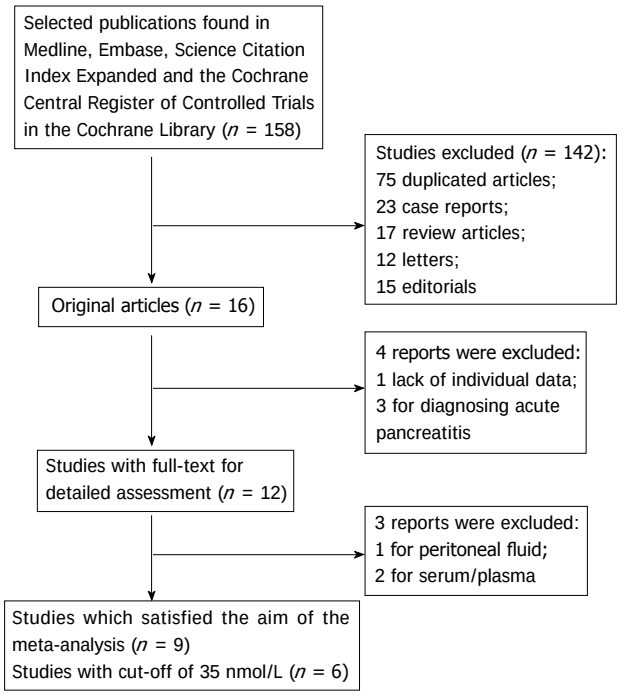

Figure 1 Flow diagram illustrating the process of identification of relevant studies.

TN were also extracted for serum CRP and APACHE Ⅱ score at the highest diagnostic values during the first 2 d after admission if these were reported in the included studies. The quality of the included studies was assessed independently by two reviewers using the Standards for Reporting of Diagnostic Accuracy (STARD) initiative guidelines[19]. Studies with a STARD score of ⩾ 16 were considered as high quality studies.

Statistical analysis

The meta-analysis was performed with Meta-DiSc 1.4 software (Hospital Ramón y Cajal, Madrid, Spain). Pooled sensitivity, specificity, and diagnostic odds ratio (DOR) with diagnostic value Q were calculated. The mentioned parameters were pooled respectively with a corresponding 95%CI. Receiver operating characteristics were also generated and expressed by area under curve (AUC). The AUC represents the accuracy of diagnosis and DOR indicates its diagnostic capability for differentiating disease groups from negative groups[20]. Heterogeneity was evaluated using Cochran's Q test and a P value of 0.1 was considered significantly different. I^2 statistics were used to measure the percentage of total variation across the studies due to heterogeneity (I^2 of 50% or more indicating the presence of heterogeneity)[21]. The publication bias of included studies was assessed using a funnel plot of the effect of effective sample size weighted regression tests of asymmetry[22]. The meta-analysis was performed using a fixed-effect model if there was no heterogeneity among the studies, otherwise the random effects model was used[23]. The sensitivity analyses were undertaken by excluding each study from the analysis to ascertain its effect on the overall results. Subgroup analyses were dependent on the following items: high quality studies, sample size ⩾ 50 in each study, single center studies and severity defined by the 1992 Atlanta Classification[18].

RESULTS

Description of included trials in the meta-analysis

Details of the literature research are shown in Figure 1 and 16 clinical studies were identified. Seven studies were excluded: one due to lack of data for analysis[24], one studied peritoneal fluid TAP[25], two studied serum/plasma TAP[26,27], three for diagnosing acute pancreatitis, but not for assessing the severity[28] or post-ERCP pancreatitis[29,30]. Of the 9 studies[15,31-38] that were potentially useful for analysis, only six had the cut-off of 35 nmol/L; it being variable in the remaining three and therefore, these six studies were included in the final analysis.

Study and patient characteristics

Table 1 describes the included studies and patient characteristics. All of the six included studies were prospectively designed and were of high quality (STARD score ⩾ 16). There were 2 multicenter[32,37] and 4 single center[33-35,38] trials. Five studies[32-35,38] defined the severity of acute pancreatitis by the 1992 Atlanta Classification, in which severe cases included the moderate and the severe groups according to the revised Atlanta Classification[39]. One

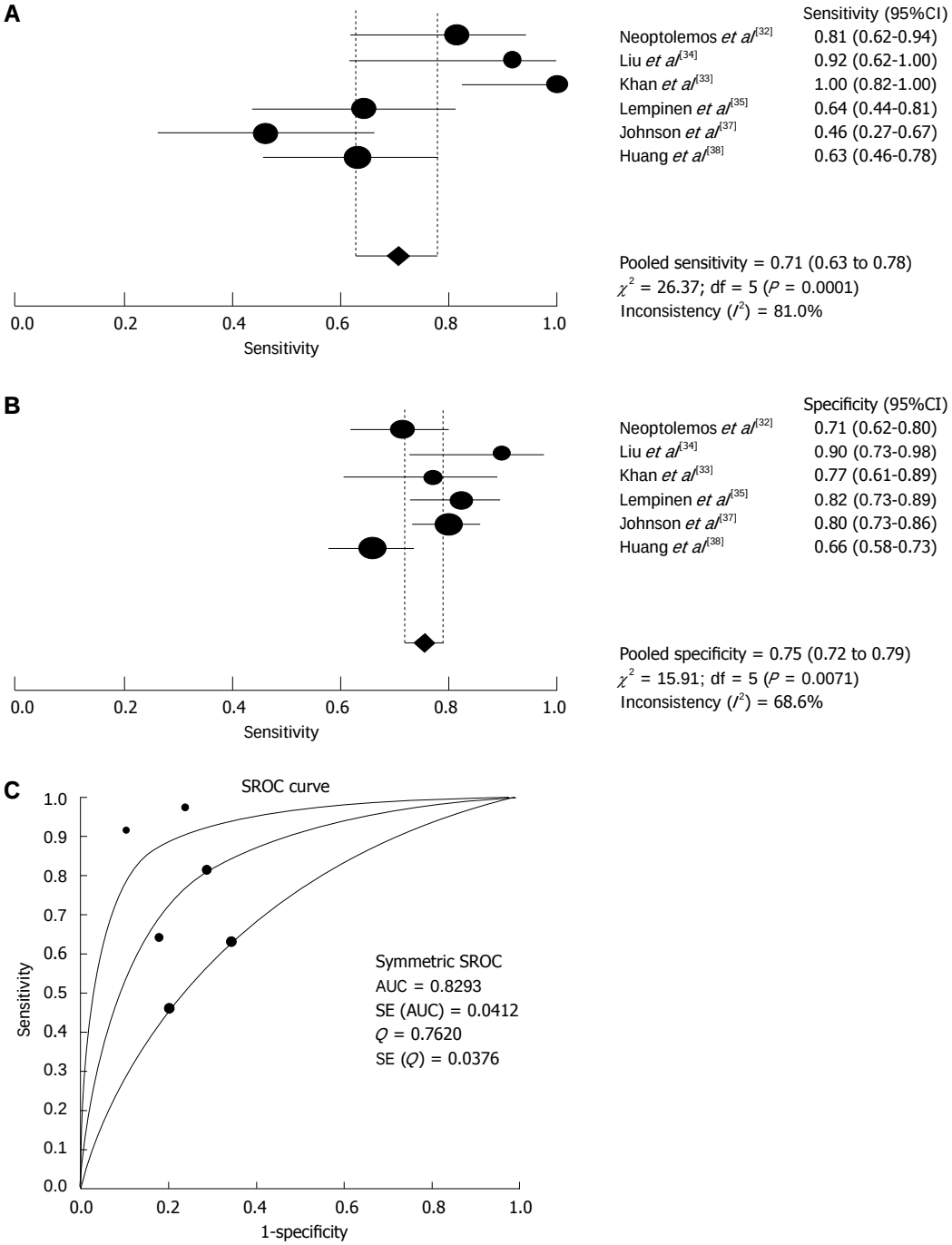

Figure 2 Forest plots of sensitivity (A), specificity (B), and summary receiver operating characteristic curve (C) for on admission urinary trypsinogen activation peptide in predicting severe acute pancreatitis. SROC: Summary receiver operating characteristic; AUC: Area under the curve.

Table 2 Diagnostic parameters of included studies

Ref.	Patients (*n*)	Patients analyzed (mild/severe)	TP	FP	FN	TN
Neoptolemos *et al*[32]	172	132 (105/27)	22	30	5	75
Liu *et al*[34]	41	41 (29/12)	11	3	1	26
Khan *et al*[33]	58	58 (39/19)	19	9	0	30
Lempinen *et al*[35]	127	118 (90/28)	18	16	10	74
Johnson *et al*[37]	190	190 (164/26)	12	33	14	131
Huang *et al*[38]	187	187 (149/38)	24	51	14	98

TP: True positive; FP: False positive; FN: False negative; TN: True negative.

study defined severe acute pancreatitis as the presence of local complications or the presence of persistent organ failure that was more than 48 h[37]. The predominant etiology in recruited patients was biliary in origin followed by alcoholic, ERCP and idiopathic.

Meta-analysis results

Results of the data extraction are shown in Table 2 and results of the meta-analysis are shown in Figures 2 and 3, and summarized in Table 3.

Table 3 Meta-analysis outcomes of included studies

	Trials (*n*)	Patients (*n*)	AUC	DOR (95%CI)	*Q*	*P* value	I^2
All studies	6	726	0.83	8.67 (3.70-20.33)	15.88	0.0072	68.50%
Study subgroups							
Sample size ⩾ 50	5	685	0.80	6.48 (3.05-13.74)	10.28	0.0360	61.10%
Single center	4	413	0.86	14.25 (3.39-59.80)	12.92	0.0048	76.80%
1992 Atlanta Classification	5	536	0.84	11.97 (4.17-34.36)	13.65	0.0085	70.70%

AUC: Area under the curve; DOR: Diagnostic odds ratios.

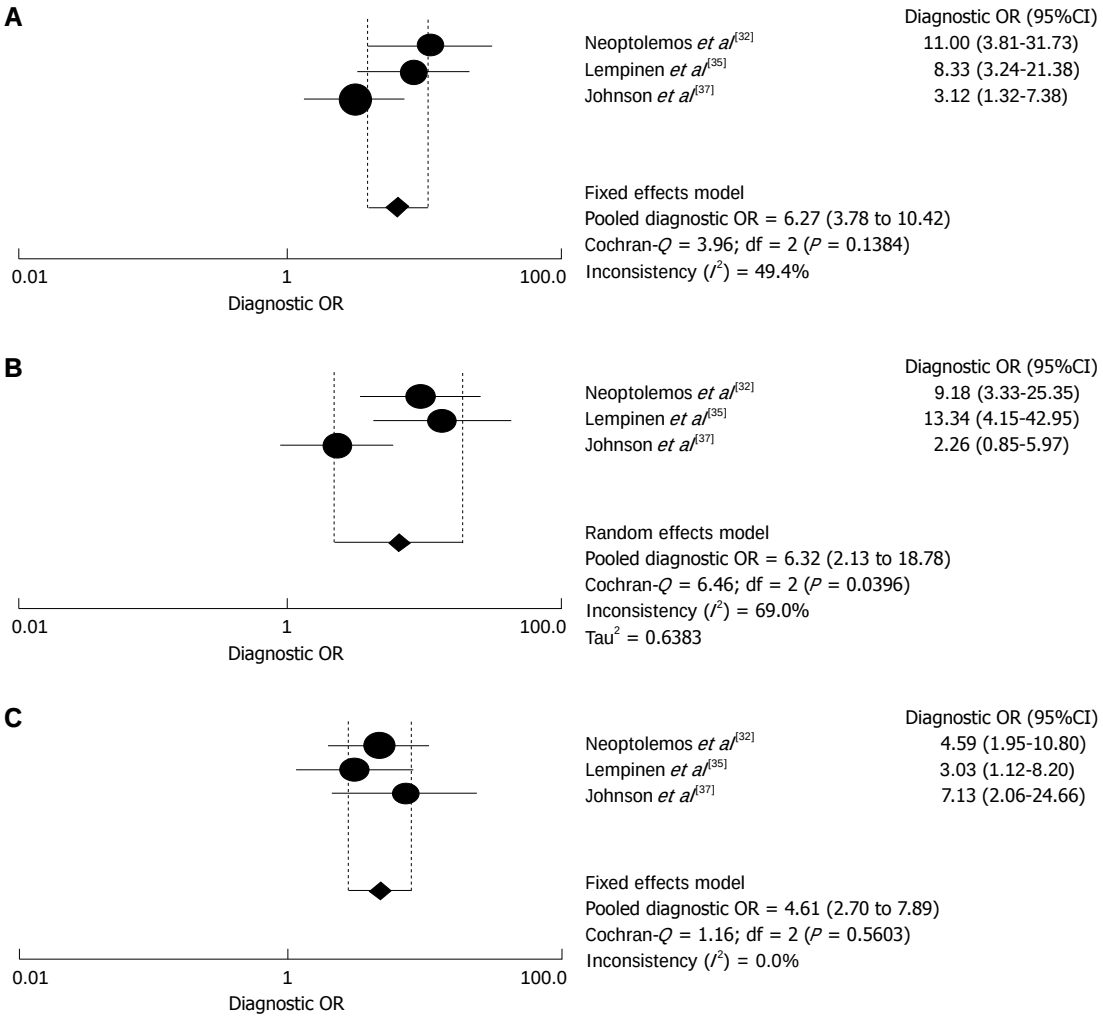

Figure 3 Forest plots of diagnostic OR for urinary trypsinogen activation peptide *vs* serum C-reactive protein and urinary trypsinogen activation peptide *vs* Acute Physiology and Chronic Health Evaluation Ⅱ score in predicting severe acute pancreatitis. The pooled diagnostic odds ratios of on-admission urinary trypsinogen activation peptide (A), plasma C-reactive protein at 48 h (B) and Acute Physiology and Chronic Health Evaluation Ⅱ score at 48 h (C).

On admission uTAP for predicting the severity of acute pancreatitis: Data from the six included studies (775 patients with 726 analyzed) revealed that the pooled sensitivity, specificity, AUC and DOR were 71% (95%CI: 63-78), 75% (95%CI: 72-79), 0.83 and 8.67 (95%CI: 3.70-20.33), respectively. Data are shown in Figure 2 and Table 3. These data suggest that uTAP has the potential to predict the severity of acute pancreatitis.

uTAP *vs* plasma CRP for severity stratification: There were 3 studies[32,35,37] that compared the prognostic value of on admission uTAP (440 patients analyzed) with plasma CRP (458 patients analyzed) within the first 48 h after admission in the severity stratification for acute pancreatitis. The pooled sensitivity, specificity, AUC value and DOR were 0.64 *vs* 0.67, 0.77 *vs* 0.75, 0.82 *vs* 0.79 and 6.27 *vs* 6.32, respectively (Figure 3A and B) for uTAP and CRP (best diagnostic values at 48 h). As suggested by the data, prognostic efficacy of the two markers was found to be similar.

uTAP *vs* APACHE-Ⅱ score for severity stratification: There were 4 studies[32,34,35,37] that compared the prognos-

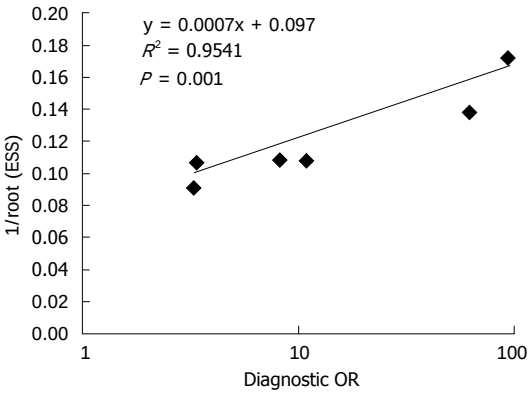

Figure 4 Funnel plot of the effect of effective sample size weighted regression tests of asymmetry for included studies. ESS: Effective sample size.

tic value of on admission uTAP with APACHE Ⅱ score within first 48 h after admission. Of these, 3 studies[32,35,37] used an APACHE Ⅱ score ⩾ 8 (422 patients analyzed) for defining severity and compared with uTAP (440 patients analyzed). The pooled sensitivity, specificity, AUC value and DOR were 0.64 *vs* 0.69, 0.77 *vs* 0.61, 0.82 *vs* 0.73 and 6.27 *vs* 4.61 for uTAP (values on admission) and APACHE Ⅱ score (best diagnostic values at 48 h), respectively (Figure 3A and C). These data suggest that uTAP may have a better prognostic value than the APACHE Ⅱ score in predicting the severity of acute pancreatitis.

Sensitivity and subgroup analysis: Outcomes for sensitivity and subgroup analysis are shown in Table 3. All six studies included were of high quality and sensitivity analysis demonstrated that significant heterogeneity still existed in these high quality studies (Q = 15.88, P = 0.0072, I^2 = 68.5%). Subgroup analysis showed that significant heterogeneity also existed in studies with sample size ⩾ 50 (Q = 10.28, P = 0.0360, I^2 = 61.1%), single center (Q = 12.92, P = 0.0048, I^2 = 76.8%), and severity defined by the 1992 Atlanta Classification (Q = 13.65, P = 0.0085, I^2 = 70.7%).

Publication bias: A funnel plot was created to demonstrate bias in the studies. The shape of the funnel plot showed asymmetry and this was confirmed by P = 0.001, showing that more significant results were present in smaller studies (Figure 4).

DISCUSSION

Upon admission, severity prediction of acute pancreatitis is crucial. This is still controversial, not universal and is mired by institutional differences. The current commonly used severity prediction systems include clinical assessment, biochemical markers, and both clinical and radiological scoring systems[40,41]. Clinical assessment provides a relatively high specificity (83%-98%) for ruling out mild acute pancreatitis, but has poor sensitivity (34%-64%) for the same[40]. When compared with clinical scoring systems, contrast-enhanced computed tomography (CECT) was not found to be superior in predicting outcomes of acute pancreatitis on admission[5].

Ideally, the best biomarker for predicting disease severity should be accurate, rapid, inexpensive and non-invasive. The pancreas-specific biomarkers are generally thought to be related to disease severity[14]. In 1988, a TAP assay with a detection limit of 10 picomolar concentration was developed by Hurley *et al*[42], enabling the detection of TAP in the body fluid to become more feasible. In a multicenter study conducted by Neoptolemos *et al*[32] that recruited 172 acute pancreatitis patients, uTAP concentration was found to be significantly different between mild and severe acute pancreatitis from 0-96 h after symptoms onset. Most importantly, uTAP values at both 24 and 48 h after admission provided the highest prognostic values for severe acute pancreatitis when compared to plasma CRP and clinical scoring systems (APACHE-Ⅱ, Glasgow and Ranson).

For the six studies included in this meta-analysis, the pooled results indicated that uTAP has potential for predicting the severity of acute pancreatitis upon hospital admission (AUC = 0.83, DOR = 8.67, 95%CI: 3.7-20.33). This is at least comparable with the current in-use biomarkers[6]. While most of the currently used biomarkers are non-specific in nature (specific for inflammation and other aspects), TAP is specific to the pancreas and is liberated within the first few hours after the onset of symptoms[15,32]. The prognostic value of uTAP (on admission) was similar to the APACHE-Ⅱ score obtained 24 h after admission in this meta-analysis. It is noteworthy that despite the APACHE-Ⅱ score being one of the most frequently used clinical scores to assess the severity of acute pancreatitis, it is cumbersome to use and has dubious use in certain settings; *i.e.,* in critical care environments where physiology has been corrected. Therefore, there is a need for simple and quick severity prediction techniques.

The prognostic value of an on-admission uTAP was also compared with plasma CRP (obtained 0-48 h after admission), currently the most widely used severity biomarker in acute pancreatitis and other acute inflammatory diseases[43]. uTAP had a relatively higher diagnostic value than plasma CRP, which suggested that uTAP might be a highly valuable biomarker for the quick assessment of acute pancreatitis severity on admission. It is unsurprising, therefore, that the revised Atlanta Classification[39] introduced the potential use of uTAP for severity stratification, although, as yet, it has not been widely adopted in the clinical arena.

To investigate the presence of heterogeneity, sensitivity and subgroup analyses were performed, based on sample size, study center and definition of severity. There was significant heterogeneity among studies with sample size ⩾ 50, single center and severity defined by the 1992 Atlanta Classification. It has been shown in many previous studies that multicenter studies tend to have better and more reliable results than single center studies. Similar to increasing sample size. Most of our studies used the Atlanta criteria for severity stratification, albeit one which represented a small proportion of the same cohort. From a clinical perspective, heterogeneity may also be caused by the defi-

nition of severity. All the severe acute pancreatitis cases included in this meta-analysis had two distinct entities: the moderate and the severe categories according to the revised Atlanta Classification. The proportion of moderate and severe cases may have had a significant impact on the results of uTAP. On the other hand, the proportion of patients who had pancreatic necrosis may also have an impact on the uTAP levels. Moreover, whether etiology plays a role in this regard remains unknown. Unsurprisingly, there was publication bias towards more significant effects reported in smaller sample sizes. This may have implications on the interpretation of the pooled sensitivity and specificity. These problems can only be overcome in the future by larger studies being performed.

The 1992 Atlanta Classification defines severe acute pancreatitis if organ failure/or local complications such as pancreatic necrosis, abscess, or pseudocyst are present[17]. Pancreatic necrosis, however, is only characterized by an area more than 30% necrosis non-enhanced on CECT, which might lead to false negative results of uTAP when pancreatic necrosis is less than 30%. The revised Atlanta Classification categorizes severity of acute pancreatitis into mild, moderate and severe classes[39]; the determinant-based classification stratifies severity of acute pancreatitis into mild, moderate, severe and critical categories[44]. These classifications consider pancreatic necrosis and persistent organ failure as the key determinants of outcome of acute pancreatitis. Compared to the 1992 Atlanta Classification, the new definition of pancreatic necrosis is described as the detection of any area of non-enhancement or every heterogeneous peri-pancreatic collection on CECT. These updated definitions and classifications might prove to be very useful in re-assessing the importance of an on-admission uTAP for the quick assessment of severity in acute pancreatitis. One might postulate that uTAP may have high prognostic accuracy in identifying patients with a disease course that is at least moderate to severe or for ruling out mild patients.

This review suffers from a relatively small sample size, publication bias in smaller studies and heterogeneity in some of the inclusion criteria. To the best of our knowledge, this is the most comprehensive meta-analysis on the subject to date. We have tried to summarize the existing data, identify problems in undertaking that, point out potential areas of improvement and suggest guidelines for future studies.

In summary, uTAP is a rapid assay for the assessment of acute pancreatitis severity on admission and provides good prognostic accuracy for severe acute pancreatitis based on the 1992 Atlanta Classification. New studies should assess its value in a larger patient cohort with uniform inclusion criteria and in line with the newly proposed classification systems.

COMMENTS

Background

Assessment of the severity of acute pancreatitis is crucial upon admission. Currently, clinical assessment, biochemical markers, and both clinical and computed tomography scoring systems are used individually or in combination to fulfil the need. However, a single, inexpensive and rapid biochemical marker is preferred due to practical and economic reasons. In this regard, urinary trypsinogen activation peptide (uTAP) has been developed and validated in many clinical studies, showing good diagnostic value in predicting severe acute pancreatitis. However, these results have not been systematically assessed.

Research frontiers

To conduct a meta-analysis on the value of uTAP in predicting the severity of acute pancreatitis on admission.

Innovations and breakthroughs

The Revised Atlanta Classification has introduced the potential use of uTAP in the prediction of severity stratification. However, current clinical studies regarding this topic have not been systemically analyzed to provide evidence on uTAP to ensure its wide adoption in the clinical arena. This is the first meta-analysis summarizing data obtained from six studies in which the uTAP cut-off concentration (35 nmol/L) was the same for severity stratification. The meta-analysis showed that the diagnostic value of uTAP (on admission) for the severity of acute pancreatitis was comparable to CRP (at 48 h after admission) and was potentially better than the APACHE-Ⅱ score (at 48 h after admission), the most frequently used biochemical marker and clinical scoring system in acute pancreatitis, respectively.

Applications

The results of the meta-analysis encourage the use of uTAP in routine clinical practice, although this needs to be established in further well designed studies with possible comparisons to the new severity classification systems.

Peer review

This is a well written study that provides useful data on the usefulness of uTAP in the diagnostic/staging algorithm for acute pancreatitis. It is a powerful study that essentially means that uTAP is unlikely to find a widespread place in acute pancreatitis prognostic scoring as there are other more widely used tests available that are equivalent.

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CASE REPORT

Neuroendocrine carcinoma of the extrahepatic bile duct: Case report and literature review

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Abstract

Neuroendocrine carcinoma (NEC) of the extrahepatic bile duct is rare, and only 22 cases have been reported. Only two of these were large-cell NEC (LCNEC); the vast majority were small-cell NEC. Here, we report a third case of LCNEC of the extrahepatic bile duct. A 76-year-old male presented to a local hospital with painless jaundice. Imaging studies revealed a tumor at the hepatic hilum. The patient underwent right hepatic lobectomy, bile duct resection, and cholecystectomy. The resection specimen showed a 5.0-cm invasive neoplasm involving the hilar bile ducts and surrounding soft tissue. Histologically, the tumor consisted of nests of medium to large cells with little intervening stroma. The tumor invaded a large portal vein branch. All four excised lymph nodes were positive for metastasis, and metastatic deposits were also present in the gallbladder wall. The tumor was diffusely positive for synaptophysin and focally positive for chromogranin A. Approximately 70%-80% of the tumor cells were positive for Ki-67, indicating strong proliferative activity. A diagnosis of LCNEC was made. A few bile ducts within and adjacent to the invasive tumor showed dysplasia of the intestinal phenotype and were focally positive for synaptophysin and chromogranin A, suggesting that the dysplastic intestinal-type epithelium played a precursor role in this case. A postoperative computer tomography scan revealed rapid enlargement of the abdominal and retroperitoneal lymph nodes. The patient died 21 d after the operation. NEC of the bile duct is an aggressive neoplasm, and its biological characteristics remain to be better defined.

Key words: Neuroendocrine neoplasm; Large cell neuroendocrine carcinoma; Small cell neuroendocrine carcinoma; Extrahepatic bile duct; Dysplasia

Core tip: The authors report a case of large-cell neuroendocrine carcinoma (LCNEC) of the hilar bile duct. Concurrent dysplasia with intestinal and neuroendocrine differentiation was suggested to be a precursor in this case. Neuroendocrine carcinoma (NEC) of the bile duct occurs more frequently in men (male:female ratio 1.9:1). The mid-portion of the common bile duct appears to be the commonest site of involvement. All three reported cases of LCNEC died within 12 mo and the prognosis of NEC of the bile duct appears to be equally poor in both small-cell NEC and LCNEC. Multimodal treatment may improve outcome in this highly aggressive cancer.

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INTRODUCTION

Neuroendocrine neoplasms of the extrahepatic bile ducts are rare, with carcinoid tumors representing the most

common type. Neuroendocrine carcinoma (NEC) is defined as a poorly differentiated, high-grade, malignant neuroendocrine neoplasm, which is classified as either small-cell NEC (SCNEC) or large-cell NEC (LCNEC)[1]. NEC of the extrahepatic bile ducts is exceedingly rare, and only 22 cases have been reported in the literature (Table 1)[2-23]. Of these, most cases are SCNEC, and only two cases of LCNEC have been reported to date. Since LCNEC was first described in the lung by Travis *et al*[24] in 1991, similar lesions have been described in extrapulmonary sites, including the gastrointestinal tract[25]. LCNEC shows immunohistochemical evidence of epithelial and neuroendocrine differentiation and is characterized by a diffuse growth pattern or neuroendocrine architecture (organoid, palisaded, rosettes, or trabeculae)[24,26]. Large cell size, low nuclear to cytoplasmic ratio, and frequent nucleoli are key cytologic features that help distinguish LCNEC from SCNEC. LCNEC of the biliary tract was first described in the gallbladder by Papotti *et al*[27] a little more than a decade ago; however, reported cases are still few, particularly in the extrahepatic bile ducts. Because of the paucity of cases, patient prognosis and responsiveness to anticancer treatments for LCNEC of the biliary tract largely remain to be elucidated. Most of the reported cases of LCNEC of the biliary tracts showed an aggressive course and short survival times; however, one case of LCNEC arising in the gallbladder achieved long survival (69 mo from the initial diagnosis) following multimodal treatment that included surgery, chemotherapy, and radiation therapy[28].

The origin of neuroendocrine neoplasms of the biliary tracts is unclear. They may arise from metaplastic epithelia, where there are a variety of epithelial cells (including neuroendocrine cells, goblet cells, and gastric-type epithelial cells) that are not found in the normal biliary epithelium. In fact, intestinal and/or gastric-type metaplasias are not uncommon in non-neoplastic mucosa adjacent to a variety of neuroendocrine neoplasms of the gallbladder, including carcinoid tumor, SCNEC, and LCNEC[27,29,30].

Here, we report a case of LCNEC of the hilar bile duct. The bile duct mucosa adjacent to the LCNEC showed high-grade dysplasia of the intestinal phenotype, which may have been the precursor in this case. While the presence of concurrent nearby dysplasia was described in 2 previously reported cases of SCNEC[4,16], neuroendocrine differentiation or metaplastic change has not been demonstrated in dysplastic epithelium. Therefore, we aimed to perform detailed immunophenotypic characterization of the dysplastic epithelium and demonstrated intestinal and neuroendocrine differentiation in the dysplastic epithelium. Another objective of this article is to provide a comprehensive literature review on NEC of the bile duct. Despite the paucity of cases, an attempt was made to compare the clinicopathologic characteristics and outcomes between SCNECs and LCNECs.

CASE REPORT

A 76-year-old male presented to a local hospital with a 2 wk history of increasing yellowish discoloration of the skin and dark-colored urine. The patient had no abdominal pain, nausea, or vomiting. He had a history of hypertension, rheumatoid arthritis, peptic ulcer disease, status post-stent placement for ischemic heart disease, and status post-right inguinal hernia repair. The patient denied any family history of cancer. His medication included metoprolol and benazepril. The patient was admitted to the local hospital for 7 d, during which time an abdominal computed tomography (CT) scan revealed a tumor involving the hepatic hilum. Endoscopic retrograde cholangiopancreatography demonstrated a stricture at the common hepatic duct, and two biliary stents were placed. The imaging studies suggested a malignant biliary stricture, but this could not be confirmed with biopsy. After the stent placement, the patient's serum bilirubin, which was initially reported to be 10 mg/dL, decreased to approximately 5 mg/dL and the yellowish discoloration of his skin normalized. The patient was transferred to the department of Surgery at the University of Pittsburgh Medical Center for further work-up and treatment. On admission, the patient's vital signs were within normal limits. The patient was obese with a body mass index of 34 kg/m^2. The sclerae were slightly icteric. The patient had normal cardiac and respiratory examinations and did not have cervical, axillary, or supraclavicular lymphadenopathy. Examination of the abdomen showed mild epigastric tenderness to deep palpation with no rebound tenderness or guarding. The right inguinal area had a well-healed scar from an inguinal hernia repair in the 1950s. The patient's complete blood count and serum biochemistry data on admission were as follows: white blood cells, 11300/mm^3; hemoglobin, 13.4 g/dL; hematocrit, 39.5%; platelets, 377000/mm^3; blood glucose, 122 mg/dL; total bilirubin, 3.5 mg/dL; aspartate aminotransferase, 106 IU/L; alanine aminotransferase, 88 IU/L; and gamma-glutamyl transpeptidase, 80 IU/L. Carbohydrate antigen 19-9 was 32.9 U/mL. Chest X-ray was normal. The patient underwent an operation in October 2011 with the presumed diagnosis of hilar cholangiocarcinoma (Klatskin tumor). The tumor of the hepatic hilum was resected with a right hepatic lobectomy, bile duct resection, and cholecystectomy. The patient had markedly enlarged lymph nodes in the hepatic hilum and retropancreatic area, which were also resected. There was no evidence of extrahepatic disease other than lymphadenopathy. Reconstruction was performed with a Roux-en-Y hepaticojejunostomy. In the resection specimen, the neoplasm diffusely involved the perihilar bile ducts and the surrounding portal connective tissue and measured 5.0 cm at its greatest dimension (Figure 1). The tumor had also invaded a large, right portal vein branch and formed an intraluminal mass. Histologically, the tumor cells were poorly differentiated with no evidence of glandular or squamous differentiation. The tumor cells were arranged in cellular nests and sheets with a small amount of intervening fibrovascular stroma (Figure 2A). The tumor cells had medium to large hyperchromatic nuclei with fine to coarse granular chromatin and occasional small nucleoli. The tumor cells had a small to

Table 1 Neuroendocrine carcinoma of the extrahepatic bile ducts-review of the literature

No.	Ref.	Age (yr)	Sex	Histology	Location	Maximal dimension (cm)	Treatment	Follow-up information	Other findings
1	Sabanathan *et al*[2]	67	M	SCNEC	Bm	5	Palliative bypass and chemotherapy	Alive 6 mo	
2	Miyashita *et al*[3]	85	F	SCNEC	Bi	3	Palliative bypass	DOD 5 mo after surgery	
3	Kuraoka *et al*[4]	75	M	SCNEC	Bi	4.5	Resection	Alive 5 mo after surgery	Dysplasia
4	Hazama *et al*[5]	60	M	SCNEC	CBD	0.3	Neoadjuvant chemotherapy and resection	DOD 12 mo after surgery	
5	Arakura *et al*[6]	70	F	SCNEC	Bm	3	Resection and chemotherapy	DOD 14 mo after surgery	
6	Park *et al*[7]	60	F	SCNEC	Bs-Bm	3	Resection	DOD 5 mo after surgery	
7	Thomas *et al*[8]	54	M	SCNEC	Bh-CBD	NA	Resection	Alive With Metastasis, 6 mo	Clonorchis sinensis infestation
8	Viana Miguel *et al*[9]	76	M	SCNEC	Bm	NA	Resection, chemotherapy and irradiation	Alive 5 mo after surgery	Gallstone
9	Jeon *et al*[10]	65	M	SCNEC	Bs-Bm	2	Presection and chemotherapy	DOD 12 mo after surgery	
10	Nakai *et al*[11]	32	M	SCNEC	CBD	NA	NA, diagnosed by autopsy	NA	
11	Arakura *et al*[12]	75	M	SCNEC	Bh, Bs, Bm, Bi	6.5	Chemotherapy and irradiation	DOD 10 mo after therapy	
12	Hosonuma *et al*[13]	69	F	SCNEC	Bs-Bm	3	Biliary drainage	Alive 2 mo after biliary drainage	
13	Okamura *et al*[14]	62	M	SCNEC	Bm	3	Preoperative chemotherapy, resection and irradiation	DOD 20 mo after surgery	
14	Yamaguchi *et al*[15]	77	F	NEC	Bi	NA	Resection and chemotherapy	Alive 27 mo	
15	van der Wal *et al*[16]	55	M	SCNEC + atypical carcinoid + AD	Bm	4	Resection	NA	Dysplasia/ carcinoma *in situ*
16	Nishihara *et al*[17]	64	M	SCNEC + AD	Bh-Bs	1.9	Resection	Alive 8 mo after surgery	
17	Yamamoto *et al*[18]	71	F	SCNEC + AD	Bh	6	Resection	DOD 7 mo after surgery	Common bile duct stones
18	Kim *et al*[19]	64	M	SCNEC + AD	Bm	3	Resection	Alive 30 d after surgery	Clonorchis sinensis infestation
19	Edakuni *et al*[20]	82	F	SCNEC + AD	Bm	6	Resection	Alive 45 mo after surgery	
20	Kaiho *et al*[21]	66	F	SCNEC + AD	Bm	3.5	Resection and chemotherapy	DOD 8 mo after surgery	
21	Sato *et al*[22]	68	M	LCNEC + AD	Bi	2	Resection and chemotherapy	DOD 3 mo after surgery	
22	Demoreuil *et al*[23]	73	M	LCNEC + AD	Bh-Bs	3	Resection and chemotherapy	DOD 12 mo after surgery	
23	Current report, 2013	76	M	LCNEC	Bh-Bs	5	Resection	DOD 21 d after surgery	Dysplasia, intestinal type

SCNEC: Small cell neuroendocrine carcinoma; NEC: Nneuroendocrine carcinoma, not otherwise specified; AD: Adenocarcinoma; LCNEC: Large cell neuroendocrine carcinoma; Bm: Mid portion of common bile duct; Bi: Inferior or distal common bile duct; CBD: Common bile duct, not otherwise specified; Bs; Superior or proximal common bile/hepatic duct; Bh: Hilar bile duct; DOD: Died of disease. N/A: Information not available; F: Female; M: Male.

moderate amount of amphophilic cytoplasm (Figure 2B). They showed brisk apoptotic activity and frequent mitotic figures (15-18 mitoses per 10 high-power fields). The tumor showed perineural and angiolymphatic invasion. All four lymph nodes were positive for metastatic tumor. The resected gallbladder had microscopic metastatic deposits in the perimuscular layer. No gallstones were present. The tumor cells were negative for mucicarmine stain. Immunohistochemical stains revealed that the tumor cells were strongly positive for synaptophysin (Figure 2C). They were also focally positive for chromogranin A, pancytokeratin, cytokeratin (CK) 7, CK 19, and MOC-31. Fewer than 1% of the tumor cells were weakly positive for thyroid transcription factor-1. They were negative for napsin-A, surfactant apoprotein A, alpha-fetoprotein, vimentin, CK5/6, p63, leukocyte common antigen, and S100. Approximately 70%-80% of the tumor cells were positive for Ki-67 (Figure 2D). The histologic findings and the immunoprofile of the neoplasm were consistent with LCNEC. A few foci of intermediate- to high-grade dysplasia were also identified

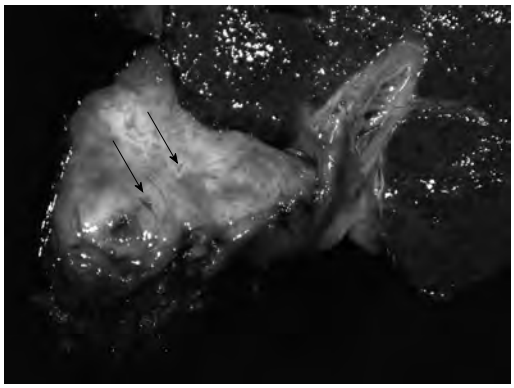

Figure 1 Gross appearance of the hilar neoplasm. The resected specimen showed a firm, tan-grey tumor measuring 5.0-cm in greatest dimension with extensive involvement of the perihilar bile ducts and surrounding soft tissue. Some of the small ducts and vessels within the lesion showed severe luminal narrowing (arrows).

in the perihilar bile ducts located within and adjacent to the invasive neoplasm (Figure 3A). The dysplastic epithelium contained some goblet cells. The dysplastic epithelial cells were immunoreactive for CK19, CK20, and CDX2 (Figure 3B and C). The goblet cells were positive for mucin (MUC)2 and negative for CK7, MUC1, MUC5AC, and MUC6. The immunoprofile of the dysplastic epithelium, together with the presence of goblet cells expressing MUC2 was consistent with an intestinal phenotype. The epithelial cells of this lesion were also positive for synaptophysin (Figure 3D) and chromogranin A but with less extensive and intense immunoreactivity compared to the invasive neoplasm. The findings of the in situ component were suggestive of a premalignant or preinvasive lesion rather than intraepithelial spread from the invasive neoplasm. The patient's immediate postoperative course was uncomplicated; however, a CT scan of the abdomen eight days after the operation revealed extensive enlargement of his abdominal and retroperitoneal lymph nodes, which was suggestive of rapid metastasis. The patient had poor oral intake, hypovolemia, and significant back pain. Postoperative chemotherapy or radiation therapy was not performed because of the patient's poor general condition. The patient died 21 d after operation. An autopsy was not performed.

DISCUSSION

According to the most recent World Health Organization (WHO) classification, neuroendocrine neoplasms of the digestive system are classified into three general categories based on histologic features and proliferation fraction[1,31]. These include NET, NEC, and mixed adenoneuroendocrine carcinoma (MANEC). A NET is defined as well-differentiated neuroendocrine neoplasm with mild to moderate nuclear atypia and a low proliferation fraction (≤ 20 mitoses per 10 high-power fields or ≤ 20% Ki67 index). This category encompasses neoplasms termed carcinoid tumors. A NEC is a poorly differentiated, high-grade malignant neuroendocrine neoplasm composed of either small or intermediate to large cells with marked nuclear atypia and a high proliferation fraction (> 20 mitoses per 10 high-power fields or > 20% Ki67 index). This category includes SCNEC and LCNEC, but a dichotomous subclassification of small cell *vs* non-small cell may also apply[25]. MANEC has a phenotype that is morphologically recognizable as both gland-forming epithelial and neuroendocrine carcinomas. Arbitrarily, at least 30% of either component should be identified to qualify for this definition.

Neuroendocrine neoplasms of the extrahepatic bile ducts are rare, and the majority of the reported cases are NET/carcinoid, which represent 0.1%-0.2% of all gastrointestinal carcinoids/NET[32]. According to the data obtained from the Surveillance, Epidemiology, and End Results program of the National Cancer Institute, there were 31 cases of carcinoid, 17 of SCNECs, and 10 of NECs of not-otherwise-specified type of the gallbladder and extrahepatic bile ducts between 1973 and 2005[33]. Thus, NEC of the extrahepatic bile duct is exceedingly rare and is probably less frequent than NET/carcinoid. According to the literature, 23 cases of NEC of the extrahepatic bile ducts, including the case described here, have been reported. The most common histologic subtype of NEC of the extrahepatic bile ducts is SCNEC (19 of 23 cases; Table 1). Only two cases of LCNEC of the common bile duct were previously reported (Table 1, cases 21 and 22); therefore, this is the third reported case of LCNEC arising in the extrahepatic bile duct. The reported cases of NEC include 15 males and 8 females, with a male to female ratio of 1.9: 1. Patient age ranged from 32-85 years with a mean age of 67.2 years. The mean age of LCNEC was higher than that of SCNEC (72.3 years *vs* 65.9 years), but this difference was not statistically significant. NEC can occur anywhere in the extrahepatic bile duct, but the mid portion of the common bile duct appears to be the most common site of involvement. Two cases had biliary stones (Table 1, cases 8 and 17). Concurrent *Clonorchis sinensis* infestation was seen in two cases (Table 1, cases 7 and 18). The presence of concurrent nearby dysplasia was described in three of 23 cases, including ours (Table 1, cases 3, 15 and 23). Eight cases were composite neuroendocrine and adenocarcinoma (Table 1, cases 15-22). Some of these composite cases may be classified as MANEC rather than NEC according to the current WHO classification system, depending on the proportion of the adenocarcinoma component. When NEC coexisted with adenocarcinoma, a gradual transition between areas of NEC and adenocarcinoma was observed in six of eight cases (Table 1, cases 15-17 and 19-21). In three of eight cases (Table 1, cases 18, 19 and 21), the adenocarcinoma component was located in the superficial portion of the tumor, and the NEC component was located mainly in the deeper portion of the tumor. No other particular spatial relationships between NEC and adenocarcinoma components have been described.

NEC of the gastrointestinal tract can show a spectrum of morphologic features ranging from classic SCNEC to LCNEC, and some cases have features between

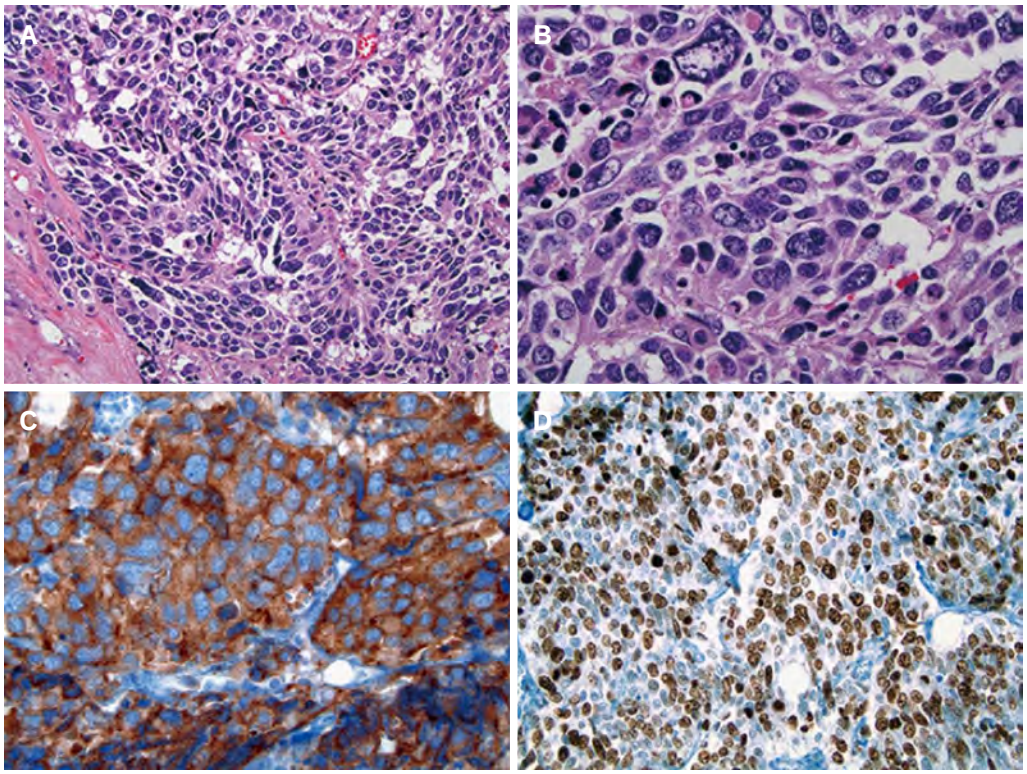

Figure 2 Histologic features of the hilar neoplasm. A: The tumor cells were arranged in cellular nests and sheets with little intervening fibrovascular stroma [Hematoxylin and eosin (HE), × 200]; B: The tumor cells had a small to moderate amount of amphophilic cytoplasm and medium to large hyperchromatic nuclei with fine to coarse granular chromatin and occasional small nucleoli (HE, × 400); C: The tumor cells were strongly positive for synaptophysin (× 400); D: 70%-80% of the tumor cells were positive for Ki-67 (× 200).

these two types. As Shia *et al*[25] previously pointed out, there were no criteria for classifying NEC of the GI tract with non-small cell morphology before the most recent WHO classification. Therefore, NEC of the extrahepatic bile ducts and gallbladder with non-small cell morphology may have been diagnosed inconsistently. In fact, histologic features of some of the previously reported cases of SCNEC of the extrahepatic bile ducts are not morphologically typical of SCNEC. For example, the NEC tumor component reported as a part of composite adenocarcinoma and SCNEC of the hilar bile duct by Yamamoto *et al*[18] (Figure 2B in their manuscript) showed prominent nucleoli and moderately abundant cytoplasm, which is not typical of SCNEC (Table 1, case 17). Thus, some of the non-small cell type NECs or LCNEC may have been diagnosed or reported as SCNEC because of the previous lack of a distinct diagnostic category.

LCNEC of the extrahepatic bile ducts is extremely rare. Both of the previously reported cases contained a minor component of adenocarcinoma (10%-20% of the entire tumor). One tumor was located in an intra-pancreatic portion of the common bile duct (Table 1, case 21), and another was located in the perihilar bile duct (Table 1, case 22). The adenocarcinoma component of one case (Table 1, case 21) showed focal expression of a neuroendocrine marker (chromogranin A), and the other case did not express neuroendocrine markers (Table 1, case 22). The coexistence of adenocarcinoma in these cases, as well as in the aforementioned six cases of composite adenocarcinoma and SCNEC suggests that NEC of the common bile duct may arise from pluripotent progenitor cells. This idea is further supported by the observation of transitional zones between NEC and adenocarcinoma components in the majority of cases. The invasive tumor described here was composed entirely of LCNEC. Although no adenocarcinoma component was identified, a few perihilar bile ducts located within and adjacent to the LCNEC showed dysplasia of the intestinal phenotype with focal endocrine differentiation. Although data on the histogenesis of neuroendocrine neoplasm of the biliary tracts in the literature are limited, neuroendocrine neoplasm of the bile duct and gallbladder may arise from neuroendocrine cells in intestinal or gastric metaplasia, where there may be progenitor cells with a greater ability to differentiate into neuroendocrine cells[29,34]. The presence of intestinal type dysplasia in our case suggests that LCNEC may have arisen from metaplastic epithelium, although we cannot exclude the possibility that the metaplastic/neuroendocrine phenotype may have been acquired at the time of or subsequent to the dysplasia. Regardless, the progression from dysplasia with an intestinal/neuroendocrine phenotype to an aggressive NEC may have occurred in the case described.

The prognosis of NEC of the bile duct appears to be poor. Among the 21 cases with follow-up data, 57% (12/21) of the patients died of disease 3 to 20 mo after surgery, and only two patients have been reported to survive more than 2 years (Table 1, cases 14 and 19). Among

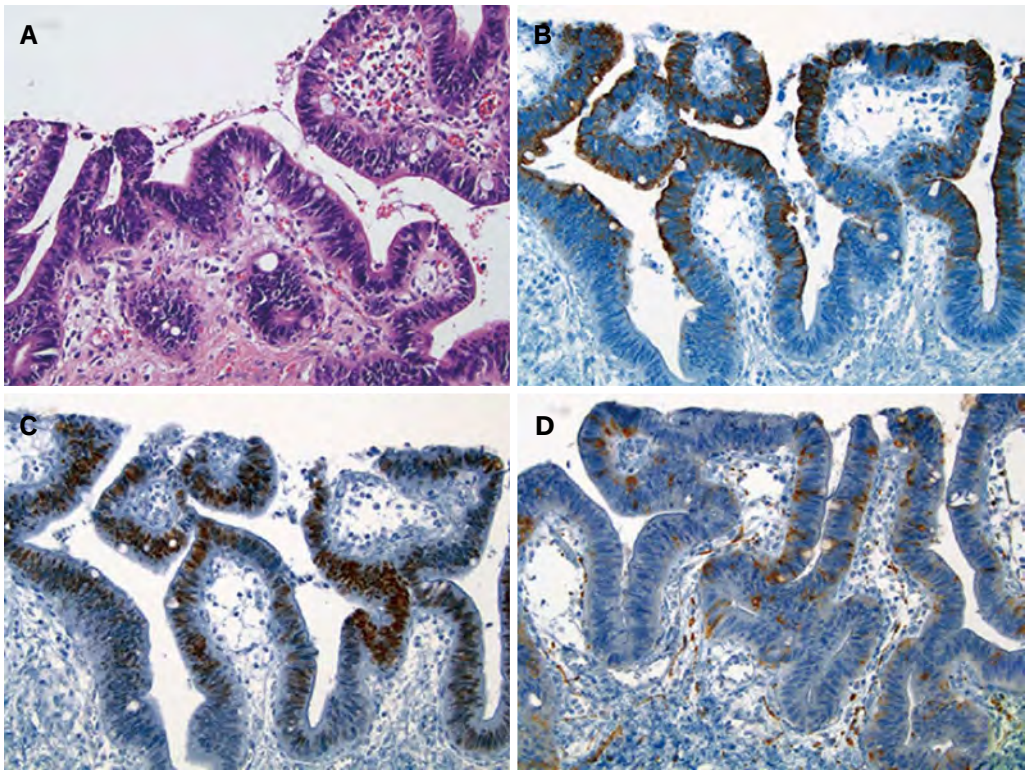

Figure 3 Histologic features of the dysplastic epithelium found in a medium-sized perihilar bile duct located within the invasive neoplasm. A: The dysplastic epithelium contained some goblet cells [Hematoxylin and eosin (HE), × 200]; B, C: The dysplastic epithelial cells were immunoreactive for CK20 (B, × 200) and CDX2 (C, × 200); D: Some of the dysplastic epithelial cells were positive for synaptophysin with less extensive and weak immunoreactivity compared to the invasive neoplasm (× 200).

the seven patients who survived at least 12 mo, five were treated with multidisciplinary treatment, including surgical resection, adjuvant or neoadjuvant chemotherapy, and radiation. The longest survival was 45 mo in a patient who was treated with surgical treatment alone (Table 1, Case 19). According to Edakuni *et al*[20], that tumor was a composite adenocarcinoma (-40%) and SCNEC (-60%). They speculated that the reason for the long survival may have been the low proliferative fraction (9.6% Ki-67-positive tumor cells) of the SCNEC. Recently, most neuroendocrine neoplasm grading systems rely extensively on the proliferation rate, which has been shown to provide significant prognostic information[31]. Based on the current WHO grading system, the NEC component of the case reported by Edakuni *et al*[20] may be best classified as intermediate-grade NET rather than SCNEC, and this appears to at least in part explain the long survival time. According to Iype *et al*[35], LCNECs appear to have a worse prognosis than SCNEC in the gallbladder. Although only two cases of LCNEC of the bile duct have been previously reported, both patients died within 12 mo of surgery despite postoperative chemotherapy (Table 1, cases 21 and 22). In our case, the patient died 21 d after the operation with radiographic evidence of rapid progression of metastatic disease. Thus, the survival for patients with LCNEC of the extrahepatic bile duct appears to be equally poor as for those with SCNEC. Multidisciplinary management appears to be effective and provide longer survival time for SCNEC of the bile duct (Table 1, cases 13 and 14). Recently, a long surviving case of LCNEC of the gallbladder was reported by Shimono *et al*[28]. That patient received multimodal treatment consisting of chemotherapy, radiation therapy, surgical resection, and γ-knife irradiation for brain metastases, which resulted in 69 mo of survival. This case suggests that multimodal treatment is potentially effective in treating LCNEC patients. The effectiveness of chemotherapy and radiotherapy in both SCNEC and LCNEC of the biliary tract needs to be further investigated in a larger number of cases to confirm this observation.

In summary, we reported a case of high-grade neuroendocrine neoplasm arising in the perihilar bile ducts that was best classified as LCNEC. The coexistent dysplasia with intestinal and neuroendocrine differentiation may represent a LCNEC precursor. We feel that histologic subtyping of NECs into SCNEC and LCNEC (or even non-small cell NEC), rather than grouping all types of high-grade neuroendocrine neoplasms together as NECs, is necessary because biologic characteristics of each subtype need to be more clearly defined for better prognostication and selection of therapy.

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CASE REPORT

Retroperitoneal cavernous hemangioma resected by a pylorus preserving pancreaticoduodenectomy

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Abstract

A retroperitoneal hemangioma is a rare disease. We report on the diagnosis and treatment of a retroperitoneal hemangioma which had uncommonly invaded into both the pancreas and duodenum, thus requiring a pylorus preserving pancreaticoduodenectomy (PpPD). A 36-year-old man presented to our hospital with abdominal pain. An enhanced computed tomography scan without contrast enhancement revealed a 12 cm × 9 cm mass between the pancreas head and right kidney. Given the high rate of malignancy associated with retroperitoneal tumors, surgical resection was performed. Intraoperatively, the tumor was inseparable from both the duodenum and pancreas and PpPD was performed due to the invasive behavior. Although malignancy was suspected, pathological diagnosis identified the tumor as a retroperitoneal cavernous hemangioma for which surgical resection was the proper diagnostic and therapeutic procedure. Reteoperitoneal cavernous hemangioma is unique in that it is typically separated from the surrounding organs. However, clinicians need to be aware of the possibility of a case, such as this, which has invaded into the surrounding organs despite its benign etiology. From this case, we recommend that combined resection of inseparable organs should be performed if the mass has invaded into other tissues due to the hazardous nature of local recurrence. In summary, this report is the first to describe a case of retroperitoneal hemangioma that had uniquely invaded into surrounding organs and was treated with PpPD.

Key words: Retroperitoneal tumor; Retroperitoneal cavernous hemangioma; Cavernous hemangioma; Pancreaticoduodenectomy; Pylorus preserving pancreaticoduodenectomy

Core tip: A retroperitoneal cavernous hemangioma is a rare disease. This case of retroperitoneal hemangioma had uniquely invaded into the duodenum and pancreas head, and thus required treatment with pylorus preserving pancreaticoduodenectomy. Although hemangiomas are typically benign, clinicians should be aware of the possibility of invasion into the surrounding organs such as with this case. In the event of invasion, we recommend a combined resection of both the tumor and affected organs to reduce the chance of local recurrence that may be associated with inadequate resection.

Hanaoka M, Hashimoto M, Sasaki K, Matsuda M, Fujii T, Ohashi K, Watanabe G. Retroperitoneal cavernous hemangioma resected by a pylorus preserving pancreaticoduodenectomy. *World J Gastroenterol* 2013; 19(28): 4624-4629 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i28/4624.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i28.4624

INTRODUCTION

A retroperitoneal hemangioma is an uncommon disease in adulthood[1-3]. Only 23 cases of adult retroperitoneal hemangioma have been reported in the literature since 1950. Among those, five cases, including the case detailed in this report, needed combined resection of surrounding organs because of adhesion of the tumor. In this report, we describe the diagnosis and treatment of a retroperitoneal hemangioma that had uniquely invaded into the pancreas and duodenum and required a pylorus preserving pancreaticoduodenectomy (PpPD).

CASE REPORT

A 36-year-old man came to a local hospital with right upper quadrant pain since the day before admission. He had no specific medical history or family history. A screening-enhanced computed tomography (CT) scan revealed a bulky tumor between the dorsal side of the pancreatic head and the right kidney. The tumor was diagnosed as a retroperitoneal sarcoma and the patient was referred to Toranomon Hospital for the operation. On physical examination, the patient was found to have no palpable mass. The results of urine, blood, and adrenal cortex functional tests were within normal limits. An abdominal enhanced CT scan showed a 12 cm × 9 cm tumor without marked contrast enhancement, pushing the pancreas to ventral side (Figure 1A). The mass was distinct from the surrounding organs, including the duodenum, pancreas, kidney and retroperitoneal spaces as observed from the CT scan and therefore appeared to be resectable. An abdominal ultrasound showed an uneven echoic lesion in the same area as observed by CT. T1-weighted image of magnetic resonance imaging (MRI) showed low and a few part of relatively high intensity area inside the tumor (Figure 1B). On fat suppression examination of the MRI image, the tumor was not suppressed to any degree. T2-weighted image also showed heterogeneous finding; there were high intensity area with a few part of intermediate signal intensity area. However, there were no typical findings which suggest the type of retroperitoneal tumor on diagnostic images (Figure 1C). Based on the qualitative assessments, the tumor was diagnosed as a retroperitoneal mesenchymal tumor. Because of the high rate of malignancy associated with retroperitoneal tumors, surgical resection was performed.

Intraoperatively, the mass measured 12 cm × 9 cm in diameter. We initially tried to perform a tumor excision without combined resection of surrounding organs. However, the intraoperative findings revealed that the mass encroached on the head of the pancreas and duodenum (Figure 2). As such, we performed Kocher's mobilization of the duodenum and tried to separate the tumor from the duodenum and pancreas; however, the tumor could not be successfully separated from these organs. After the tumor was lifted from the retroperitoneal space, we tried again to separate the tumor from the pancreas head and duodenum. The tumor adhered strongly to both tissues and could only be removed completely by PpPD. The duration of surgery was 4 h and 16 min and the total blood loss was 561 mL. The patient was discharged from the hospital on postoperative day 24 and was in good health without recurrence over two and a half years after the operation.

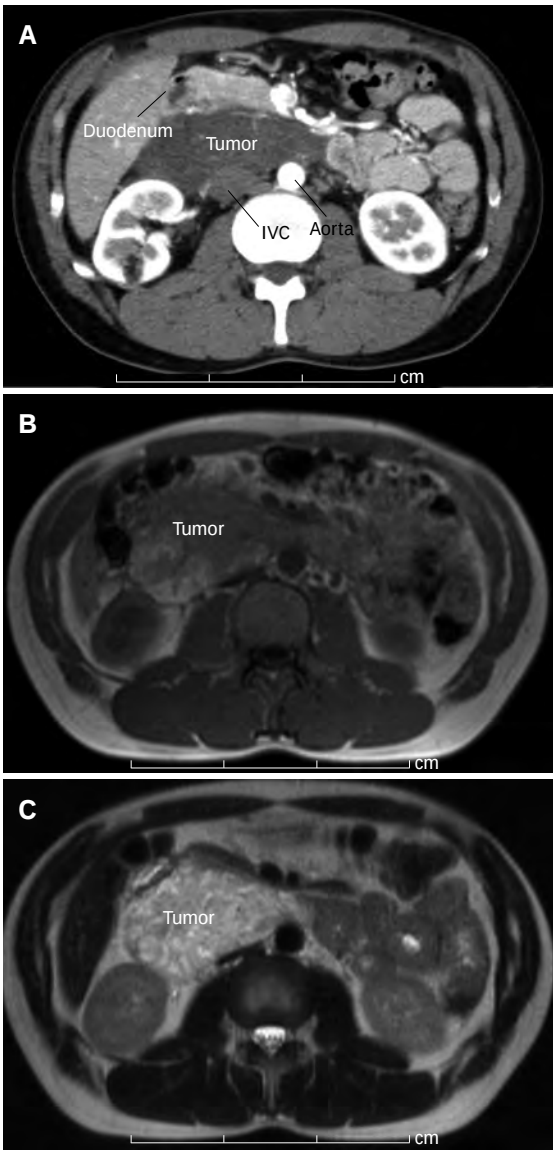

Figure 1 12 cm × 9 cm tumor was detected by computed tomography and magnetic resonance imaging. A: Abdominal enhanced computed tomography in early phase showed tumor without marked contrast. The tumor had pushed the pancreas to the ventral side; B: T1-weighted image of magnetic resonance imaging showed low and relatively high intensity area inside the tumor; C: T2-weighted image showed high intensity area with a few part of intermediate signal intensity area. IVC: Inferior vena cava.

Gross pathologic examination revealed a 120 mm × 95 mm × 50 mm hemangioma composed of multioculated cysts containing intra-cystic hemorrhages (Figure 3). The diagnosis on pathological examination was a cavernous hemangioma with a few focal areas of venous hemangioma (Figure 4A). As defined by the pathologist, the lesion had invaded into the muscle layer of the

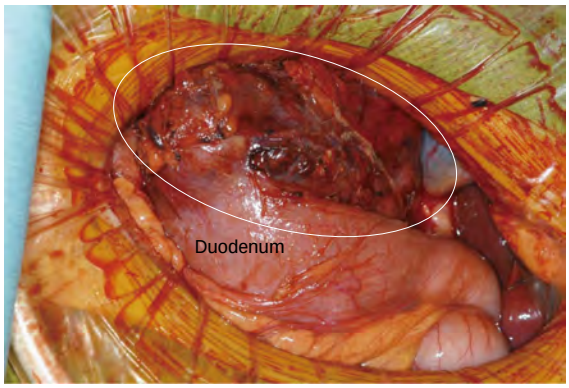

Figure 2 Macroscopic view of intraoperative findings. The mass was attached to both the duodenum and the pancreas head and required surgical resection by pylorus preserving pancreaticoduodenectomy. The circle denotes the tumor.

duodenum and the pancreas head (Figure 4B-D). Immunohistochemical analysis of tissue sections revealed that the lumina were positive for CD31 and CD34, markers of endothelial cells (Figure 4E), and partially weak positive for podoplanin/D2-40 (Figure 4F), a marker of lymphatic endothelial cells. Less angiogenic invasion was observed toward the retroperitoneal side of the tumor than toward the pancreas and duodenum. Interestingly, both macroscopically and microscopically, the tumor extended into both the pancreas and duodenum and not into the retroperitoneal space.

DISCUSSION

In this report, we describe a patient with retroperitoneal hemangioma that required PpPD. A retroperitoneal tumor is a very rare tumor that accounts for less than 0.2% of all tumor types[1]. Among malignant tumors located in the retroperitoneal space, liposarcomas and leiomyosarcomas are the most frequent, while teratomas, cysts and neurinomas are common benign masses. In the current case, the tumor was diagnosed initially as a retroperitoneal sarcoma; retroperitoneal liposarcomas are the most common malignant tumors of the retroperitoneal soft tissue[2]. Indeed, accurate diagnosis of a retroperitoneal hemangioma is classically difficult preoperatively and prior to pathological examination of the tissue.

An analysis of other retroperitoneal tumor types reveals subtle, yet distinct, differences in symptom presentation, localization, and radiographic features. Among retroperitoneal tumors, imaging studies of leiomyosarcomas demonstrate a non-specific mass but are helpful in delineating the relationship to adjacent structures[3]. Angiosarcomas classically present with cutaneous involvement of the head and neck region in elderly patients[4]. The solid growth pattern and epithelioid cytology can be easily confused with poorly differentiated carcinoma[5]. Most patients with lymphanginoleiomyomatosis (LAM), characterized by proliferation of smooth muscle cells, present with pulmonary symptoms, whereas extrapulmonary LAM is rare and typically presents in premenopausal females[6]. Cystic lymphangioma is a well-known benign tumor and its cystic abnormalities of the lymphatic vessels are predominantly congenital. By CT scan, the tumor is typically well-circumscribed and polycystic with thin septa similar in appearance to the cystadenomas[7]. Kaposiform hemangioendothelioma mainly occurs during childhood. MRI of the affected region is accepted as the

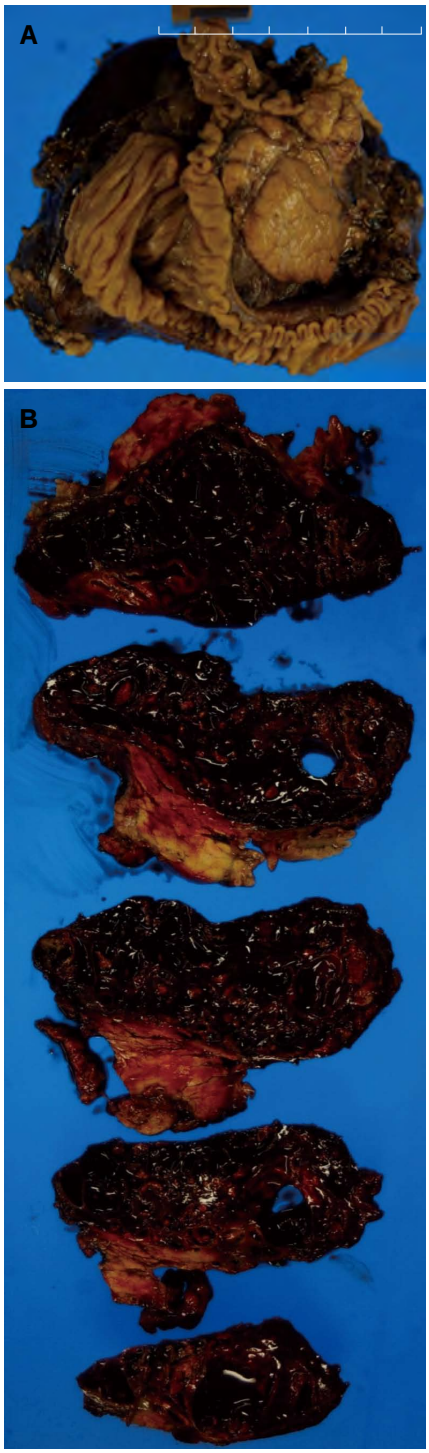

Figure 3 Macroscopic findings of the resected specimen. A: A 120 mm × 95 mm × 50 mm tumor was resected. Scale bar, 70 mm; B: The tumor contained multi-oculated cysts containing intra-cystic hemorrhages.

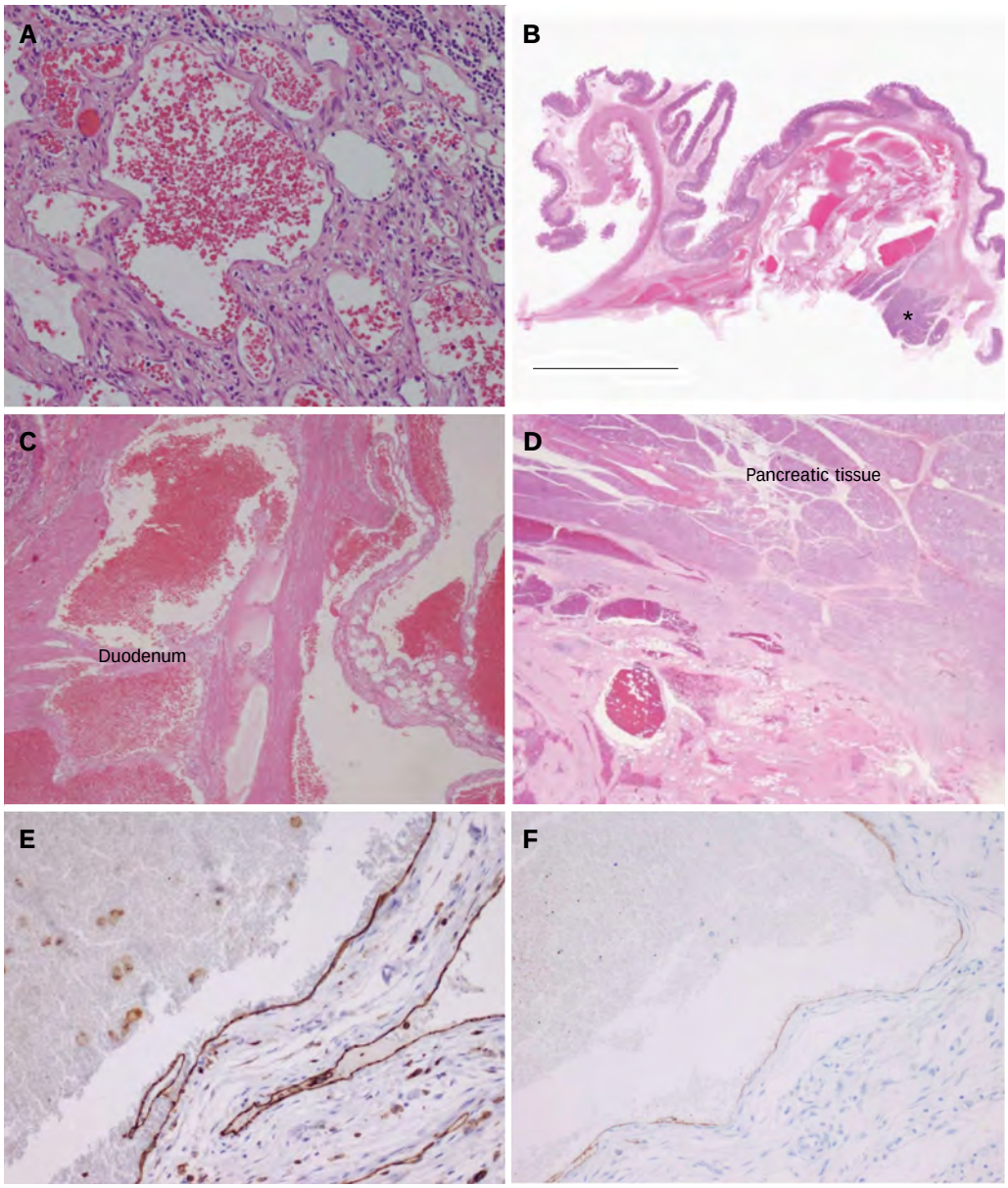

Figure 4 Pathological analysis by hematoxylin and eosin staining and immunohistochemistry. A: A representative tissue section from the main region of the cavernous hemangioma [hematoxylin and eosin (HE), magnification × 200]; B: The loupe view demonstrates a portion of cavernous hemangioma infiltrating the pancreatic head (asterisk) and duodenal wall (HE). The scale represents 10 mm; C: Tumor invasion into the muscle layer of the duodenum (HE, magnification × 4); D: Tumor invasion into the pancreas head (HE, magnification × 1); E: Positive immunostaining for CD31 supports the diagnosis of hemangioma (magnification × 20); F: The lumen showed partial and weakly positive staining for podoplanin/D2-40 (magnification × 20).

diagnostic imaging technique of choice[8]. Concerning hemangiomas arising from other tissues, pancreatic and mesenteric hemangiomas have been reported[9,10], which are extremely rare. MRI shows a common characteristic of pancreatic hemangiomas[9]. Mesenteric hemangiomas shows heterogeneous enhancement by enhanced CT and changes its shape during intestinal peristalsis[10].

As for pathological findings, gross pathological findings showed the tumor included multi-oculated cysts filled with blood. This cyst is suggested to be a pseudocyst which is a result of repeats of hemorrhage[11]. D2-40, reliable podoplanin antibody clone, has been described in a variety of lymphovascular neoplasms including lymphangioma, Kaposi sarcoma, and hemangioendothelioma[12]. Because lymphatic endothelial cells express high levels of podoplanin[13], we suggest that the weakly positive findings of D2-40 in the current case do not support a diagnosis of lymphangima. In addition to macroscopic and immunohystological findings, microscopic finding of almost all of lumen are filled with red blood cells supported the diagnosis as hemangioma.

Retroperitoneal hemangioma in the adult is extremely rare and confirmed in only 1%-3% of all retroperitoneal tumors[14]. Only 23 cases of adult retroperitoneal hemangioma have been reported in literature since 1950.

Retroperitoneal tumors are difficult to diagnose preoperatively[15] because there are usually no initial symptoms until tumors have grown large enough to produce

Table 1 Reported cases of retroperitoneal hemangioma with combined resection of surrounding organs

Ref.	Age (yr)	Sex	Tumor size (cm^3)	Hyper-vascularity	Curative resection	Pathological diagnosis	Organ(s) of combined resection
Ogura *et al*[21]	73	M	23 × 14 × 9	-	+	Cavernous	Left kidney
Takaha *et al*[20]	55	M	10 × 9 × 9	+	+	Cavernous	Spleen, diaphragm, chest wall
Syo *et al*[22]	72	F	9 × 7 × 7	-	+	Cavernous	Right ovarian artery
Tseng *et al*[18]	61	F	NA	+	-	Venous	NA
Hanaoka *et al*, 2013	36	M	12 × 19 × 5	-	+	Cavernous and Venous	Duodenum, pancreas head

Five cases, including this, needed combined resection of surrounding organs. In four cases, a complete combined resection of surrounding organs was performed, while in one case a subtotal resection was performed (Tseng *et al*[18]). NA: Not available.

patient discomfort[11]. Furthermore, retroperitoneal cavernous hemangiomas have features similar to ischemic tumors, but differ from hemangiomas arising from other tissues such as the skin or liver[11].

As for imaging studies of retroperitoneal cavernous hemangiomas, because they are usually only discovered when large enough to develop thrombi and organization at the center[16], these tumors often show slight to no enhancement in normal enhanced CT[11,14,15]. In the present case, the CT scan revealed a cystic mass with minor contrast enhancement, similar to cases reported previously[17,18]. In addition, retroperitoneal cavernous hemangiomas typically lack the complete fill-in or cotton-wool appearance in enhanced CT or high echoic areas with the same density as the abdominal echo, which is usually only seen in cavernous hemangiomas of the liver[11]. On T1-weighted image of MRI, relatively high intensity area inside the tumor is suggested to be hemorrhage and hyalinization of the tissue. A part of high intensity area of T2-weighted image suggests blood contain and relatively high signal intensity area shows hyalinization and fibrillization. However, these findings were suggested to be secondary change of structure, which don't indicate any typical tumors. With few clues for diagnosis, very few cases of retroperitoneal hemangiomas have been diagnosed preoperatively. Therefore, surgical resection is a choice for both diagnostic and therapeutic procedures.

One feature of a cavernous hemangioma is that it may be locally destructive by virtue of the pressure exerted on neighboring tissues[19]. In the present case, the pressure of the tumor affected the duodenum and pancreas, leading to invasion and destruction of these organs. Among the 23 reported retroperitoneal hemangiomas, five cases, including ours, needed combined resection of surrounding organs because of an adhesion (Table 1)[18,20-22]. Among the five cases, four cases performed complete combined resection of surrounding organs. In one case, subtotal resection was performed due to technical difficulties caused by firm adherence to the adjacent organs and the major blood vessels[22]. Like this case, which did not demonstrate findings typical of hemangioma from the contrast-enhanced CT, three cases including ours showed hypovascularity, while two cases showed hypervascularity. Pathologically, four cases, including this, were diagnosed as cavernous hemangiomas and one was diagnosed as a venous hemangioma. Hence, we conclude that vascularity from CT analysis and pathological diagnosis are not always directly correlated with adhesion to other organs.

The recommended treatment for retroperitoneal hemangioma has been surgical resection[11,15,23]. Hemangiomas are non-malignant, but patients run the risk of rupture and bleeding[24]. Therefore, surgical treatment is recommended for high-risk tumors, such as with those of large masses[25]. Although hemangiomas are benign, local recurrence has been reported with inadequate resection[26]. In this case, the decision was made to combine the tumor resection with resection of the surrounding organs to avoid local recurrence in the pancreas and duodenum from residual tumor tissue. Therefore, the PpPD procedure for this case was appropriate. From a treatment standpoint, we support a combined resection of both the tumor and the compromised organs if the tumor is invasive and cannot be removed cleanly because of adhesion.

In conclusion, a retroperitoneal cavernous hemangioma is an uncommon disease. Clinicians need to be aware of the possibility of a case that has invaded into surrounding organs despite its benign pathology. This case of a retroperitoneal hemangioma had uniquely invaded into the duodenum and pancreas and this is the first report of treatment using PpPD.

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CASE REPORT

Unusual upper gastrointestinal bleeding: Ruptured superior mesenteric artery aneurysm in rheumatoid arthritis

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Abstract

This case report describes an unusual case of upper gastrointestinal (UGI) bleeding caused by a ruptured superior mesenteric artery (SMA) aneurysm in the duodenum in a patient with rheumatoid arthritis. The patient presented with UGI bleeding and hemorrhagic shock. Emergency UGI endoscopy could not identify the source of the bleeding because of excessive blood clots under the second portion of the duodenum. An SMA aneurysm with active contrast extravasation was diagnosed by computed tomography. The aneurysm, together with the fourth portion of the duodenum and the proximal portion of the jejunum, was surgically resected, and the SMA was skeletonized. On postoperative day 15, the patient was discharged from hospital under satisfactory conditions. Rheumatoid arthritis has been known to cause a wide spectrum of manifestations, and an SMA aneurysm is an unusual extra-articular manifestation. An SMA aneurysm rupture presenting as upper gastrointestinal bleeding is a rare complication with a high mortality rate. The clinician must be alert to this potential issue to achieve rapid diagnostic confirmation, and immediate surgical or radiological intervention.

Key words: Gastrointestinal bleeding; Aneurysm; Rheumatoid arthritis; Endoscopy; Duodenum

Core tip: Gastrointestinal bleeding is a common medical emergency. Ruptured superior mesenteric artery aneurysm is an uncommon cause of gastrointestinal bleeding, with few cases reported previously. Diagnosis is difficult before surgery. We reported the successful diagnosis and treatment of such a case.

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INTRODUCTION

Upper gastrointestinal (UGI) bleeding is a common medical emergency. Patients usually present with hematemesis or melena, and are diagnosed after endoscopic evaluation. Peptic ulcers and esophagogastric varices account for most causes of UGI bleeding. A visceral aneurysm is an uncommon cause of gastrointestinal bleeding, and diagnosis is usually made after surgery. Here, we report an interesting case with pre-operative diagnosis of a ruptured superior mesenteric artery (SMA) aneurysm in the duodenum of a patient with rheumatoid arthritis.

CASE REPORT

A 27-year-old man with a history of rheumatoid arthritis was brought to the emergency department because of an episode of coffee ground vomitus and dizziness. He suffered from melena for 2 d. His blood pressure was 89/52 mmHg and his heart rate was 106/min. He presented with alert consciousness in the emergency department. A nasogastric tube lavage revealed coffee ground material, and further investigation revealed hemoglobin

levels of 8.1 g/dL. Emergency UGI endoscopy was performed after his hemodynamic status was stabilized with fluid hydration and blood transfusion. UGI endoscopy found excessive blood clots below the second portion of the duodenum. To identify the source of the bleeding, abdominal contrast-enhanced computed tomography (CT) was performed. The CT scan revealed an aneurysm about 3.9 cm in diameter at the jejunal mesentery; with active contrast extravasation into the fourth portion of the duodenum (Figure 1). The aneurysm, together with the fourth portion of the duodenum and the proximal portion of the jejunum, was surgically resected, and the SMA was skeletonized. Pathology revealed an aneurysm measuring 3.8 cm × 3.5 cm × 2.0 cm at the mesenteric side, with blood clots within the cystic space, as well as a focal rupture into the lumen of the small intestine.

The patient was subsequently able to eat on postoperative day 7, and his condition was satisfactory. He was discharged from the hospital on postoperative day 15.

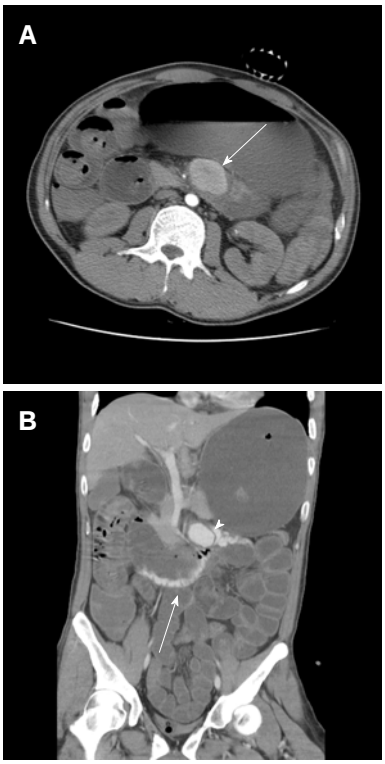

Figure 1 Computed tomography scans. A: Computed tomography (CT) scanning revealed a superior mesentery artery aneurysm about 3.9 cm in diameter at the jejunal mesentery (white arrow); B: CT scan showing a superior mesentery artery aneurysm (arrow head) with active contrast extravasation into the fourth portion of the duodenum (white arrow).

DISCUSSION

UGI bleeding is a common medical condition that results in high patient morbidity. UGI bleeding commonly presents with hematemesis or melena. It is important to take a careful history and perform a thorough physical examination to identify the possible source and etiology of the bleeding. Common causes of UGI bleeding include peptic ulcers, esophagogastric varices, erosive gastritis, Mallory-Weiss syndrome, angiodysplasia or Dieulafoy's lesion. Peptic ulcers are responsible for approximately half of UGI bleedings[1].

Our patient with an SMA aneurysm rupture in the duodenum presenting as UGI bleeding was extremely rare; there have been only a few reported cases[2-4]. Overall, only 0.2% of the general population is found to have an aneurysm of the visceral arteries, and SMA aneurysms represent only 5.5% of all visceral aneurysms[5]. Unlike other splanchnic artery aneurysms, which are mostly asymptomatic, more than 90.0% of SMA aneurysms are symptomatic, manifesting primarily as nonspecific abdominal pain[6]. Rarely, an abdominal mobile pulsatile mass or abdominal bruit maybe found on physical examination. Common causes of SMA aneurysms include septic emboli, atherosclerosis, pancreatitis and trauma. Septic emboli account for about one-third of SMA aneurysms. Other uncommon causes are collagen vascular disease, connective tissue disease and neurofibromatosis[7]. Abdominal ultrasonography and CT are useful in identifying the type of aneurysm. However, up to 50.0% of SMA aneurysms present with a rupture, where hypovolemic shock, hemoperitoneum or acute abdomen are the first manifestations. Once an SMA aneurysm ruptures, a high intra-operative mortality rate of more than 30.0% has been reported in previous studies[8]. Therefore, in patients diagnosed with an SMA aneurysm, surgical resection or radiological intervention should be recommended before the aneurysm ruptures.

An SMA aneurysm is an unusual extra-articular manifestation of rheumatoid arthritis, and our patient had no other history contributing to the formation of an aneurysm, including septic embolic, trauma or atherosclerosis. Rheumatoid arthritis can cause a wide spectrum of manifestations, from clinically insignificant to life-threatening. In postmortem examinations, rheumatoid vasculitis occurred in approximately 25.0% of all patients with rheumatoid arthritis, whereas less than 1% of rheumatoid arthritis patients developed clinical signs of vasculitis[9]. Among these patients, 10%-38% will have gastrointestinal manifestations of vasculitis and this may be the first manifestation[10,11]. Cases of abdominal aneurysm rupture with syncope and hemorrhage, bowel infarction, and intestinal perforation have been described [10-13].

Therefore, in patients with rheumatoid arthritis who have UGI bleeding, diagnosis of a ruptured SMA aneurysm in the duodenum should be considered, and the clinician should be alert to this issue to achieve rapid confirmation and to save lives.

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Conscious sedation: A dying practice?

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Abstract

Sedation practices vary according to countries with different health system regulations, the procedures done, and local circumstances. Interestingly, differences in the setting in which the practice of gastroenterology and endoscopy takes place (university-based *vs* academic practice) as well as other systematic practice differences influence the attitude of endoscopists concerning sedation practices. Conscious sedation using midazolam and opioids is the current standard method of sedation in diagnostic and therapeutic endoscopy. Interestingly, propofol is a commonly preferred sedation method by endoscopists due to higher satisfaction rates along with its short half-life and thus lower risk of hepatic encephalopathy. On the other hand, midazolam is the benzodiazepine of choice because of its shorter duration of action and better pharmacokinetic profile compared with diazepam. The administration of sedation under the supervision of a properly trained endoscopist could become the standard practice and the urgent development of an updated international consensus regarding the use of sedative agents like propofol is needed.

Key words: Sedation; Conscious; Endoscopy; Propofol; Fentanyl; Meperidine

Core tip: Even though the most recent "Multisociety sedation curriculum for gastrointestinal endoscopy" guidelines addressed the upper-limit range of Midazolam for proper sedation in prolonged endoscopic procedures, there are no clear-cut guidelines on the upper limit of Fentanyl dosing, especially with the risk of rigid chest syndrome and drug accumulation in skeletal muscle at high doses. Additionally, we also raised the question of whether Propofol or other agents used for deep sedation should be a routine indication for patients with chronic opioid use.

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TO THE EDITOR

While the controversy regarding the administration of sedation during gastrointestinal (GI) endoscopy continues, Triantafillidis *et al*[1] in their recent article in January 2013, provided a concise and thorough overview of the current knowledge regarding this topic. In summary, the authors concluded that the administration of sedation under the supervision of a properly trained endoscopist could become the standard practice and stressed urgent development of an updated international consensus regarding the use of sedative agents, especially propofol[1]. We would like to share some comments with the authors.

In a short survey of about sedative use in GI endoscopies conducted among thirty endoscopists at our large community-based hospital setting, Fentanyl/Midazolam and Meperidine/Midazolam are the most commonly used

sedatives for elective outpatient procedures in 63% and 36% of instances respectively. Usually, patients do not require more than 6 mg of Midazolam and 200 μg of Fentanyl to achieve moderate sedation. In a minority of patients who require more than usual dose of sedatives, 73% of the surveyed endoscopists are hesitant to go beyond the above-mentioned limits for the fear of side effects, leading to either inadequate evaluation or premature termination of the procedure. According to the recent "Multisociety sedation curriculum for gastrointestinal endoscopy" guidelines[2], the dose of Midazolam could be increased up to 6 mg and even more for prolonged endoscopic procedures[2]. However, there are no clear-cut guidelines on the upper limit of Fentanyl dosing and the risk of rigid chest syndrome[3]. During regular outpatient colonoscopies, few patients would not achieve moderate sedation despite receiving more than doses mentioned above. In such situations, 57% of the physicians responded that they would terminate the procedure and reschedule it again and 43% noted that they would proceed with increasing doses of sedatives to achieve moderate sedation. As Fentanyl can cause delayed side effects through accumulation in skeletal muscle, increasing the dose might be a concern in high-risk patients.

The second issue is with people who are chronic narcotic users. Ninety percent of the surveyed endoscopists responded that they would prefer Propofol administration in patients with chronic opioid use due to high tolerance observed in these patients. Propofol administration is usually by the anesthesia service thus adding to the patient's costs. This leads to a clinical question whether Propofol or other agents used for deep sedation should be a routine indication for patients with chronic opioid use.

Overall, this study provided a concise overview of the current knowledge and issues concerning sedation during digestive endoscopy and the authors are to be commended on their work.

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FIELD OF VISION

Stool therapy may become a preferred treatment of recurrent *Clostridium difficile*?

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Abstract

Fecal enemas were first reported to successfully treat life threatening enterocolitis in 1958, but fecal therapy to treat *Clostridium difficile* (*C. difficile*) infection has remained esoteric and not well investigated until recently. In the past few years, systematic reviews of case series and case reports of fecal microbiota transplant for recurrent *C. difficile* infection have become available and validate use of fecal transplant for *C. difficile* enterocolitis. Methods of fecal transplant reported in the literature include: nasogastric tube, gastroscope, duodenal tube, colonoscopy, rectal tube, and fecal enemas administered at home; no method has been shown to be superior. A recent randomized study published in *New England Journal of Medicine* found fecal transplant to be superior to oral vancomycin alone in treatment of recurrent *C. difficile* enterocolitis. The significance of this trial cannot be underestimated as it lends credibility to the idea of intentionally using microbes to combat disease, providing an alternative to the older paradigm of disease eradication through use of antimicrobials.

Key words: *Clostridium difficile*; Enterocolitis; Pseudomembranous; Fecal transplantation; Vancomycin

Core tip: Recurrent *Clostridium difficile* has been a challenge for patients, clinicians and hospital alike. Drug therapy for this epidemic is still not very effective. A more traditional method of fecal transplant has been discussed in this article, but it has been an uphill task to execute. We are discussing this first randomized control study, showing overarching benefits of stool transplant over traditional drug treatment. More studies needed with similar results, before making a strong recommendation in favor of it.

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COMMENTARY ON HOT TOPICS

Recurrent *Clostridium difficile* (*C. difficile*) is a growing epidemic with high rate of recurrence despite use of antibiotics. Fecal therapy to treat enterocolitis has been discussed in the literature since the late 1950's; despite anecdotal evidence suggesting its safety and efficacy, fecal therapy has remained an esoteric treatment. We read the recent article by van Nood *et al*[1] describing the results of the European randomized study investigating fecal therapy *vs* oral vancomycin for treatment of recurrent *C. difficile* infection with great interest. This is first randomized study investigating and validating use of fecal transplant for treatment of recurrent *C. difficile* infection.

C. difficile infection is identified as the cause of 25%-55.4% cases of antibiotic-associated diarrhea[2,3],

costs over $1 billion dollars annually in the United States to treat[4], and is a growing epidemic with twice as many cases reported in 2003 as in 1996 in part due to emergence of the more virulent, flouroquinolone-resistant NAP1/BI/027 strain[4,5]. *C. difficile* infection has a risk of initial recurrence rate following treatment with antibiotics of 20%-35%[6,7]; risk of recurrence is increased by use of antibiotics for other infections, being female, having initial infection in the spring, and having number of previous *C. difficile* infection recurrences[8]. Up to 65% of patients with recurrent disease ultimately develop pattern of recurrent *C. difficile* infection[6,8]. Patients with recurrent *C. difficile* infection are at risk of developing antibiotic resistance, and complications from *C. difficile* infection including: colitis, pseudomembranous colitis, toxic megacolon, and death[7,9]. Current therapies to treat recurrent *C. difficile* infection include tapered or pulsed dose oral vancomycin or metronidazole; these therapies are associated with high recurrence rates making it important that an effective treatment option for recurrent *C. difficile* infection become available[6].

Regardless of the method, fecal transplant for recurrent *C. difficile* infection appears to resolve symptoms in 83%-96% patients with most patients having durable response following single treatment[2,5,6,9]. Of patients requiring retreatment, 87.5% of patients experience symptom resolution[6]. Fecal transplant for pseudomembranous colitis appears promising with 72%-88% patients reporting improvement in symptoms[9]. Additionally, a case series found fecal transplant to be effective at treating the more pathogenic NAP1/BI/027 *C. difficile* strain in 89% patients[10]. Fecal transplant was associated with few and infrequent adverse events related to the procedure in all available case series and reports[2,6].

However promising the data from the systematic reviews of the case studies and case reports, it is not a substitute for data from prospective randomized controlled clinical trial data, as case reports and series are subject to bias from retrospective review of the data, subject selection and possible underreporting of adverse events. Results from a 3 arm randomized controlled clinical trial was recently reported by van Nood *et al*[1] in the *New England Journal of Medicine* comparing fecal transplant to vancomycin ± bowel lavage. More than 50% of patients enrolled in the trial had experienced multiple episodes of recurrent *C. difficile* infection and had been previously exposed to tapered vancomycin. The trial was stopped after an interim analysis showing superiority of fecal transplant arm to the other arms; 94% patients on the fecal transplant arm experienced symptom resolution - 81% (13/16) following initial infusion, and 66% (2/3) having symptom resolution following second infusion from another donor, *vs* 31% (4/13) patients with symptom resolution on the vancomycin alone arm and 23% (3/13) patients with symptom resolution on the vancomycin and bowel lavage. Adverse events from this trial confirm fecal transplant to be well tolerated with most common events experienced to be diarrhea (94% patients), abdominal cramping (31%), and belching (19%) immediately following fecal transplant and resolving within 3 h; and constipation (19%) as major adverse event reported during follow-up[1]. This study is far from perfect as it enrolled a small number of participants (16 on fecal transplant arm, 13 each on vancomycin and vancomycin and bowel lavage arms), was not blinded, patients on vancomycin ± bowel lavage arms frequently crossed-over following recurrence of *C. difficile* infection (non-protocol directed) and received fecal transplant, and although it enrolled primarily elderly patients it excluded many patients at higher risk of recurrent *C. difficile* infection including: patients with prolonged immunodeficiency, critically ill intensive care unit patients, and patients requiring antibiotics to treat another infection. Despite the studies limitations, it appears to favor fecal transplant for treatment of recurrent *C. difficile* infection with results similar to previous systematic reviews of case reports and case series available in the literature.

Data from the randomized trial by van Nood *et al*[1] provides further evidence that the efficacy of fecal transplant is not likely due to bowel preparation as it included a vancomycin and bowel lavage arm, but appears to be due to reconstitution of microbes in the gastrointestinal tract. As noted by observations the early 1980's, *C. difficile* growth can inhibit growth of certain strains of *Peptococcus, Peptostreptococcus,* and *Bacteroides* and its growth can also be inhibited by certain strains of *Staphylococcus, Pseudomonas, Bacteroides* and *Lactobacillus*, and recurrent *C. difficile* infection is likely due to germination of spores before balance of large bowel flora restored[11], or reinfection with a new strain of *C. difficile* due to the lack of protective bacteria in the colon[5]. Fecal transplant likely works by repopulating normal gut flora and preventing colonization with pathogenic *C. difficile* bacteria[5].

Fecal microbiota transplant has had empiric evidence demonstrating effectiveness and safety in treating recurrent *C. difficile* infection and pseudomembranous colitis enduring for over 50 years and is relatively less cost than other treatment options, so why has it remained an esoteric treatment for these disorders and not been investigated in randomized controlled trials until recently? Some possibilities include concerns of transmitting infections from donors to recipients *via* fecal material, no clear fecal transplant protocol as several methods have been described in the literature, and the idea of transplanting fecal material from one individual to another is aesthetically unappealing[9,11,12]. It should be noted that patients are reportedly receptive to the idea of fecal transplant following frustration at repeated antibiotic failure[13] and high out of pocket medical expenses to treat recurrent *C. difficile* infection[14].

Given the growing epidemic of *C. difficile* infection, cost and complications of treating recurrent disease, increasing antibiotic resistance, and growing body of evidence to support fecal microbiota transplant as a cost-effective and widely available therapy to treat recurrent *C. difficile* infection it is important that further research on fecal transplant be performed to identify methods and

indications for its use. The significance of the randomized study by van Nood *et al*[11] cannot be underestimated as it lends credibility to the idea of intentionally using microbes to combat disease, providing an alternative to the older paradigm of disease eradication through use of antimicrobials.

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REVIEW

Strategies to rescue steatotic livers before transplantation in clinical and experimental studies

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Abstract

The shortage of donor livers has led to an increased use of organs from expanded criteria donors. Included are livers with steatosis, a metabolic abnormality that increases the likelihood of graft complications post-transplantation. After a brief introduction on the etiology, pathophysiology, categories and experimental models of hepatic steatosis, we herein review the methods to rescue steatotic donor livers before transplantation applied in clinical and experimental studies. The methods span the spectrum of encouraging donor weight loss, employing drug therapy, heat shock preconditioning, ischemia preconditioning and selective anesthesia on donors, and the treatment on isolated grafts during preservation. These methods work at different stages of transplantation process, although share similar molecular mechanisms including lipid metabolism stimulation through enzymes or nuclear receptor *e.g.*, peroxisomal proliferator-activated receptor, or anti-inflammation through suppressing cytokines *e.g.*, tumor necrosis factor-α, or antioxidant therapies to alleviate oxidative stress. This similarity of molecular mechanisms implies possible future attempts to reinforce each approach by repeating the same treatment approach at several stages of procurement and preservation, as well as utilizing these alternative approaches in tandem.

Key words: Liver transplantation; Steatosis; Donor liver; Clinical; Experimental

Liu Q, Izamis ML, Xu H, Berendsen T, Yarmush M, Uygun K. Strategies to rescue steatotic livers before transplantation in clinical and experimental studies. *World J Gastroenterol* 2013; 19(29): 4638-4650 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i29/4638.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i29.4638

INTRODUCTION

Liver transplantation (LTx) is a successful therapy for end-stage liver disease, but it is severely restricted by the donor organ shortage[1-4]. In an effort to increase the size of the donor pool, livers from expanded criteria donors (ECD), including steatotic livers, are increasingly being used[3]. The current contribution of steatotic livers is marginal however, since the majority has an increased risk of ischemia-reperfusion injury (IRI) after LTx[3]. Here we review the clinical and experimental attempts to minimize this risk by modifying the quality of livers at different stages, on donors and isolated liver grafts during preservation, before LTx. We begin by briefly introducing the etiology, pathophysiology, categories and experimental models of hepatic steatosis, provide a summary of the techniques applied on donors and liver grafts, and con-

clude by providing future perspectives for these experimental approaches.

ETIOLOGY OF HEPATIC STEATOSIS

Livers are defined as steatotic or fatty when they have excessive (above 5% of wet liver weight) accumulation of lipids, mainly triglycerides. Steatosis occurs when lipid ingestion and synthesis exceed export and consumption in livers[5]. Based on the patient's alcohol consumption[5,6] fatty liver disease is classified as either alcoholic or non-alcoholic in origin (AFLD and NAFLD). Alcohol can decrease fatty acid oxidation and lipoprotein excretion, and increases the esterification of fatty acid to triglycerides *via* alpha-glycerophosphate[6]. Several factors contribute to NAFLD including dietary lipid overload; insulin resistance, which results in abnormal lipid metabolism; and ingestion of drugs or toxins such as carbon tetrachloride, which induces a decrease in apoprotein synthesis and lipid export[5-8].

PATHOPHYSIOLOGY OF HEPATIC STEATOSIS

A complicated pathophysiology has been exposed although the exact pathways have not been completely elucidated. Briefly, a "two-hit" theory is the current consensus, with the first "hit" being the initial abnormal fat accumulation and the second "hit" being the consequent inflammation ("steatohepatitis") leading to fibrosis and cirrhosis[6]. In the first "hit", excessive fat accumulates in vacuoles within hepatocytes, increasing cell volume and narrowing sinusoidal lumens[5,9]. This impairs microcirculation and decreases nutrient, oxygen and waste exchange. Excessive non-esterified fatty acids in hepatocytes inhibit β-oxidation thereby reduce acetyl-coenzyme A production[10]. Mitochondria uncoupling protein-2 is upregulated and associated with a dysfunction of adenosine triphosphate (ATP) synthesis[11-13]. Fat-induced hyperactivity of cytochrome P-450 enzymes increases the production of reactive oxygen species (ROS)[6,14]. ROS in turn lead to lipid peroxidation, phospholipid depletion and membrane dysfunction[6,14,15] as well as the release of inflammatory cytokines such as tumor necrosis factor (TNF)-α[14,16,17]. Inflammation occurs gradually and marks a significant downturn in disease progression as the second "hit"[18,19]. Alcohol can exacerbate oxidative injury and Kupffer cell activation[20], though alcoholic and non-alcoholic steatohepatitis (ASH and NASH) are thought to progress similarly[18].

Steatotic livers have reduced tolerance to ischemia due to low ATP stores, and are thus prone to early onset of acidosis and cellular edema during standard liver preservation method, static cold storage (SCS)[21]. Edema significantly impairs hepatic microcirculation further than the preceded impairment induced by excessive fat. Moreover, steatosis-induced inflammation is not addressed in the present liver SCS preservation solutions (Table 1).

Upon reperfusion, a complex inflammatory response involving Kupffer cells, lymphocytes, neutrophils, numerous cytokines[22] and nuclear factor kappa-B (NFκB)[23], is inevitably worse in steatotic livers compared to non-steatotic livers. Oxidative stress is also exacerbated *e.g.*, through xanthine oxidase[24,25]. The microcirculation is deteriorated further due to adherence of platelets in the sinusoids[22]. Strategies to minimize fat content and ameliorate the inflammatory and oxidative injury of steatotic livers are essential for improving these organs for transplantation.

CATEGORIES OF HEPATIC STEATOSIS

Besides AFLD and NAFLD in etiology, steatosis is also classified as "macro-" or "micro-" in histology based on the size and number of the fat vacuoles and on the location of the nucleus in the hepatocytes[5,26]. Macrosteatosis has a single fat vacuole larger than the nucleus filling the majority of the cell and pushing the nucleus to the periphery. Microsteatosis has many small fat vacuoles surrounding the nucleus in the central zone of the hepatocytes, and has more LTx success than macrosteatosis[3,27]. Steatosis can also be classified based on the proportion of hepatocytes affected, being mild (< 30%), moderate (30%-60%), or severe (> 60%), with incremental risk of graft dysfunction after LTx[5,26].

CLINICALLY APPLIED STRATEGIES

Approaches to improve steatotic livers before LTx have been tested in a handful of pilot clinical studies on living donors or donors after brain death (DBD) (Table 2). The interventions focused on reducing excessive fat (the first "hit") through limiting lipid intake and increasing lipolysis, or stimulating factors likely to be protective against inflammation and oxidative stress (the second "hit") of steatohepatitis. Living donors, though a minority in western countries[1,2], are used extensively in Asia[28] and are theoretically amenable to therapies before procurement after ethical concern is taken into account[29]. DBD livers, which comprise the majority of donor organs for LTx in western countries, could be treated between brain death declaration and organ procurement since circulation is maintained until procurement. Livers from donors after cardiac death (DCD) are seldom utilized when they have steatosis because they experience a period of warm ischemia (WI) before procurement[3] and thus were normally considered as unacceptable with two defects (steatosis and WI). Currently there are no attempts to rescue steatotic DCD livers.

Physical exercise and dietary intervention

Physical exercise and dietary restriction are general therapies for NAFLD patients, independent of whether or not they are organ donors[30,31]. But this treatment normally needs several months and might be risky to increase the mortality of recipients during the waiting time for treat-

Table 1 Intrinsic composition of preservation solutions in this review

		UW	HTK	Celsior	IGL-1	UW-gluconate	Kreb-Henseleit	MEM
Electrolyte	Na	25	15	100	125	125	143	143.4
(mmol/L)	K	120	9	15	30	25	5.9	5.4
	Mg	5	4	13	5	5	1.2	0.8
	Ca		0.0015	0.25		0.5	1.25	1.4
	Cl	20	32	42		1	125.2	124
	SO4	5			5		1.2	0.8
Buffer	Phosphate	25			25	15	1.2	1
(mmol/L)	Bicarbonate					25	25	26.2
	HEPES					10	20	
	Histidine		198	30				0.27
Antioxidant	Glutathione	3		3	3	3		
(mmol/L)	Allopurinol	1			1	1		
	Mannitol		38	60				
	NAC						5	
	Vit C							0.25
	Glucose					10	5	5.5
Metabolic Substrates	Adenosine	5			5	5		
(mmol/L)	Adenine					5		
	Ribose					5		
	Tryptophan		2					
	Ketoglutarate		1					
	Glutamate			20				
	Amino acid							0.7
Impermeants	Lactobionate	100		80	100			
(mmol/L)	Gluconate					95		
	Raffinose	30			30	30		
Colloid	HES	50						
(g/L)	PEG				1	50		
Other intrinsic	Insulin	100 U						
compounds	Dexamethason	8 mg						
	Penicillin	40 U						
	Phenol-red (mmol/L)							0.03
Osmolarity		340	300	363	330	360	320	310

UW: University of Wisconsin; HTK: Histidine-trypotphan-ketoglutarate; IGL-1: Institut Georges Lopez-1; MEM: Minimum essential cell culture medium; HEPES: N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid; NAC: N-acetylcysteine; HES: Hydroxyethyl starch; PEG: Polyethylene glycol.

ing donors[32]. An intensive protocol might be a solution, which was already reported to successfully reduce macrosteatosis on obese human living donors in 2-8 wk, through exercise burning 600 kcal/d, a protein-rich diet of 1000 kcal/d, and bezafibrate, an anti-hyperlipidaemia drug, at 400 mg/d[33]. However, supplementary glucose to donors a few hours before living donor liver transplantation was recommended to supply additional energy reserves, since fasting before procurement can induce glycogen depletion, decrease glycolytic ATP generation, and compromise graft transplantability[34].

The lack of omega-3 polyunsaturated fatty acids (PUFA) has been recognized in the development of NAFLD because they can activate peroxisomal proliferator-activated receptor (PPAR)-α, suppress sterol regulatory element-binding protein-1, improve microcirculation, and reduce Kupffer cell activity and inflammation[35-43]. The mechanism on microcirculation might work through reducing TXA2 synthesis after manipulating the composition of hepatic lipid (omega-3: omega-6 PUFA ratio)[43]. Based on experimental success on rodents[35-43], omega-3 PUFA was shown to be effective on NAFLD clinically after treatment at 1-2 g/d for 6-12 mo[44-46]. This has not been applied specifically on living donors but is expected to be a safe and promising approach.

Pharmacological preconditioning

Many drugs are being used clinically to treat NAFLD by decreasing lipid intake[47-49], stimulating lipid metabolism[50-53], or improving insulin sensitivity[54-57]. Ursodeoxycholic acid, a natural bile acid, was used as a non-specific hepato-protector to treat NAFLD in a pilot clinical study[58], but afterward was revealed to be controversial[59]. Pentoxifylline was used against NASH and ASH in patients[60,61] due to the effect of reducing TNF-α by inhibiting phosphodiesterase[62] and lessening oxidative stress by increasing glutathione[63]. But to date, only bezafibrate was used to treat human living donors for LTx[33]. This drug works through activating PPAR-α and β/δ to stimulate lipid metabolism and decrease fat content in livers[64,65]. While there are other candidate drugs that could potentially be taken by living donors, concerns of significant side effects are limiting their use[66,67].

Ischemic preconditioning

Though extended ischemia is deleterious to organs, it has been recognized since the 1980s that a short period of ischemia with subsequent reperfusion triggers natural de-

Table 2 Overview of the clinical and experimental strategies in this review

	Clinically applied	Experimentally applied
Donors	Dietary	Pharmacological
	Pharmacological	Heat shock
	Ischemic (except remote ischemic)	
	Anesthetic	
Liver grafts		SCS preservation
		New solution
		Pharmacological additives
		Additional oxygen (VSOP)
		MP preservation
		MP solely
		MP + pharmacological additives
		Flushing
		Pharmacological additives

SCS: Simple cold storage; MP: Machine perfusion; VSOP: Venous systemic oxygen persufflation.

fense mechanisms against future ischemic insults and protects the organ against IRI[68]. Ischemic preconditioning (IP) was first observed in kidneys and hearts[69,70], and then employed for clinical liver resections and transplantation[68]. It can be applied intermittently[71], or as a single short period (5-10 min) of ischemia followed by 10-15 min reperfusion before cold flush during liver procurement[72,73]. Franchello *et al*[73] have used the technique clinically on marginal DBD livers including steatotic livers, and observed a reduction of hepatocyte swelling and enzyme release in recipients after LTx.

IP is protective because ATP consumption during the short ischemic period increases endogenous adenosine and nitric oxide[74]. Adenosine protects sinusoidal endothelial cells through adenosine A2 receptor[75]. Cyclic adenosine monophosphate (cAMP) worked as the second messenger, but whether increasing or blocking cAMP would be beneficial was still controversial[75,76]. Nitric oxide is a vasodilator, and further it attenuates the release of TNF-α, decreases the injurious interleukin (IL)-1β and increases the anti-inflammatory IL-10[77]. Another effect of the intermittent ATP consumption is to increase the level of adenosine monophosphate (AMP), which stimulates AMP-activated protein kinase (AMPK). AMPK can regulate an energy-conserving state, decrease inflammation through inhibiting NFκB, and induce the synthesis of nitric oxide as well[78-80]. Overall, the advantages of IP were an improved microcirculation[81,82], mitochondrial permeability transition and mitochondrial function[83], cytochrome oxidase C activity and tissue oxygenation[82,84], and the reduction of oxidative stress such as the xanthine accumulation and xanthine oxidase activity[85,86].

Interestingly, IP can work remotely, *e.g.*, liver IP decreased lung IRI[25,87] and limb IP decreased liver IRI[88-90]. This possibly works through some protective agents, *e.g.*, heme oxygenase-1, endothelial nitric oxide synthase, and nuclear protein High Mobility Group-Box 1[88-90]. But foreseeable ethical concerns exist with the logistics of implementing this technique in human donors.

Anesthesia selection

During clinical liver resection, Beck-Schimmer *et al*[91] observed the superiority of volatile anesthesia using sevoflurane in the prevention of hepatic injury after reperfusion compared to intravenous anesthesia with propofol. The mechanism was suggested to be the increased synthesis of nitric oxide with sevoflurane, which alleviates some of the effects of IRI as discussed above. Moreover they observed that patients with steatosis did benefit more[91]. This method could be easily applied during the procurement of steatotic livers from both DBD and living donors.

EXPERIMENTAL STRATEGIES

Induction of steatosis in animal models

Steatosis in animal models are established with or without alcohol and classified also as micro- *vs* macro- or as mild, moderate, *vs* severe. The timeline to develop the different classifications largely depends on strain and method used (Table 3).

A common rodent NAFLD model induces cerebral deficiency of the leptin receptor through a genetic mutation that causes the animals to become obese through overeating[92-98]. The methods inducing lipid overload with high fat or cholesterol diets are also quite common. A high fat diet (50% dextrose, 18% casein, 25% lipids, 4% minerals, 1% cholesterol, 0.5% sodium cholate, 0.2% choline, and 1% vitamins) for 7 d induces severe steatosis in rats[99]. A high-cholesterol (2%) diet for 12 wk in rats[82] and 8 wk in rabbits results in moderate steatosis[84]. A cafeteria diet (65% of fat) for 4-15 wk was also used to create NAFLD on rats[100,101]. Recent studies suggested it reflects human metabolic syndrome better than high-fat diet[100,101]. A choline/methionine-deficient diet (CMDD) was another rather common method to develop steatosis, for 4-6 wk or short as 7 d in rodents[5,27,102,103]. Choline is essential for the formation of phosphatidyl-choline and very low density lipoprotein needed for lipid export, while methionine is a good source of methyl groups for the endogenous synthesis of choline. This model could be criticized for not being clinically accurate because NAFLD patients due to CMDD are rather unrealistic. The most rapid induction of NAFLD in rats was with a high-starch, fat-free diet [saccharose (40%), starch (40%), casein (16%), and a mineral and vitamin mix (4%)] administered for 2 d after fasting for 2 d, which can lead to mild to moderate steatosis[104-106]. While none of these models are unanimously agreed to be ideal in replicating clinical NAFLD, the high fat or cholesterol diet model was the most widely used to mimic steatosis in humans.

There are fewer large animal NAFLD models, which usually combine more than one method described above to achieve steatosis. Takahashi *et al*[107] established a dog model using a diet rich in fat and deficient in choline, which produced moderate to severe macrosteatosis after 8-12 wk feeding. Lee *et al*[108] used a high fat and high cholesterol diet (20% kcal from fructose, 46% kcal from fat,

Table 3 Animal models of hepatic steatosis for liver transplantation

Disease	Approaches	Description	Animals	Treatment time
NAFLD	Genetic	Cerebral leptin receptor deficiency	Rodent	
	Dietary	High fat	Rodent	7 d
		High cholesterol	Rodent, rabbits	8-12 wk
		Cafeteria diet	Rodent	4-15 wk
		Choline/methionine-deficient	Rodent	7 d-6 wk
		High starch and fat free after fasting	Rodent	4 d
		High fat and choline deficiency	Dog	8-12 wk
		High fat and high cholesterol, plus choline deficiency or not	Miniature pig	24 wk
		High fat and carbohydrate with streptozotocin for a diabetic state	Landrace pig	5 wk
AFLD	Dietary	Ethanol in liquid diet, intragastric infusion or gavage	Rodent	20 h-9 wk
		Ethanol and high fat diet	Rodent	6 wk
		Ethanol and deficient folate diet	Micropig	12 wk

NAFLD: Non-alcoholic fatty liver disease; AFLD: Alcoholic fatty liver disease.

2% cholesterol and 0.7% cholate by weight) for 24 wk to develop microsteatosis on miniature pigs. When using lowered choline content simultaneously, severe steatosis with fibrosis was observed with increased TNF-α and oxidative stress. A recent study on Landrace pigs used a diet rich in fat (20% in volume) for 5 wk together with intravenous streptozotocin (125 mg/kg) to induce a diabetic state in the last 2 wk; but this treatment led only to mild steatosis[109].

In rodent AFLD models, ethanol was provided in a liquid diet[110-114] or *via* intragastric infusion[115,116]. Different degrees of alcohol exposure have been reported: 5%-8% in the concentration of liquid diet; 35%-40% of total energy consumption; 8-16 g/kg per day; or 150-300 mg/dL of blood ethanol[110-116]. Normally several days are needed for the animals to adapt to the alcoholic diet, and an additional 4-9 wk to observe steatosis. Acute responses to ethanol have also been reported as early as 20 h after feeding 6 g/kg ethanol by gavage on rats[117]. Combined ethanol with high-fat diet to develop steatosis on rats was also reported[118]. Large animal AFLD models are uncommon though micropigs have been fed a diet with ethanol and a deficiency of folate, as a substrate for methionine synthase, with some efficacy[119,120].

Experimental strategies applied to donors

Pharmacological preconditioning: Reduction of oxidative and inflammatory activity with heme oxygenase-1, a microsomal enzyme[121-123], was used intravenously or intraperitoneally on AFLD and NAFLD rats 24 h before liver procurement. It decreased macrophage infiltration, improved portal venous blood flow, bile production, and survival rate after LTx[121-123]. Bortezomib, a NFκB inhibitor, was used intravenously on obese donor rats and reduced IRI after LTx[23]. N-acetylcysteine, a precursor of glutathione, was injected through the mesenteric vein of CMDD rats 15 min before liver procurement and showed a protective effect on IRI in an isolated reperfusion system[124]. The subcutaneous injection of IL-6 for 10 d was observed to be protective against IRI after *in situ* partial ischemia-reperfusion on NAFLD and ALFD mice[92]. The mechanism might be the prevention of cell death and the reduction of TNF-α[125], in addition to stimulating PPAR-α, β-oxidation of fatty acids, and the export of triglycerides and cholesterol[92,125]. Theaflavin, a polyphenol substance extracted from black tea, was tested on CMDD mice and observed to have antioxidant, anti-inflammatory, and anti-apoptotic effect[126]. A multi-drug approach was reported by von Heesen *et al*[127] including N-acetylcysteine as an antioxidant, pentoxifylline for anti-inflammation, glycine to stabilize Kupffer cells, deferoxamine as an iron chelator to reduce ROS, and erythropoietin, melatonin and simvastatin to protect against IRI. In the treated rats they observed no inflammatory response with significantly reduced parenchymal dysfunction and injury compared to the untreated rats.

Heat shock preconditioning: An intriguing experimental method to improve the quality of steatotic donor livers has been to induce protective heat shock proteins (HSPs) endogenously by exposure to heat. Termed "heat shock preconditioning" and applied at 3-48 h before organ procurement by exposing anesthetized donor animals to warm (42 ℃) bath water for 10-15 min[128-132], obese and CMDD rats showed an increased expression of HSP-32 (heme oxygenase-1), -72 and -90[128,129]. These HSPs can decrease TNF-α production[129], improve microcirculation through producing carbon monoxide, and inhibit platelet aggregation[62,64]. Our group has also reported the inhibition of CD4+ T lymphocytes in CMDD rats after LTx with heat shock preconditioning[130]. Other factors might be involved in the treatment since studies on normal and WI rat livers showed IL-6, inter-cellular adhesion molecule-1, and some neutrophil chemo-attractants were also impacted[131,132].

Strategies applied on liver grafts during ex vivo preservation

Obviously, strategies to improve steatotic liver quality during preservation are more desirable than those on donors, as they have no effect on the donor's other organs, and are practical when it's not possible to work on the donor. The clinical standard for liver graft preservation has been SCS with University of Wisconsin (UW)

solution for more than 20 years[3,21]. In the past decade, histidine-trypotphan-ketoglutarate (HTK) solution[133-135] and Celsior solution[136,137] were recognized as having similar efficacy and safety as UW solution; Institut Georges Lopez-1 (IGL-1) was reported to be comparable to UW for healthy human livers[138], and better for steatotic rat livers[139]. Besides the arising new solutions, many adaptations have been suggested through enriching the intrinsic composition of the solutions with additives, or replacing SCS by machine perfusion (MP) preservation in experimental studies to rescue steatotic livers.

Pharmacological additives during SCS preservation: Liver preservation solutions normally comprise electrolytes, pH buffers, antioxidants, metabolic substrates, impermeants with or without colloid, insulin, dexamethasone, and antibiotics (Table 1)[139-143]. The additives for improving steatotic liver preservation were intended to ameliorate metabolism or suppress oxidative injury and inflammation. They were reported to be effective during SCS, despite the reduced metabolic rate of liver grafts during hypothermic preservation.

Addition of IL-6 into UW solution for donor liver flushing and SCS was tested by Sun *et al*[144], leading to improvement in microcirculation and reduced IRI after LTx of NAFLD and AFLD rats. Arnault *et al*[99] added pentoxifylline into UW solution and also observed a benefit to the microcirculation, but the exact mechanism is yet to be identified.

Tolba *et al*[106] added L-carnitine into HTK solution for SCS preservation of steatotic rat livers and observed a reduction of IRI in an isolated reperfusion system. L-carnitine is a nonessential amino acid but is essential for transporting fatty acids through the inner mitochondrial membrane and for β-oxidation[106]. It has also been reported to function as an antioxidant and to stabilize the membrane fluidity and stability *in vitro* and *in vivo*[145].

Ben Mosbah *et al*[146] added carvedilol, a cardiologic drug to block α- and β-adrenergic receptor, into UW solution for SCS of obese rat livers, and reduced oxidative stress and mitochondrial damage after isolated reperfusion. The mechanism might be an enhanced release of nitric oxide that facilitates vasodilatation and ROS scavenging[147]. AMPK activators, trimetazidine and aminoimidazole-4-carboxamide ribonucleoside (AICAR) were also tested as UW additives on obese rat livers by this group. Increased bile production, decreased enzyme release and vascular resistance, and reduced oxidative stress after isolated reperfusion were observed. It was noted that combination of trimetazidine and AICAR was not necessary[95].

Zaouali *et al*[148] tested the use of epidermal growth factor and insulin-like growth factor-I as UW additives and observed that each additive resulted in the improvement of fatty rat liver function after LTx. The mechanisms are suggested to be upregulation of Akt, a cytoprotector[149], and the subsequent over-expression of PPAR-γ. They also tested melatonin as additive in IGL solution and reported its protective role through generating nitric oxide and decreasing oxidative stress and inflammation[150].

Venous systemic oxygen persufflation during SCS preservation: In 1990s, Minor *et al*[151] developed a new method, called venous systemic oxygen persufflation (VSOP) to supply gaseous oxygen to livers during SCS preservation. The oxygen was introduced into hepatic vasculature *via* the suprahepatic vena cava and allowed to exit *via* several small pin pricks on the liver capsule made using an acupuncture needle. This technique was employed on steatotic rat livers for 24 h, and resulted in improved preservation of mitochondria and sinusoidal endothelial linings, less Kupffer cell activation and reduced hepatocellular enzyme release compared to SCS preservation[105]. Recently, by assessing the enzyme release, energy storage, bile production, and cell death during isolated reperfusion, it was demonstrated that application of VSOP for 90 min may rescue steatotic livers after extended (18 h) SCS preservation[152].

MP preservation: MP is an alternative preservation method to SCS[153], which can be further categorized based on the temperature employed[154]. Hypothermic (4 ℃) machine perfusion (HMP) preservation has proven to be superior to SCS for human kidneys[155], and feasible for normal human livers[156]. Normothermic (32 ℃-37 ℃) and sub-normothermic (20 ℃-30 ℃) machine perfusion (NMP and subNMP) preservation have been reported in experimental studies on livers, but mostly on their advantages for DCD models[143,157-162]. MP preservation of steatotic livers is limited, but also reported to be beneficial on preserving energy content and liver function experimentally[109,143,163,164]. The advantages of MP preservation result from continuously supplying nutrients, removing waste products, and maintaining microcirculation[154]. Because MP, especially NMP, provides a physiologically-relevant environment to the isolated donor organ, the quality of liver grafts can be manipulated more efficiently than those simply stored in an ice-box during SCS. Another advantage of MP is the considerable convenience for non-invasively evaluating liver viability, a key issue when ECD livers are used[153].

Bessems *et al*[163] employed HMP preservation with UW-gluconate solution on steatotic rat livers for 24 h and alleviated IRI compared to SCS. Vairetti *et al*[143] preserved steatotic rat livers by subNMP (20 ℃) with Kreb-Henseleit solution for 6 h and obtained similar results. The longest preservation of steatotic livers was the NMP preservation for 48 h in a pig model by Jamieson *et al*[109], who employed blood containing additional insulin and vasodilators as perfusate, and observed a mild reduction of steatosis from 28% to 15%. This NMP setting provided the most physiological environment to liver grafts and lead to an activated function of the isolated organs with sufficient oxygen and nutritional support. This is expected to be the best preservation method in spite of the

highest logistic restriction.

Recently our group has combined NMP and pharmacological preconditioning for decreasing steatosis[96]. A "defatting cocktail" was developed with 6 compounds to activate nuclear receptors such as PPARs, pregnane X receptor, and constitutive androstane receptor, to exert an insulin mimetic effect and to stimulate intracellular cAMP. This cocktail was added into Minimum Essential cell culture medium as a perfusate to stimulate lipid metabolism of obese rat liver grafts preserved at NMP. A significant decrease (50%) in steatosis was observed after 3 h NMP[96].

Other experimental approaches

The solution used for SCS preservation needs to be flushed out of the donor organ prior to implantation to remove possible air bubbles, and preservative components such as high potassium, which are deleterious to the recipient. This provides an opportunity for treatment of ischemic injury, although it has been very little explored. In one study, polyphenols, an antioxidant extracted from Camellia sinensis (green tea), was added to the flushing solution and improved the hepatic injury and survival rate after LTx in a steatotic rat model[117].

Another option is applying preconditioning at several phases. While it appears little explored, in one study Ye *et al*[118] injected glutathione intraperitoneally in rats with hepatic steatosis 2 d before procurement and preserved the livers by SCS with VSOP and additional glutathione. Remarkable improvement on survival rate, liver function, and oxidative stress in liver tissues after transplantation was observed.

SUMMARY AND FUTURE PERSPECTIVES

Based on the understanding on pathophysiology, current strategies to rescue steatotic donor livers work through ameliorating the abnormal lipid metabolism (the first "hit"), and the oxidative stress and inflammation (the second "hit"). Each approach employs various methods at different phases of organ recovery and preservation but generally targets similar molecular mechanisms. Notably, it may be possible to attack the same mechanism but reinforce the effects by applying the treatment at multiple points of organ recovery and preservation process, thereby producing a stacking effect. The use of glutathione on donors and in SCS solution by Ye *et al*[118] is a good example in this direction that demonstrates the therapeutic effects may stack. Stacking other medications, *e.g.*, IL-6 and pentoxifylline could also be promising as these could be given to the donors prior to recovery or readily added into preservation solutions to treat liver grafts. Whether the stacking approach works for different targets in lipid metabolism, oxidative stress or inflammation remain to be elucidated.

Similar to stacking the same/similar pharmacological agents at different stages, it is a reasonable idea to attack the two "hits" simultaneously by stacking medications targeting different pathways. Development of such combinations is usually shunned because it exponentially increases the complexity of development and clinical testing, but given that the disease itself is a very complex phenomenon spanning multiple pathways, it may be unavoidable, and a single silver bullet to treat steatosis simply nonexistent.

An intriguing alternative in development is the efforts to use MP for liver preservation. Especially in near normothermic conditions, MP provides a combined opportunity to improve energy storage, maintain microcirculation, and support pharmacological approaches to decrease fat content and treat IRI. While machine perfusion by definition is more complex than simple storage on ice, it is a very promising approach available in the near future and could be the ultimate solution to rescue steatotic as well as ischemic livers.

Both steatotic livers and DCD livers are highly susceptible to IRI. Therefore, potentially, a method to rescue DCD livers could be also applicable to steatotic livers even with the fat content intact. For instance, MP preservation was able to rescue DCD livers and steatotic livers[143,157-164]. Similarly, perfluorocarbon as an artificial oxygen carrier to improve SCS preservation of DCD livers[165] could also be tested on steatotic livers. If successful, we would then secure both DCD and fatty livers for transplantation, which would boost the organ availability dramatically and resolve donor liver shortage for a decade or more.

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REVIEW

Clinical utility of anti-*p53* auto-antibody: Systematic review and focus on colorectal cancer

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Abstract

Mutation of the *p53* gene is a key event in the carcinogenesis of many different types of tumours. These can occur throughout the length of the *p53* gene. Anti-*p53* auto-antibodies are commonly produced in response to these *p53* mutations. This review firstly describes the various mechanisms of *p53* dysfunction and their association with subsequent carcinogenesis. Following this, the mechanisms of induction of anti-*p53* auto-antibody production are shown, with various hypotheses for the discrepancies between the presence of *p53* mutation and the presence/absence of anti-*p53* auto-antibodies. A systematic review was performed with a descriptive summary of key findings of each anti-*p53* auto-antibody study in all cancers published in the last 30 years. Using this, the cumulative frequency of anti-*p53* auto-antibody in each cancer type is calculated and then compared with the incidence of *p53* mutation in each cancer to provide the largest sample calculation and correlation between mutation and anti-*p53* auto-antibody published to date. Finally, the review focuses on the data of anti-*p53* auto-antibody in colorectal cancer studies, and discusses future strategies including the potentially promising role using anti-*p53* auto-antibody presence in screening and surveillance.

Key words: *p53* gene; *p53* mutation; Anti-*p53* auto-antibody; Cancer; Colorectal cancer

Core tip: Anti-*p53* auto-antibodies are commonly produced in response to *p53* mutations. Anti-*p53* auto-antibody titres generally increase with tumour load, but not all patients who are initially sero-negative develop an auto-antibody response despite disease progression and metastases. Conversely, sero-positive patients do not lose their anti-*p53* auto-antibodies despite the cancer being completely excised. In general, cancers with the highest *p53* mutation rate, *e.g.*, oesophageal and ovarian, demonstrate the highest anti-*p53* auto-antibody rates; conversely, melanoma and testicular carcinoma with the lowest mutation rate have the lowest serum auto-antibody levels. Measurement of anti-*p53* auto-antibodies may be useful in screening or monitoring for tumour recurrence.

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INTRODUCTION

The *p53* gene is located on the distal band of the short arm of chromosome 17p13[1,2]. It consists of approximately 20000 base pairs spread over 11 exons[2-5]. *p53* was initially discovered in 1979 as a protein binding to a viral

oncogene, Simian Vacuolating 40 (SV40) large T-antigen, and hence was thought to be an oncogene itself[6-8]. It has since been established that it has a critical role as a tumour-suppressor gene[9-11]. *p53* inactivation predisposes cells to malignant transformation in rodent models and in human clinical diseases such as Li-Fraumeni syndrome; the latter being characterized by germline mutations of *p53*[12-14]. The tumour suppressive role of *p53* is so crucial that it is referred to as "the guardian of the genome"[15,16]. It is the most common mutation found in cancers and is present in half of all solid tumours thus emphasising its importance in protecting cells from carcinogenesis[3]. The frequency of mutation varies in individual cancers ranging from 5%-12% in cervical and haemopoietic malignancies to 40%-50% in colorectal and ovarian cancer[1,5]. Additionally, the remaining cancers with no detectable *p53* mutation are still thought to have dysfunctional *p53* caused by mechanisms other than mutation[9,17-20]. The most recent advances in colorectal cancer (CRC) treatment have been in the field of immunology with the use of antibodies against potent growth factors including epidermal growth factor receptor (EGFR) and vascular endothelial growth factors (VEGF)[21,22]. As such, *p53*, with its diverse immuno-regulatory role maintains a vital role in future of management of cancer and benign diseases. This review begins with the description of the normal *p53* gene function and mechanisms of *p53* inactivation in cancer, followed by a systematic review of the association between the anti-*p53* auto-antibody response and underlying *p53* mutations, and finally a clinical focus on the current evidence and potential future role of anti-*p53* auto-antibody in colorectal cancer.

p53 GENE AND GENE FUNCTION

p53 acts as a tumour suppressor by preventing propagation of defective cells. It is up-regulated by various upstream factors in response to cellular stress or damage such as DNA damage, hypoxia, telomere shortening and oncogenic stimulation or radiation[2,11]. Activated *p53* modifies downstream gene expression and co-factor transcription, which in conjunction with *p53*, lead to growth arrest (*e.g.*, *via* $p21^{WAF1}$) or apoptosis (*e.g.*, *p53*-upregulated modulator of apoptosis, PUMA)[19,23,24].

The *p53* gene encodes for a 393 amino-acid, 53 kDa, phospho-protein which is divided into 3 domains-an amino (-NH_2) terminal region (approximately amino acids 1-100), a central "core" domain (amino acids 100-300) and a terminal carboxyl (-COOH) region (amino acids 320-360)[25-27]. Almost all mutations are harboured in the central "core" which contains the DNA-binding regions. Thus *p53* dysfunction is most likely caused by mutations that alter DNA binding behaviour. However, most anti-*p53* auto-antibodies do not recognise central core mutations but rather recognise epitopes in the 2 terminal regions. An interesting observation is that these terminal regions which contain the least mutations are also found on the wild-type and the mutant p53 protein[25,27,28]. This suggests anti-*p53* is not only produced in response to mutation but also elevated levels of normal *p53*. This is discussed later (see Anti-*p53* Auto-antibody).

Wild-type p53 protein expression is intra-nuclear with a half-life of 5-30 min and is subject to complex regulation[29]. The most important regulator is thought to be Murine Double Minute 2 (Mdm2)[29,30]. Mdm2 is an ubiquitin-dependant E3 ligase which targets wild-type p53 protein for nuclear and cytoplasmic proteasome-mediated degradation[31,32]. When up-regulated *p53* binds to the Mdm2 promoter leading to increased levels of mdm-2 transcription; the *Mdm2* gene product then inhibits *p53* thus creating a negative feedback loop. This feedback process is complex and regulated by multitude of factors. Mdm-2 in itself is subject to modifications mainly self-degradation by (1) auto-ubiquitination[33,34]; (2) small Ubiquitin-like Modifier (SUMO)-ylation[35]; (3) acetylation[36]; (4) post-translational upstream kinases (*e.g.*, ATM kinase phosphorylation of Mdm2 and Mdm-X)[37-39]; (5) Mdm-2 in conjunction with Mdm-X (also known as Mdm-4) can form a Mdm2-MdmX-p53 complex which represses *p53* activity; and (6) Mdm-X can furthermore act independently of Mdm-2 and repress *p53*-bound chromatin, without Mdm2, most likely by direct binding. The combinations of these mechanisms, regulate *p53* accumulation in response to various cellular stresses (Figure 1).

p53 increases in response to cellular stress caused by a variety of insults including DNA damage, oncogene activation, ribosomal stress and hypoxia[11] by several mechanisms: (1) increased transcription; (2) increased intra-nuclear accumulation of active *p53*; (3) increased extra-nuclear export of Mdm-2[40,41]; (4) down-regulation of Mdm2-Mdmx which usually represses chromatin-bound-*p53*[24]; (5) various downstream post-translational modifications of both *p53* and its regulators *e.g.*, Mdm2[17]; and (6) raised cytosolic *p53*[42-45].

Active *p53* has tumour suppressive activity by causing cell cycle arrest, apoptosis and autophagy. Cell cycle arrest initially provides additional time for the cell to repair damaged DNA. However, cells unable to repair damage are directed towards apoptosis by shifts in the balance between pro-arrest, pro-autophagy and pro-apoptotic factors severe cellular damage, the cell pushed directly towards apoptosis by the relative increase in pro-apoptotic markers relative to cell-cycle arrest promoters[19].

Autophagy is an evolutionary catabolic process of mass lysosomal self-degradation of cytosol/proteins/organelles which are sequestered into a double membrane vesicle which is then fused with lysosomes for bulk degradation[42,43]. *p53* plays a dual role in activating and/or inhibiting autophagy by transactivating numerous autophagy regulators including mammalian target of rapamycin (mTOR)[46], activated protein kinase (AMPK) and tuberous sclerosis protein (TSC2)[43,47]. *p53* is also able to influence the decision between apoptosis and autophagy by selectively activating pro-autophagy proteins such as AMPK, death-associated protein kinase 1 (DAPK-1) and damage-regulated autophagy modulator (DRAM)[48]. Alternatively, *p53* also promotes apoptosis by activating pro-apoptotic markers such as B-cell Lymphoma 2

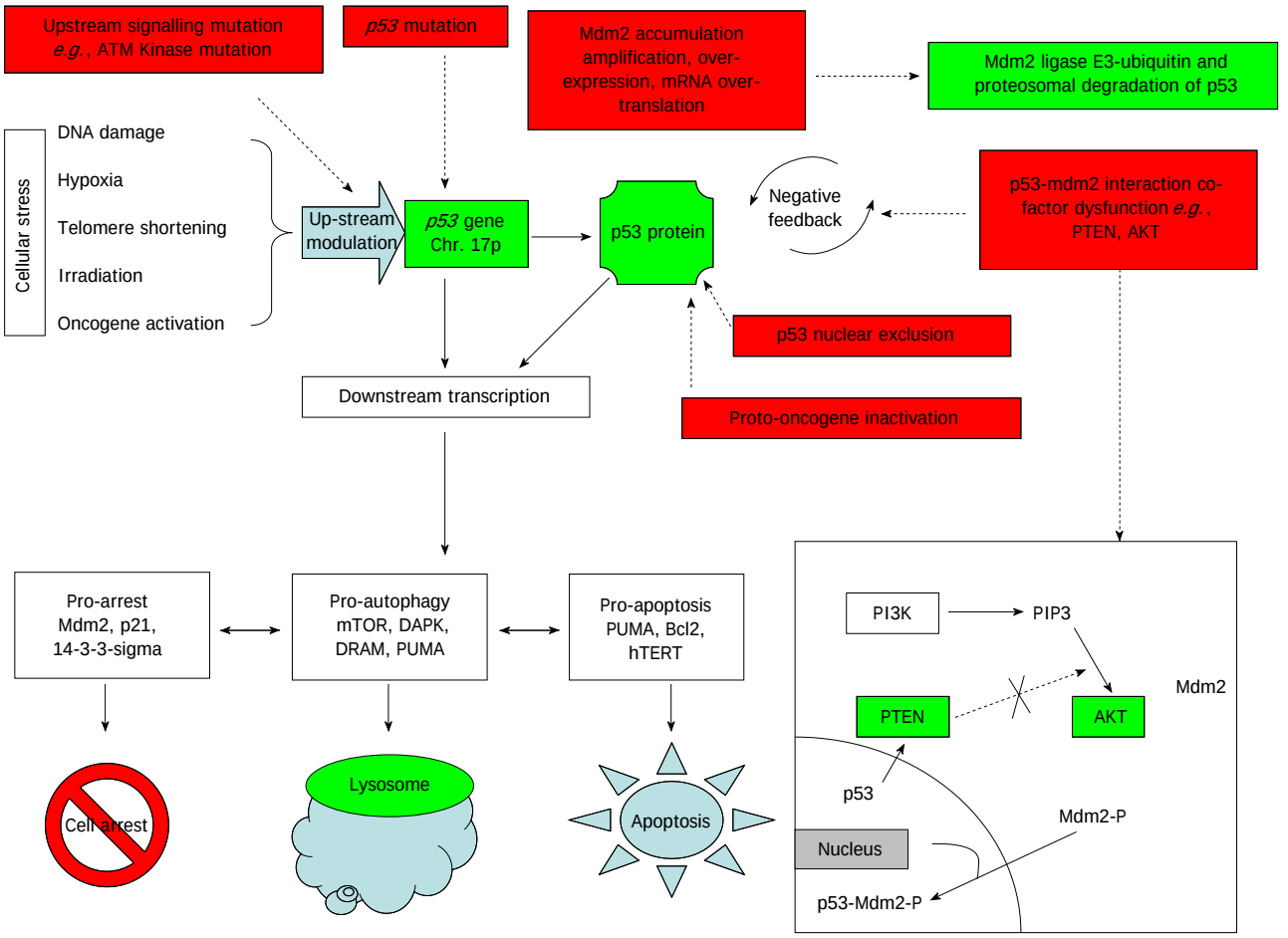

Figure 1 Normal *p53* function highlighted in green boxes and blue figures as described. Red boxes show mechanisms for inactivation; Normal *p53* function highlighted in green and blue figures. PI3K: Phosphatidylinositol-3-kinase; PTEN: Phosphatase and tensin homolog; AKT: Protein kinase B; ATM: Ataxia telangiectasia mutated kinase; mTOR: Mammalian target of rapamycin; DAPK: Death-associated protein kinase; DRAM: Damage-regulated autophagy modulator; PUMA: p53-upregulated modulator of apoptosis; hTERT: Human telomerase reverse transcriptase.

(Bcl-2), Bcl-2 associated death protein (BAD), Bcl-2 associated X-protein (BAX), *p53*-upregulated modulator of apoptosis (PUMA)[49] and autophagy inhibitors such as TP-53 induced glycolysis and apoptosis regulator proteins (TIGAR)[42].

Disruption of *p53* gene transcription function and subsequent production of an inactive mutant p53 protein allows cells to escape the cellular arrest/apoptosis controls. This allows unregulated propagation of abnormal cells and a predisposition to malignant transformation. It is important to be aware that *in vivo p53* behaviour can be different from *in vitro* response. This could be due to different stress types[50,51], cell types[52] and immune responses[24,53,54].

MECHANISMS OF *p53* INACTIVATION

The most common cause of *p53* inactivation is mutation which most frequently occurs within the *p53* core, and furthermore 70% occur at "hot spots"-amino acids 132-142, 151-159, 172-179, 237-249 and 272-286[26,55]. The International Agency for Research on Cancer (IARC) TP53 database similarly reports the most frequent *p53* mutations at codons 175, 245, 248, 249, 273 and 282; which is further corroborated by the UMD-TP53 mutation database, another international database that spans over 25 years[1,3,56]. The most common type of mutation is mis-sense (73%) followed by frame shift (9%), non-sense (8%), silent (4%) and others (6%)[1,3,57].

p53 is also inactivated by mechanisms other than *p53* gene mutation and are described below (Figure 1).

Proto-oncogene activation

Proto-oncogenes are normal proteins that become oncogenic with relatively minor modifications[6]. These proto-oncogenes are usually important cell cycle regulators (*e.g.*, $p14^{ARF}$)[58]. Human papilloma virus (HPV) 16/18 E6 protein which causes cervical cancer is able to inactivate *p53* without mutation. This explains the relatively low incidence of *p53* mutation in cervical cancer[59,60]. The SV40 large-T-antigen is another viral oncogene which is able to inactivate wild-type *p53*[61].

Mdm2 over-expression

Mdm2 is a negative regulator of *p53* and reduces the cell's ability to trigger the pro-arrest/apoptotic pathway in the event of cellular damage[62,63]. Mdm2 over-expression can occur by gene amplification, gene over-expression or

mRNA over-transcription[20,56]. Mdm2 over-expression is classically observed in soft tissue sarcomas[64,65]. Interestingly, instead of a decrease in p53 expression, the levels of both Mdm2 and p53 expression are increased. This suggests Mdm2 may have an additional *p53*-independent oncogenic mechanism (in addition to *p53* suppression by negative feedback) which can promote tumour growth.

Dysfunction of regulators of the p53-Mdm2 loop

Mutations of in the *p53*-Mdm-2 feedback loop such as AKT Kinase, Phosphatidylinositol-3-kinase (PI3K), Phosphatase and Tensin Homolog (PTEN) and Ataxia Telangiectasia Mutated (ATM)-Kinase can inappropriately influence levels of *p53* (see detailed description below). *p53* disruption has also been associated with inactivation of other tumour suppressors *e.g.*, BRCA1, Bcl-2, transforming growth factor (TGF)-β. AKT-kinase not only influences p53 levels but forms an apoptotic pathway with mTOR, an autophagy marker, in the PI3K/AKT/mTOR pathway demonstrating the complex interplay between *p53* and the relative levels of its regulators in deciding cell fate[19,42,43]: (1) AKT-kinase phosphorylates Mdm2 and induces migration of phosphorylated-Mdm2 into the nucleus where it inactivates *p53*. AKT over-expression has been shown to occur in cancer cells[66,67]; (2) PTEN is tumour-suppressive and activated in response to stress leading to *p53* up-regulation. Wild-type PTEN inhibits AKT-kinase phosphorylation of Mdm-2 and thus, intra-nuclear Mdm2 migration which suppresses *p53* activity[29,68,69]. In contrast mutated PTEN is unable to inhibit AKT-kinase which leads to continuous Mdm2- phosphorylation and Mdm-2 intra-nuclear migration leading to reduced *p53* tumour suppressive ability[69,70]; and (3) Cell stress (*e.g.*, irradiation) activates factors up-stream of *p53* such as ATM kinase and checkpoint Kinase-2[54,65]. Mutated ATM-kinase is unable to activate *p53* in response to radiation-induced stress.

Nuclear exclusion and cytoplasmic p53

Extrusion of *p53* into cytoplasm has been observed in certain tumours such as breast[71], colon[72], neuroblastoma[73] and malignant melanoma[74]. Nuclear extrusion prevents *p53* from performing its intra-nuclear interactions.

Gain of oncogenic function

Mutant *p53* has an impaired ability to regulate cell cycle which is referred to as "loss of function"[3,6,75]. In addition to this, mutated *p53* can also exhibit conformational changes which result in acquisition of new pro-oncogenic abilities; this is known as "gain of function". Such functions include increased transcription of tumour-promoting factors such as MYC and VEGF[76] and disruption of protective pro-apoptotic factors such as p73[7,77].

ANTI-*p53* AUTO-ANTIBODY

An anti-*p53* auto-antibody response was first reported by Crawford *et al*[78] in 1982 in 9% (14/155) of patients with breast cancer. Further interest in this anti-*p53* auto-antibody response declined due to the lack of accurate quantification methods and no observable clinical relevance. Research into the auto-antibody was invigorated in the 1990s when the critical role of *p53* gene in carcinogenesis was recognized. The exact cause of induction of anti-*p53* auto-antibody production is unknown but is thought to be associated with the presence of *p53* mutation and p53 protein over-expression.

An anti-*p53* auto-antibody is not normally produced wild-type p53 protein induces tolerance of the host[32,79]. In abnormal cells, mutant p53 protein is stabilised as discussed above which cause relatively high intra-nuclear p53 protein accumulation which then escapes into the cytoplasm. The resulting high cytoplasmic p53 levels increase the likelihood of p53 protein being degraded by proteasomes and presented on cell surfaces to be recognised by T-cells in a MHC I response[16]. Auto-antibodies recognises epitopes on the terminal regions of the protein, and hence auto-antibody production can theoretically be triggered by either the wild-type or the mutant *p53*, provided sufficiently high levels of these immunodominant epitopes are present at the cell surface[80]. Another probable antigen presentation mechanism is where cancer cells containing high cytoplasmic concentrations of *p53* undergo necrosis and release *p53* into the blood and lymphatic system where appropriate B-cells can interact. These antigens are also captured by Antigen Presenting Cells (APC) in their normal scavenging role and are presented in association with MHC class Ⅱ response causing a Th2-like cell response[16] (Figure 2).

P53 mutation alone is insufficient to trigger anti-*p53* auto-antibody production as evidenced by several observations. Firstly, only 20%-50% of patients with detectable *p53* mutations produce detectable auto-antibodies[81,82]. This is attributed to the type of mutation, *e.g.,* mis-sense mutations are associated with higher auto-antibody production compared with other mutations[5,56,57,83]. This is probably because mis-sense mutations are more likely to produce a stable mutant p53 protein which is more likely to accumulate to sufficient levels to increase the likelihood of antigen presentation. Other mutations such as non-sense, frameshift and deletions often lead to truncated mRNA and unstable protein sequences which are less likely to accumulate, and thus less likely to induce auto-antibody production[84]. Secondly, anti-*p53* auto-antibodies most frequently recognise terminal epitopes but not the central domain with the majority of mutations[25,27,28,81]. Thirdly, large SV40 T-antigen stabilises p53 protein leading to accumulation of the wild-type protein which also induces auto-antibody production. Together these observations suggest that humoral response is triggered by elevated p53 protein levels *per se* (mutated and/or wild-type) rather than specifically directed at a mutated sequence.

Discrepancy between anti-p53 auto-antibody and p53 mutation

There are discrepancies between the presence of *p53* mutation, p53 protein product expression and anti-*p53*

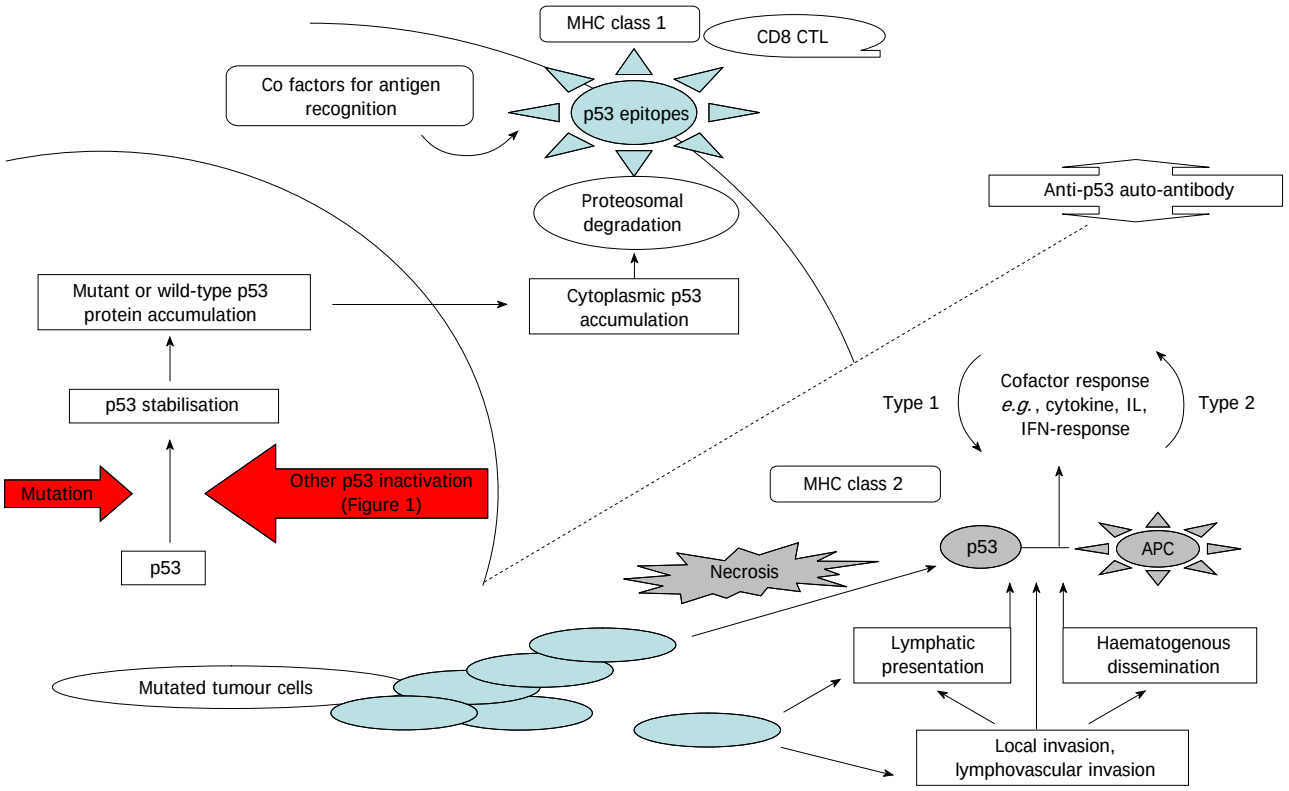

Figure 2 Proposed mechanisms of anti-*p53* auto-antibody induction. MHC: Major histocompatibility complex; CD8: Cluster of 8 differentiation; CTL: Cytotoxic T-cell; IL: Interleukin; APC: Antigen presenting cell.

auto-antibody production. This is largely attributed to the methodological differences of detection. Initial gene screening studies reported that most *p53* mutations were localised to exons 5-8 and to a lesser extent 4, 9, 10. Subsequent studies then only tended to screen these regions leading to substantial screening bias. It is now known that at least 10% of *p53* mutations occur outside these areas[84,85]. Another source of methodological difference is p53 protein detection which is inherently subject to tissue sampling and biopsy errors. Older studies (pre-1999) had different immuno-histochemical, fixation, paraffinization, antigen and antibody retrieval and observer scoring techniques. Finally, the antibody used to detect the mutant p53 protein affects sensitivity of p53 protein detection in IHC and also the detection of anti-*p53* auto-antibody in ELISA as described below.

Historically, the auto-antibody was initially detected using immunoblots or in-house enzyme linked immunosorbent assay (ELISA). These ELISA used different cut-off values leading to a vast range of reported frequencies of anti-*p53* auto-antibody within individual cancers in many older studies. Although standardised commercial ELISA are now widely available leading to an increase in anti-*p53* ELISA studies (2000 onward), auto-antibody detection can still vary depending on different manufacturers' product[86]. Most importantly, these ELISAs only measure an antibody response against those *p53* epitopes, which are expressed by the recombinant proteins used as the coating antigen. This may account for the reason that there are minor variations in commercial ELISA studies in different populations but when the same ELISA is used in the same population, inter and intra-coefficient of variations of 0.3%-2.7% are extremely reliable[82].

Finally, the differences in individual's immune systems cannot be ignored. The humoral response is dependent on an individual's unique MHC presentation as shown by several observations. Firstly, patients with similar cancers containing the same *p53* mutations do not necessarily mount the same immune response[81]. Secondly, whilst anti-*p53* auto-antibody titres increase in response to tumour load, all patients who are initially sero-negative do not develop an auto-antibody response despite disease progression and metastases. Conversely, patients who are sero-positive at diagnosis do not sero-convert to a negative anti-*p53* auto-antibody status even after the cancer is completely excised. It seems that once the patient's immune system has been primed, there is sufficient *p53* antigen available to maintain a long-term anti-*p53* humoral response[28,87,88].

MATERIALS AND METHODS: SYSTEMATIC REVIEW

Literature searches were performed using Medline and PubMed up to January 2012. Keywords used were "*p53*", "anti-*p53*", "antibody", "auto-antibody", "cancer" and combinations. No language or time restrictions were applied. All abstracts were reviewed and the relevant articles retrieved. The results of all published anti-*p53* auto-antibody cancer studies were accumulated and compiled in Table 1 with relevant key findings. The anti-*p53* auto-

Table 1 Cumulative reported frequencies of anti-*p53* auto-antibody (anti-*p53*) in controls and individual cancers *n* (%)

Group	Ref.	Anti-p53 positive	Summary of study and tumour type
Healthy/Benign	Park *et al*[107]	4/79 (5)	Comparative study with lung cancer
	Wu *et al*[133]	9/879 (1)	Case-control study of anti-*p53* in various cancers
	Kulić *et al*[134]	1/20 (5)	Comparative study with breast carcinoma
	Suppiah *et al*[130]	0/28 (0)	Comparative study with colorectal carcinoma
	Cai *et al*[125]	0/30 (0)	Comparative study with oesophageal carcinoma
	Atta *et al*[135]	5/29 (17.2); 13/26 (50)[1]	Comparative study with hepatocellular carcinoma
	Mattioni *et al*[136]	0/64 (0)	Comparative study with gastric carcinoma
	Akere *et al*[137]	4/45 (8.9)	Comparative study with hepatocellular carcinoma
	Müller *et al*[123]	0/57 (0); 0/379 (0)[2]	Single study of anti-*p53* in various cancers
	Chang *et al*[85]	0/40 (0)	Comparative study with colorectal carcinoma
	Fonseca *et al*[95]	0/15 (0)	Comparative study with glioma
	Shimada *et al*[82]	10/205 (6.3); 13/189 (7)[3]	Multi-institutional study of anti-*p53* in various cancers
	Neri *et al*[138]	0/51 (0)	Comparative study with lung carcinoma
	Numa *et al*[139]	0/9 (0)	Comparative study with uterine, ovarian, cervical carcinoma
	Mack *et al*[140]	1/46 (2.2)	Comparative study with SCLC
	Chow *et al*[141]	1/28 (3.6)	Comparative study with head and neck carcinoma
	Moch *et al*[142]	2/130 (1.5)	Comparative study with skin carcinoma (SCC/BCC)
	Hofele *et al*[143]	0/80 (0)	Comparative study with oral SCC
	Hagiwara *et al*[144]	0/13 (0)	Comparative study with oesophageal carcinoma
	Ralhan *et al*[145]	4/50 (8)	Comparative study with lung carcinoma
	Bielicki *et al*[111]	0/28 (0)	Comparative study with colorectal carcinoma
	Soussi[90]	35/2404 (1.5)	Literature review of anti-*p53* in various cancers (1979-1999)
	Total	102/4924 (2.1)	
Oesophageal	Blanchard *et al*[146]	24/97 (28)	Correlates with decreased overall and disease free survival
	Wu *et al*[133]	4/29 (13.8)	Case-control study of anti-*p53* in various cancers
	Cai *et al*[125]	18/46 (39.1)	Correlates with advanced histological grade, stage, lymph node metastases and decreased tumour response following radiotherapy
	Müller *et al*[123]	10/50 (20)	No correlation with stage or prognosis
	Bergström *et al*[147]	31/42 (73.8)	No correlation with clinico-pathological parameters, tumour size or survival
	Shimada *et al*[82]	90/301 (29.9)	Multi-institutional study of anti-*p53* in various cancers
	Kozłowski *et al*[148]	20/75 (26.6)	No correlation with stage, lymph node metastases or size.
	Shimada *et al*[99]	14/35 (40)	Correlates with tumour p53 protein expression but not clinico-pathological parameters
	Hagiwara *et al*[144]	13/46 (28)	Correlates with increased stage and tumour p53 protein expression but not prognosis
	Ralhan *et al*[145]	36/60 (60)	Correlates with tumour p53 protein expression and missense mutations but not clinico-pathological parameters.
	Soussi[90]	85/274 (31)	Literature review of anti-*p53* in various cancers (1979-1999)
	Total	345/1055 (32.7)	
Head/Neck[16]	Wu *et al*[133]	1/20 (5.0)	Case-control study of anti-*p53* in various cancers
	Shimada *et al*[82]	10/31 (32.3)	Multi-institutional study of anti-*p53* in various cancers
	Chow *et al*[141]	23/75 (31)	Correlates with nodal metastases but not prognosis
	Total	34/126 (27.0)	
Oral	Wu *et al*[133]	5/15 (33.3)	Case-control study of anti-*p53* in various cancers
	Hofele *et al*[143]	19/102 (18.6)[4]; 12/24 (50)[5]	Correlates with poor prognosis
	Castelli *et al*[149]	3/61 (18.7); 9/13 (69.2)[3]	Serum anti-*p53* is useful as a screening tool in pre-malignant lesions
	Soussi[90]	309/1062 (29.1)	Literature review of anti-*p53* in various cancers (1979-1999)
	Total	348/1219 (28.5)	
Ovary	Wu *et al*[133]	5/12 (41.6)	Case-control study of anti-*p53* in various cancers
	Qiu *et al*[150]	36/92 (39.1)	Correlates with p53 expression, not clinico-pathological parameters
	Shimada *et al*[82]	2/27 (7.4)	Multi-institutional study of anti-*p53* in various cancers
	Numa *et al*[139]	8/30 (27)	Correlates with *p53* tumour expression and poor prognosis
	Abendstein *et al*[151]	28/113 (25); 21/113 (19)[6]	Correlation between serum and ascitic anti-*p53*. No correlation with stage or grade. Anti-*p53* in ascites associated with poor prognosis
	Soussi[90]	140/635 (22)	Literature review of anti-*p53* in various cancers (1979-1999)
	Total	219/909 (24.1)	
Colorectal (detailed results in Table 2)	Wu *et al*[133]	11/66 (16.7)	Case-control study of anti-*p53* in various cancers
	Suppiah *et al*[130]	20/92 (21.7)	No correlation with stage or prognosis
	Nozoe *et al*[97]	17/36 (47.2)	Correlates with advanced lymph node status and stage
	Müller *et al*[123]	63/197 (32)[7]; 7/46 (15.2)[8]	No correlation with stage or prognosis
	Chang *et al*[85]	47/167 (28.1)	*p53* mutation, not anti-*p53*, correlates with poor prognosis
	Lechpammer *et al*[88]	40/220 (18.2)	? Correlation with stage or prognosis in Dukes' A/B1
	Shimada *et al*[82]	46/192 (23.9)	Multi-institutional study of anti-*p53* in various cancers
	Forslund *et al*[84]	24/88 (27.3)	Correlates with *p53* mutation
	Tang *et al*[89]	130/998 (13)	Correlates with advanced lymph node involvement but not prognosis
	Broll *et al*[152]	20/130 (15.4)	No correlation with stage or prognosis
	Takeda *et al*[98]	17/27 (63)	95% negative sero-conversion within 3 wk post-surgery
	Shiota *et al*[112]	18/71 (25.4)	Correlates with advanced stage and poor prognosis

	Bielicki *et al*[111]	30/145 (20.7)	? Correlation with Dukes' A →B
	Soussi[90]	307/1244 (24.7)	Literature review of anti-*p53* in various cancers (1979-1999)
	Total	797/3719 (21.4)	
HCC	Wu *et al*[133]	15/93 (16.1)	Case-control study of anti-*p53* in various cancers
	Atta *et al*[135]	28/41 (68.3)	Correlates with advanced stage and shorter survival.
	Akere *et al*[137]	5/41 (12.2)	Correlates with increased Okuda stage
	Müller *et al*[123]	19/80 (23.8)	Non-significant trend towards poor prognosis
	Charuruks *et al*[153]	26/141 (18.4)	Correlates with stage but not tumour p53 protein expression
	Tangkijvanich *et al*[154]	16/121 (13.2)[17]	Preliminary report of Charuruks *et al* (2001). No correlation with severity, stage or prognosis. Survival too short for survival analysis (3 mo *vs* 4 mo)
	Sitruk *et al*[155]	19/159 (12)	Correlates with multinodular, infiltrative tumour but not survival
	Soussi[90]	82/387 (1.2)	Literature review of anti-*p53* in various cancers (1979-1999)
	Total	210/1063 (19.8)	
	Wu *et al*[133]	0/11 (0)	Case-control study of anti-*p53* in various cancers
Bladder	Müller *et al*[123]	3/24 (12.5)	No correlation with prognosis
	Watanabe *et al*[156]	17/63 (27)[9]	Correlates with higher grade, stage, lymph node metastases and tumour p53 protein expression, but not prognosis
	Gumus *et al*[157]	14/80 (17.5)	Correlates with tumour p53 protein expression and poor prognosis.
	Gumus *et al*[158]	25/76 (33)	Negative sero-conversion post-treatment (35%, 8/23) associated with good prognosis.
	Shimada *et al*[82]	4/33 (12.1)	Multi-institutional study of anti-*p53* in various cancers
	Morita *et al*[159]	12/100 (12)	Correlates with stage, and p53 protein expression but not prognosis
	Wunderlich *et al*[160]	4/32 (12.5)	Correlates with tumour protein p53 expression but not stage.
	Soussi[90]	8/29 (27.6)	Literature review of anti-*p53* in various cancers (1979-1999)
	Total	70/385 (18.2)	
Lung	Park *et al*[107]	28/82 (34.1)	Sensitivity study with other markers for lung cancer
	Wu *et al*[133]	13/95 (13.7)	Case-control study of anti-*p53* in various cancers
	Bergqvist *et al*[161]	14/84 (16.6)	No correlation with tumour volume. Correlates with survival in adenocarcinoma, but not SCC
	Bergqvist *et al*[162]	12/58 (20.7)	No correlation with tumour volume or lymph node metastases
	Neri *et al*[138]	2/30 (6.7)[10]; 8/48(16.7)[11]	No correlation with stage, histology or prognosis. Non-significant increased survival in LC but not MM
	Cioffi *et al*[163]	35/109 (32.1)	Low sensitivity, but high specificity (100%) and accuracy (69%). Only 14% agreement with other tumour markers (CEA/TPA, CYFRA21-1, NSE.)
	Zalcman *et al*[126]	20/97 (20.6)	Correlates with poor prognosis in limited stage SCLC, but not all SCLC
	Mack *et al*[140]	4/35 (11.1)[12]; NSCLC 13/99 (13.3)[13]	Correlates with stage and prognosis in NSCLC but not SCLC
	Shimada *et al*[82]	18/125 (14.4)	Multi-institutional study of anti-*p53* in various cancers
	Soussi[90]	219/1282 (17.1)	Literature review of anti-*p53* in various cancers (1979-1999)
	Total	373/2049 (18.2)	
Cervix	Shimada *et al*[82]	10/53 (18.9)	Multi-institutional study of anti-*p53* in various cancers
	Numa *et al*[139]	12/86 (14)	No correlation with tumour p53 protein expression or prognosis
	Total	22/139 (15.8)	
	Wu *et al*[133]	7/43 (16.3)	Case-control study of anti-*p53* in various cancers
Gastric	Qiu *et al*[150]	19/61 (31.1)	Correlates with tumour size but not prognosis.
	Mattioni *et al*[136]	17/111 (15.3)	Correlates with tumour p53 protein expression, prognosis and survival
	Lawniczak *et al*[164]	16/71 (22.5)	Correlates with tumour type and age, but not stage or prognosis
	Müller *et al*[123]	14/122 (11.5)	No correlation with prognosis
	Shimada *et al*[82]	13/123 (10.6)	Multi-institutional study of anti-*p53* in various cancers
	Nakajima *et al*[165]	13/81 (16)	Correlates with lymph node metastases but not stage or prognosis
	Maehara *et al*[166]	23/120 (19.2)	Correlates with increased stage and tumour p53 protein expression but not prognosis
	Soussi *et al*[90]	105/727 (14.1)	Literature review of anti-*p53* in various cancers (1979-1999)
	Total	227/1459 (15.6)	
	Nozoe *et al*[167]	15/42 (35)	Correlates with grade 3 and triple negative cancer
Breast	Wu *et al*[133]	9/25 (16)	Case-control study of anti-*p53* in various cancers
	Kulić *et al*[134]	21/61 (35)	Correlates with decreased 5 year survival
	Müller *et al*[123]	17/50 (34)	Non-significant trend towards poor prognosis
	Gao *et al*[168]	31/144 (21.5)	Correlates with stage, lymph node metastases, ER negative, c-erb-2 and tumour p53 protein expression
	Shimada *et al*[82]	13/71 (18.3)	Multi-institutional study of anti-*p53* in various cancers
	Volkmann *et al*[169]	18/165 (10.9)	Poor concordance between recombinant/native *p53* ELISA, immunoblot and immunofluorescence
	Metcalfe *et al*[87]	155/1006 (15.4)	No correlation with stage and prognosis
	Soussi[90]	296/2006 (14.8)	Literature review of anti-*p53* in various cancers (1979-1999)
	Total	539/3467 (15.5)	
Uterus	Wu *et al*[133]	1/13 (7.7)	Case-control study of anti-*p53* in various cancers
	Shimada *et al*[82]	5/22 (22.7)	Multi-institutional study of anti-*p53* in various cancers
	Numa *et al*[139]	5/41 (12)	No correlation with tumour p53 expression/prognosis (see Cervix, Ovary)
	Total	11/79 (13.9)	
Pancreas	Wu *et al*[133]	0/17 (0)	Case-control study of anti-*p53* in various cancers

	Müller *et al*[123]	5/22 (22.7)	Increase sensitivity in conjunction with CA19-9. No correlation with prognosis.
	Shimada *et al*[82]	3/28 (10.7)	Multi-institutional study of anti-*p53* in various cancers
	Ohshio *et al*[170]	19/82 (23.2)	No correlation with tumour *p53* expression or prognosis
	Soussi[90]	60/650 (9.2)	Literature review of anti-*p53* in various cancers (1979-1999)
	Total	87/799 (10.9)	
Lymphoma	Messmer *et al*[171]	19/120 (15.8)	Associated with 17p deletions
	Wu *et al*[133]	0/18 (0)	Literature review of anti-*p53* in various cancers (1979-1999)
	Soussi[90]	19/248 (14.3)	Case-control study of anti-*p53* in various cancers
	Total	38/386 (9.8)	
Biliary tract[16]	Wu *et al*[133]	1/8 (6.3)	Correlates with tumour p53 protein expression but not stage
	Limpaiboon *et al*[172]	6/49 (12.2)	Multi-institutional study of anti-*p53* in various cancers
	Shimada *et al*[82]	1/6 (16.7)	Correlates with tumour *p53* mutation
	Tangkijvanich *et al*[173]	6/82 (7.3)	
	Total	14/145 (9.7)	
Haematological	Wu *et al*[133]	8/33 (25)	Case-control study of anti-*p53* in various cancers
	Shimada *et al*[82]	32/364 (6.3)[14]	Multi-institutional study of anti-*p53* in various cancers
	Soussi[90]	14/428 (3.3)[15]	Literature review of anti-*p53* in various cancers (1979-1999)
	Total	54/825 (6.5)	
Glioma	Wu *et al*[133]	1/24 (4.2)	Case-control study of anti-*p53* in various cancers
	Fonseca *et al*[95]	5/24 (20.8)	No correlation with *p53* protein but increased in patients < 16 years
	Shimada *et al*[82]	2/31 (6.5)	Multi-institutional study of anti-*p53* in various cancers
	Soussi[90]	6/144 (4.2)	Literature review of anti-*p53* in various cancers (1979-1999)
	Total	14/223 (6.3)	
Prostate	Wu *et al*[133]	1/8 (12.5)	Case-control study of anti-*p53* in various cancers
	Shimada *et al*[82]	4/23 (17.4)	Multi-institutional study of anti-*p53* in various cancers
	Soussi[90]	4/148 (2.7)	Literature review of anti-*p53* in various cancers (1979-1999)
	Total	9/179 (5.0)	
Skin	Moch *et al*[142]	3/105 (2.9)	No difference between controls and patients. Increased in aggressive SCC (8%) *vs* slow-growing BCC (1.5%)
Testicular	Soussi[90]	0/144 (0)	Literature review of anti-*p53* in various cancers (1979-1999)
Melanoma	Soussi[90]	0/58 (0)	Literature review of anti-*p53* in various cancers (1979-1999)
Total		3419/18595 (18.4)	All cancers (1979-2012)

Cancer types are listed in order of decreasing anti-*p53* auto-antibody frequency. The reported studies within each cancer type are listed in reverse chronology. Squamous cell carcinoma (SCC); basal cell carcinoma (BCC), hepatocellular carcinoma (HCC), carcino-embryonic antigen (CEA). Tissue polypeptide antigen (TPA), CYFRA21-1, Neurone-specific enolase (NSE), Oestrogen receptor (ER), c-erb-2. [1]Cirrhosis; [2]Benign disease; [3]Oral pre-malignant lesions-excluded from calculation; [4]Primary carcinoma; [5]Secondary/recurrent carcinoma; [6]Ascitic titre, not included in calculation of serum titres; [7]Colon; [8]Rectum; [9]Upper renal tract tumours, excluded from anti-*p53* titres in bladder carcinoma; [10]Malignant mesothelioma (MM); [11]Lung carcinoma (LC); [12]Small cell lung carcinoma (SCLC); [13]Non-small cell lung carcinoma (NSCLC); [14]Myeloma; [15]Leukaemia; [16]Tumour type not specified; [17]Excluded as is preliminary report of the same cohort (duplicate) reported in Charuruks *et al*.

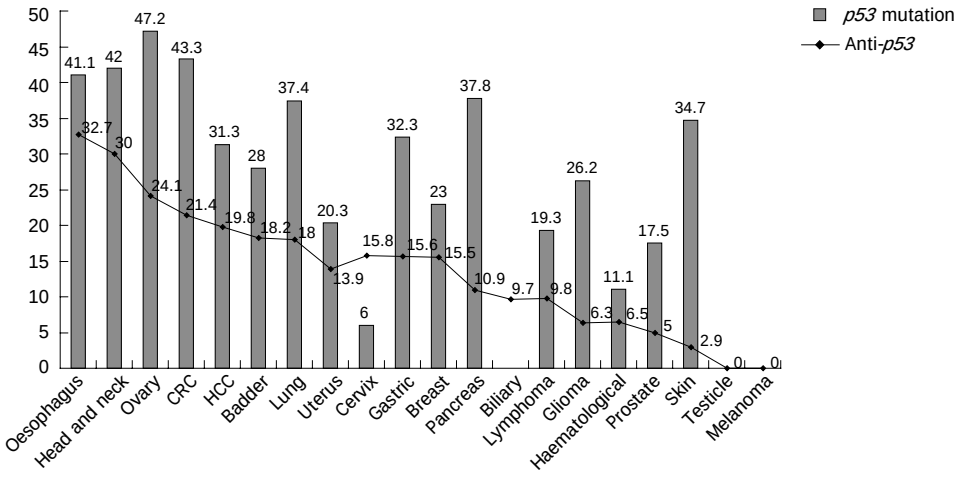

Figure 3 Percent *p53* mutations (International Agency for Research on Cancer, 2008) and percent anti-*p53* auto-antibody incidence calculated in this review. $r^2 = 0.45$, Correlation = 0.59. CRC: Colorectal cancer; HCC: Hepatocellular carcinoma.

antibody frequency from all published studies in each cancer type was calculated in this review. This calculated anti-*p53* auto-antibody frequency was then correlated with reported *p53* mutation rates to determine the associated between anti-*p53* auto-antibody presence and mutation in each cancer (Figure 3).

Methodological quality of anti-p53 and CRC publications

All published studies on anti-*p53* auto-antibody (1979-2012) were retrospective or cross-sectional case

control series with relatively small sample size (27-220 subjects tested) with a heterogeneous mix of cancer stages. The largest single study was published by Tang *et al*[89] that included a cohort of 998 CRC patients with anti-*p53* present in only small numbers (n = 130) for stage-specific analysis. An earlier non-systematic review by Soussi in 2000 recruited large numbers from various anti-*p53* studies but was study quality was limited by different cancers at various stages and different auto-antibody detection methods[90]. The primary outcome was not stated in most studies, and none was powered appropriately for survival outcomes.

ANTI-*p53* AUTO-ANTIBODY IN ALL CANCER TYPES

The reported frequency of anti-*p53* auto-antibody in individual cancer studies vary significantly due to small sample sizes, stage bias (usually a greater proportion of advanced stage tumours were included and different detection methods used. Anti-*p53* auto-antibody is usually measured in patients' sera but has also been measured in ascitic fluid of patients with ovarian cancer[68], saliva of patients with oral cancer[91] and in pleural effusions (12.5%) associated with lung, colon and pancreatic cancer[92]. In a landmark review, Soussi compiled results of 80 anti-*p53* auto-antibody studies in 18 cancer types over a 20 year (1979-1999) period[90]. The mean serum sero-positivity across all cancer types was 16.9% (1600/9489 patients, range 0%-31%) compared with 1.45% (35/2404) in controls thus demonstrating remarkable specificity (98%) but poor sensitivity. The specificity would be even higher as half the false positive subjects (17 out of 35) were from a single study reporting an extra-ordinarily high sero-positivity (24%, 17/70)[93](Table 1). When this study was excluded anti-*p53* auto-antibody specificity is near 100% for any cancer, which is confirmed by most recent reports.

Relationship between anti-p53 auto-antibody and p53 mutation

The 30-year cumulative sera anti-*p53* auto-antibody frequencies in individual cancers were calculated in this review to provide the most comprehensive anti-*p53* auto-antibody frequency in each cancer to date (Table 1). The auto-antibody frequencies are plotted (point) against the *p53* mutation rate (bars) as reported by the IARC TP53 Mutation Database to ascertain a relationship between anti-*p53* auto-antibody and *p53* mutation rates in each cancer.

The graph shows moderate correlation (r^2 = 0.45, correlation 0.59) between anti-*p53* auto-antibody and *p53* mutation (Figure 3). In general, cancers with the highest *p53* mutation rate such as oesophageal, head and neck, and colorectal demonstrate highest anti-*p53* auto-antibody rates[82,90]. Conversely, melanoma and testicular carcinoma with the lowest mutation rate have the lowest serum anti-*p53* auto-antibody rates (< 1%)[90]. The two exceptions are gliomas and skin cancers which have moderate *p53* mutation rate and low anti-*p53* auto-antibody rate (Figure 3). Proposed reasons for the low anti-*p53* auto-antibody production are poor brain antigenicity, poor *p53* antigen-presentation across the blood-brain barrier, and use of immuno-suppressive steroids (dexamethasone) in the majority of glioma cases[90,94,95]. Similar arguments about poor antigen presentation across an epithelial barrier are made for the majority superficial skin cancer. In summary, anti-*p53* auto-antibody has up to 35% sensitivity, depending on cancer type, nearly 100% specific for any malignancy but varies in individual cancer types; and demonstrates moderate correlation with *p53* mutation rate of each cancer.

ANTI-*p53* AUTO-ANTIBODY AND COLORECTAL CANCER VARIATIONS

CRC has the second highest anti-*p53* auto-antibody sero-positivity rates due in part to the high frequency of *p53* mutation. Pre-1999, eight studies used an "in-house" developed ELISA, 1 used Western blotting (WB), 1 used immuno-precipitation (IP) and another used all 3 detection methods (ELISA, WB, IP)[90]. Despite these methodological differences, most studies, 10 out of 11, reported a sero-positivity rate between 12.5% and 32%. The only study to report a discrepant and much higher sero-positivity rate (68%) used WB thus demonstrating the potential bias caused by non-standardised detection methodology[96]. New standardised commercial ELISA kits have since been developed with less variation in sero-positivity (13%-27%) with intra- and inter-assay coefficient of variation of 1.85%-2.37% and 0.3%-3.32% respectively (MESACUP anti-*p53* Test; Medical and Biological Laboratories, MBL, Nagoya, Japan)[82].

The mean sero-positivity from ELISA-only CRC studies calculated in this review was 19.9% (479/2409) with individual studies reporting of 13%-27% (Table 2). Only two studies reported inconsistently high rates of 47% and 63% in patients, and also in controls (2.6%), which suggests a lower cut-off value was used[97,98]. The same authors then reported an unusually high (40%) sero-positivity in superficial oesophageal carcinoma in another series, compared with 20%-30% in the majority of other studies. These studies used the same ELISA (Pharmacell, France)[99]. Interestingly, when the same authors later used a different ELISA (anti-*p53* EIA Kit Ⅱ, MESACUP) in a similar population, they reported a much lower sero-positivity of 30% (oesophageal cancer) and 24% (CRC) which was more consistent with other published ELISA studies[82]. This highlights potential methodological biases with anti-*p53* auto-antibody quantification even with commercially standardised ELISA kits.

Anti-p53 auto-antibody in diagnosis and screening

Cancer screening is used when early detection and intervention can lead to improved outcome for example CRC where 5 year survival in Dukes' A is 95%-100% com-

Table 2 Anti-*p53* auto-antibody in all published colorectal cancer studies and key findings *n* (%)

Ref.	Method and manufacturer	Samples	Follow-up	Key findings
Suppiah *et al*[130]	ELISA (*p53* ELISAPLUS, Calbiochem, Darmstadt, Germany)	20/92 (21.7); 0/20 (0)[1] 0/8 (0)[2]	Median 97 mo	No correlation with tumour stage, differentiation or location. Multivariate analysis show only Stage (Dukes' and TNM) to be independent prognostic factors
Nozoe *et al*[97]	ELISA (Pharmacell, France)	17/36 (47.2)	Not stated	Anti-*p53*-ab (+) associated with greater lymphatic invasion (94.1%; 16/17 *vs* 68.4%; 13/19), nodal involvement (70/6%; 12/17 *vs* 17.6%; 3/17) and advanced stage (P = 0.02). Anti-*p53* frequency higher in *p53* protein expressing tumours (74%; 14/19 *vs* 18%; 3/17). Only 3 patients with Dukes' A CRC, all sero-negative
Muller *et al*[123]	Immunoblot	Colon 63/197 (32); Rectum 7/46 (15.2); 0/57 (0)[1] 0/379 (0)[2]	CRC patients enrolled into trial with 5 year follow-up	No correlation with clinico-pathological parameters or prognosis. Trend toward higher anti-*p53* sero-positivity in N2/3 disease, poor differentiation and metastases. There were no patients with Dukes' A in this study. Anti-*p53* independent of CEA and CA19-9 with 16% information gain. This is the only study to report negative to positive sero-conversion (3.6%, 11/303)
Chang *et al*[85]	ELISA (*p53*-AK, Dianova, Hamburg, Germany)	47/167 (28.1); 0/40 (0)[1]	Median 36.3 mo (4-58)	Anti-*p53* correlates with *p53* mutation (43% *vs* 18%) but not tumour *p53* expression, clinico-pathological features or prognosis. *p53* mutations, advanced stage and pre-operative CEA > 5 ng/mL were independent prognostic factors (in that order). *p53* mutation strongly associated with advanced stage and poor differentiation
Lechpammer *et al*[88]	ELISA (ELISAPLUS Oncogene Research Products, Cambridge, United States)	40/220 (18.2); 0/42 (0)[1]	40 patients up to 20 wk; 8 patients up to 48 wk	Anti-*p53* had higher tumour *p53* expression (70% *vs* 52%). Anti-*p53* frequency shows highest increase in Dukes' A (0%, 0/28) → Dukes' B: (24%, 21/87) but no increase in progression to Dukes' C (18%,19/105). No correlation with overall tumour grade or metastases. Anti-*p53* reflects tumour load following surgery, during chemotherapy and with disease recurrence
Shimada *et al*[82]	ELISA (Anti-*p53* EIA Kit II, MESACUP anti-*p53* Test; MBL; Nagoya, Japan)	46/192 (23.9); 10/205 (4.9)[1]; 13/189 (6.9)[2]	Not reported	Validation study for MESACUP ELISA using prevalence of anti-*p53* in various cancers. Good intra- and inter-assay coefficient of variation of 1.85-2.37% and 0.3-3.2% respectively. Demonstrates stability of anti-*p53* titres at room temperature for 7 d and following 10 freeze-thaw cycles. No comment on correlation with clinico-pathological parameters or prognosis
Forslund *et al*[84]	ELISA (Dianova, Hamburg, Germany)	24/88 (27)	Not reported	Cross-sectional study on relationship between *p53* mutations and anti-*p53* presence. Frequency of *p53* mutation higher in anti-*p53* sero-positive group (92%, 22/64 *vs* 34%, 22/64) Correlation with clinico-pathological and survival parameters not reported
Tang *et al*[89]	ELISA (Calbiochem-Novabiochem, Darmstadt, Germany)	130/998 (13); 2/211 (1)[3]	Not reported	Anti-*p53* sero-positivity increases in progression from N2→ N3 (2.9%-10.6%); but not N0→N1 (11.7%-12.3%), N1→N2 (12.3%-10.6%) or M0→M1 (12%-17%). No correlation with CEA, overall TNM stage or metastases. Anti-*p53* associated with shorter survival in uni- but not multi-variate analysis. Largest study on anti-*p53* in CRC
Broll *et al*[152]	ELISA (*p53*-autoantikorpfer ELISA, Dianova, Hamburg, Germany)	20/130 (15); 0/44 (0)[1]	Median 25.5 mo	Anti-*p53* positive predictive value of 100%, but accuracy 37% and negative predictive value 29% due to poor sensitivity (15%). Anti-*p53* correlated with *p53* expression (P < 0.05), but not TNM stage, grade or location (exact numbers not shown). Approximately 70% of series Stage Ⅰ/Ⅱ CRC
Takeda *et al*[98]	Anti-*p53* EIA (PharmaCell, Paris, France)	17/27 (63); 1/38 (2.6)[3]	Up to 2 years Median not reported	Anti-*p53* correlates with *p53* protein expression and independent of CEA and CA-19-9. Sero-conversion in 94% (16/17) within 3 wk of endoscopic resection. No correlation with clinico-pathological parameters or prognosis/recurrence as all patients had early superficial CRC (23 mucosal, 4 submucosal invasion). This study reports exceptionally high anti-*p53*, especially considering very early CRC
Takeda *et al*[174]	ELISA (anti-*p53*-EIA kit, Pharmacell, Paris, France)	40 patients with anti-*p53* ab from previous studies	Up to 29 mo	No correlation between post-operative anti-*p53* sero-positivity and histological (depth, lymphatic or venous invasion) or clinico-pathological features of lymph node or liver metastases. High (96%; 27/28) sero-conversion in patients with complete tumour resection. No sero-conversion in patients with residual disease.
Shiota *et al*[112]	ELISA (GIF, Munster, Germany)	18/71 (25); 1/18 (6)[3]	Not stated, median survival 56 mo anti *p53* ab negative	Anti-*p53* correlates with TNM stage (Stage Ⅰ-Ⅲb: 9%, 4/45 *vs* Ⅳ: 56%, 14/25), Dukes' stage (A-C: 9%, 4/45 *vs* D: 56%, 14/25), CEA, CA19-9 and tumour p53 protein expression. Anti-*p53* associated with shorted survival (56 mo *vs* 20 mo) and is weak poor prognostic indicator. Anti-*p53* prognostic significance secondary to other factors, including weak factors *e.g.*, CEA and CA19-9. Only small number of Stage Ⅰ-Ⅲb patients

Bielicki *et al*[111]	ELISA (Dianova, Hamburg, Germany)	30/145 (21); 0/20 (0)[2]; 0/8 (0)[3]	Not stated. Cross sectional study	No correlation with Dukes' Stage (A/B: 22%, 16/73 *vs* C/D 19% 14/72), size, location, CEA. Highest increase in anti-*p53* frequency from Dukes' A (0%, 0/6) to Dukes B1 (28%, 5/18) but no further difference in progression to Dukes' C (19%, 7/36). Only 6 Dukes' A patients in study, all sero-negative
Soussi[90]	ELISA/WB/IP	307/1244 (24.7)	ELISA/ WB/IP	Review combining all studies with different methodologies from 1979-1999. Range of sero-positivity (12.5%-68% in 11 studies)
Total (1999-2009)		479/2409 (19.9)		All modern studies (1999 onwards) using commercial ELISA only, with one exception using Immunoblot (Muller *et al*, 2006)
Review Total (1979-2009)		786/3653 (21.5)		All studies on anti-*p53* in CRC (1979-2009)

Studies prior to 1999 used different methodology and not included (see above). Enzyme-linked immunosorbent assay (ELISA); Western blotting (WB); Immunoprecipitation (IP) [1]healthy, [2]benign disease, [3]adenoma. The study by Muller *et al* was included despite using immunoblot technique as it was a recent study with relatively large sample size. CRC: Colorectal cancer; CEA: Carcino-embryonic antigen.

pared with 5% in Dukes' D. Colonoscopy is the current gold-standard diagnostic tool but is painful, expensive and is associated with life-threatening complications such as colonic perforation (0.01%-0.3%) and haemorrhage (0.6%)[100,101]. The United Kingdom Flexible Sigmoidoscopy Screening Trial has provided evidence that one-off screening flexible sigmoidoscopy between age 55 and 64 was beneficial in reducing CRC incidence by 23%-33% and reducing mortality by 31%-43%[102,103]. Similar mortality reduction has been reproduced in other screening trials such as Norwegian Colorectal Cancer Prevention (NORCCAP)[104]. A recent meta-analysis similarly confirmed benefits of screening (endoscopy or stool-based screening) over an unscreened population in increasing detection and prognosis[105].

There are intuitive benefits of screening with serum anti-*p53* auto-antibody compared to CT, barium enemas and colonoscopy. The titre is not subject to tumour sampling error, is quicker, cheaper, easier and less traumatic, thus making it more repeatable in the general population. The auto-antibody titre itself is remarkably stable, showing no significant change when stored at room temperature for up to 7 d, or when stored at -80 ℃ for 3 years[81]. Repeated freeze-thaw cycles (up to 10 cycles) have minimal or no effect on serum levels as immunoglobulins are generally robust proteins[82]. Also, anti-*p53* auto-antibody appears to be independent of other conventional CRC tumour markers such as carcino-embryonic antigen (CEA) which means it could detect CRC in CEA-negative patients. The combined advantages of serum testing and the characteristics of anti-*p53* auto-antibody (serum stability, 95%-100% specificity, independent of current tumour markers), makes anti-*p53* auto-antibody a potentially valuable screening modality.

The role of *p53* in screening is promising its specificity for cancer, but this enthusiasm is tempered by a low sensitivity (20%-30%). It would thus be required as part of a panel of tumour markers. This panel could then be used to guide more invasive investigations such as colonoscopy. Combined serum immuno-testing for 6 markers (CEA, anti-*p53* auto-antibody, CYFRA 21-1, osteopontin, separase and ferritin) has been reported to have comparable sensitivity (> 80%) to faecal immuno-testing[106]. A similar tumour marker panel using CYFRA-21, CEA and anti-*p53* has been used in lung cancer also with 80% sensitivity[107]. Combined biomolecular and endoscopic strategies[108] are being investigated, and in conjunction with other new diagnostic non-invasive modalities (*e.g.*, CT-colonography)[109] may be able to further broaden the screening programmes for CRC and other cancers in the general population.

Anti-p53 auto-antibody and clinico-pathological parameters of CRC

p53 mutation is usually a late event in the adenoma-carcinoma sequence and hence anti-*p53* auto-antibody is unlikely to be present in early pre-invasive lesions where *p53* mutations have not occurred[110]. The largest study reports 1% (2/211) sero-positivity in adenomas which increased to 6% in carcinoma *in-situ*[89]. This 1% could be due to undetected microfoci of invasive cancer within adenoma or changes that predate microscopic detection. The increase prevalence of anti-*p53* auto-antibody to 6% in carcinoma *in situ* can be expected in these tumours which are at the end of the adenoma-carcinoma sequence with greater proportion of *p53* mutation. This would then suggest that anti-*p53* auto-antibody should increase with further growth (CRC stage) but this is not seen. Almost all studies reported no association between anti-*p53* auto-antibody and CRC stage (Tables 1 and 2). This was reported in the largest cross-sectional series, and confirmed by other long-term follow-up studies[89] (Table 2). Only a handful of studies have suggested an association between anti-*p53* and T-stage[88,111], selected nodal disease[89] and metastases[112]

Tumour depth

Two studies reported increased anti-*p53* in progression from Dukes' A to B, but not with progression from Dukes' B to C[88,111]. Lechpammer *et al*[88] reported 0% (0/28) anti-*p53* in Dukes' A which increased significantly to 9.6% (21/87) in Dukes' B, but did not increase further with Dukes C (8.6%, 19/105). Bielicki *et al*[111] similarly reported increased anti-*p53* auto-antibody from Dukes' A (0%) to Dukes' B (28% Dukes' B1, 22% Dukes' B2); but no increase in progression to Dukes C (19%). This suggests auto-antibody production is stimulated by early (Tis to T2) local invasion such as microvascular basement membrane invasion leading to antigen presentation; but not further progression. Further studies are required to

Table 3 Prevalence of anti-*p53* auto-antibody and carcino-embryonic antigen in studies reporting the presence of both tumour markers in colorectal cancer *n* (%)

	CEA	Anti-*p53*
Tang *et al*[89]	408/943 (43.3)	130/998 (13.0)
Shibata *et al*[96]	23/47 (48.9)	32/47 (68.0)
Bielicki *et al*[111]	46/148 (31.1)	29/148 (19.6)
Hammel *et al*[175]	20/54 (37.0)	14/54 (25.9)
Overall	497/1192 (41.7)	204/1247 (16.4)

CEA: Carcino-embryonic antigen.

understand the precise series of events in anti-*p53* auto-antibody production.

Nodal involvement

Anti-*p53* auto-antibody is produced in part due to response to *p53*-antigen presentation. Thus nodal involvement should also increase anti-*p53* auto-antibody production by increasing probabilities of antigen presentation to the humoral system. However, there is no correlation between anti-*p53* auto-antibody and nodal involvement in any of the studies (Table 2). Tang *et al*[89] suggested increased anti-*p53* with "advanced" nodal disease (N3: > 10 regional nodes or systemic nodal metastases) compared to N0-2 CRC in selected analysis. We re-classified the data into node "positive" and "negative" disease and found no difference in sero-positivity of 12% *vs* 14% with nodal involvement (calculation not shown).

Metastases

It would be expected that haematogenous cancer cell dissemination should invoke a further immune response but there has been no association between anti-*p53* auto-antibody status and metastatic disease except one study[112]. In this study, anti-*p53* auto-antibody had extremely high prevalence (56%, 14/25) in Stage Ⅳ disease, and unusually low prevalence in Stage Ⅰ-Ⅲ (9%, 4/45) leading to strong anti-*p53* bias towards Stage Ⅳ disease. This is the only study to report anti-*p53* auto-antibody association with stage and adverse prognosis which is discussed later (anti-*p53* auto-antibody in prognosis in CRC).

Summary of anti-p53 auto-antibody and CRC Stage

Anti-*p53* auto-antibody production is initially most likely to be produced in the final stages the adenoma-carcinoma sequence (in keeping with *p53* mutation being a relatively late event. It is likely that anti-*p53* auto-antibody production is no longer dependant on antigen-presentation, but rather now dependant on immune-recognition by (1) tumour factors *e.g.*, *p53* mutation type and conformation, presence of co-factors; and (2) patients' immune-specific factors such as MHC expression required for recognition. This response is not sufficiently consistent to justify a separate clinico-pathological parameter of its own. In the future, anti-*p53* auto-antibody may have some benefit in refining CRC stage if there is influence on prognosis or treatment, similar to k-RAS status in anti-EGFR and anti-VEGF therapy for CRC and liver metastases, or oestrogen- or progesterone-status in breast cancer.

Anti-p53 auto-antibody and carcino-embryonic antigen

CEA is the most common serum tumour marker used in CRC. It is a 180 kDa serum glycoprotein which is present at low levels in normal cells but over-expressed in adenocarcinoma, especially of the colon, rectum, breast and lung[113]. Pre-operative CEA presence has been associated with aggressive CRC and poor prognosis[114,115]. CEA has also been used as an adjunct in CRC screening, monitoring for disease recurrence following resection, or as part of tumour marker panel for metastases of unknown primary origin. CEA has high specificity (80%) with false elevations in smokers, inflammatory diseases, cirrhosis, obstructive jaundice, gastric ulcers, emphysema, diabetes and collagen vascular diseases[116-118].

CEA in isolation is not recommended for screening or detection of recurrence due to its variable sensitivity (30%-80%)[114,119]. CEA sensitivity can be modulated by changing the cut-off values for "positivity" but sensitivity has still remained low despite variations in the cut-off value used[120,121]. Despite this, The American Society of Surgical Oncology (ASCO) guidelines suggest serial CEA measurements every 3 mo in Stage Ⅱ/Ⅲ CRC for at least 3 years following diagnosis, and during treatment of metastatic disease[122].

Tumour markers used in conjunction with CEA could increase the efficacy of CRC screening in selected populations. Such tumour markers should be independent of CEA as to detect the CEA-negative CRC population and thus increase sensitivity of the tumour marker panel. The majority of studies have shown that anti-*p53* auto-antibody is independent of CEA (Table 3). The two studies which report a positive correlation had results inconsistent with other studies, with the first study having an unusually strong association between anti-*p53* auto-antibody and Stage Ⅳ disease (as discussed earlier)[112] and the second reporting the highest anti-*p53* auto-antibody frequency (68%) and used WB, not ELISA[96]. Methodological difference and sample bias are most likely responsible for the results observed.

In this review, information is compiled from all studies reporting CEA and anti-*p53* in Table 4. This shows that when used in isolation, anti-*p53* auto-antibody can CEA can detect CRC in 17% and 42% respectively. If both tumour markers are used, the sensitivity increases to 51% (as both markers are absent in 48.9%). This results in information/sensitivity gain of +9% (compared with CEA alone); and +34% (compared with anti-*p53* auto-antibody alone). The only other study to report "information gain" using anti-*p53* auto-antibody in CRC confirmed reported mean increased sensitivity of 16% with individual increased sensitivity from 55% to 71% in colon cancer and 78% to 83% in rectal cancer[123]. This report is consistent with our calculation using data from all other published anti-*p53* auto-antibody and CEA studies in CRC (Tables 3 and 4).

Table 4 Combined carcino-embryonic antigen and anti-*p53* auto-antibody rates from all studies reporting the presence of both markers (*n* = 1192) *n* (%)

	CEA normal	CEA elevated
Anti-*p53* ab present	112 (9.4)	90 (7.6)
Anti-*p53* ab absent	584 (48.9)	406 (34.1)

CEA: Carcino-embryonic antigen.

The clinical utility of this "information gain" requires examination. CRC has low prevalence in the general population and thus pick-up rates would remain low despite the use of both tumour markers. Both markers also have preponderance towards later stage CRC (as opposed to Stage I) which reduces impact of earlier detection and thus screening efficacy. It is thus likely that these 2 markers alone are insufficient and additional markers would be required, i.e panel of 6 tumour markers was used to form a panel with sensitivity similar to faecal occult testing in population screening[108]. In post-operative surveillance, small studies have demonstrated overall 4 tumour markers (CEA, TPA, CA19-9, CA72.4) panel sensitivity of 81% compared with 9%-45% using individual markers[124]. Hence, the optimal strategy would be to use other markers in addition anti-*p53* and CEA to select patients for investigations. The cost-effectiveness of these immunological-targeted strategies for general population screening, high risk population screening or post-operative surveillance requires further evaluation.

Anti-p53 Auto-antibody and monitoring for recurrence or metastases

Anti-*p53* auto-antibody may have its most promising role in post-operative monitoring for disease recurrence or distant metastases. Several, but not all, studies have demonstrated that anti-*p53* reflects tumour load, with increasing serum titres corresponding with disease recurrence/progression and decreased titres following surgery/chemotherapy[81,88,120]. Lechpammer *et al*[88] produced the most convincing series demonstrating clear decreases with post-surgery and during chemotherapy. More importantly, increases, especially during chemotherapy, predated clinical diagnosis of recurrence. Smaller subset analysis in other studies has also demonstrated fluctuations in serum titres with disease load. Similar fluctuations with resection and radiotherapy have been reported in oral, oesophageal, lung, ovarian and breast cancer[125-127].

In almost all cases, the anti-*p53* auto-antibody persists but at a much lower level. Only one study has reported complete absence of the anti-*p53* auto-antibody in a series of patients with superficial (mucosal and submucosal) CRC treated with endoscopic resection[98]. This may be because the early stage CRC had a smaller mutant *p53* load which may not have adequately stimulated the humoral system to produce a prolonged immune response following CRC removal. The other studies had more advanced CRC where there would have been prolonged antigen exposure to the humoral system[81,88,98,128].

This cost efficacy of serial anti-*p53* auto-antibody for surveillance must be considered in the light of only 20%-30% prevalence at presentation and subsequent sero-conversion (Table 5). Assuming 1% future sero-conversion and 3-monthly serum measurements for 3 years as per ASCO recommendations, this would result in 20-30 initial positives at diagnosis; and an additional 1 positive over the subsequent 3 years. This results in an initial yield 20-30 positives followed by only 1 positive out of 960 samples over next 3 years (remaining 80 patients × 4 samples per year × 3 years). We then consider this 1 positive sero-conversions out of 960 samples may not alter treatment as serum measurements may predate clinical evidence of disease, and treatment cannot be offered based anti-*p53* auto-antibody titres alone.

An alternative more cost-effective strategy of screening would be to screen all patients for anti-*p53* auto-antibody at diagnosis with further serial measurements only in patients sero-positive at diagnosis. Post-operative patients with rising titres could be selected for expedited investigations and thus increase diagnostic yield, compared to current blanket strategy of routine investigations for surveillance at fixed time intervals. Preliminary studies in small groups using tumour marker panel (CEA, TPA, CA19-9 and CA72.4) demonstrated 81% sensitivity for recurrence with mean lead times of 5.3 mo prior to radiological confirmation of recurrence[124]. A cost efficacy study would be required to ascertain the ability of anti-*p53* auto-antibody as part of a tumour marker panel to guide post-operative surveillance, improve resource allocation and prolong survival.

Anti-p53 auto-antibody and prognosis in CRC

p53 mutations have been associated with poor prognosis, possibly in part due to chemo-resistance against *p53*-dependant chemotherapy (*e.g.,* 5-fluorouracil) but reports of its prognostic significance are inconsistent[90,129]. As the anti-*p53* auto-antibody response has been associated with *p53* mutations and serum testing is easier than DNA sequencing, studies have focused on using anti-*p53* auto-antibody to predict prognosis. The majority of studies report that anti-*p53* auto-antibody response has no independent prognostic value. This was confirmed in the study with the longest follow-up which reported CRC stage, but not anti-*p53* auto-antibody, to be an independent prognostic marker in multivariate analysis[130], and also by the study with the second longest follow-up but larger sample size[85] (Table 6).

Four studies report an adverse prognostic significance but in 3 of these, the prognostic significance was in selective univariate analysis where anti-*p53* was associated with advanced stage, and prognostic significance was lost when stage was incorporated in multivariate analysis[112,131,132]. The fourth, and only study, to report anti-*p53* auto-antibody as an independent prognostic indicator in multivariate analysis strongly associated anti-*p53* auto-antibody with Dukes' D to an extent that median survival of anti-*p53* positive patients was extremely low (20 mo) compared to other studies reporting median survival up

Table 5 Anti-*p53* auto-antibody and sero-conversion in colorectal cancer

Ref.	Patients, method	Follow-up	Findings
Müller *et al*[123]	303 patients, 197 colon, 46 rectal	Median 6 mo	All cancers: 3.6% (11/303) sero(-)→ (+); 3.6% (11/303) sero(+)→ (-); Total 7.2% (22/303) sero-conversion. Colon cancer: 3% (4/137) sero(-)→ (+); 3.6% (5/137) sero(+)→ (-); Total 6.6% (9/137) sero-conversion. Rectal cancer: 6.5% (2/31) sero(-)→ (+); 3.2% (2/31) sero(+)→ (-); Total 12.9% (4/31) sero-conversion
Lechpammer *et al*[88]	Immunoblot 32 , ELISA (Oncogene, Research Products, Cambridge, United States)	Up to 20 wk; 8 patients-48 wk	Non-significant decrease at 4 wk (pre-first cycle chemo) and significant decrease at 12 wk post-surgery Significant decreases during chemotherapy and 2 patients with anti-*p53* increase at 12 wk (during chemotherapy) developed recurrence 8 patients with extended follow-up: 7/8 had decreased anti-*p53* with no recurrence. 1/8 anti-*p53* decrease post-surgery/chemotherapy but increased at 12 wk corresponding with liver metastases. Anti-*p53* fluctuates in response to tumour load but does not disappear. Anti-*p53* levels reflects tumour load even during chemotherapy
Takeda *et al*[174]	30 CUR A, 5 CUR B, 5 CUR C, anti-*p53* EIA, Pharmacell	Median 26 mo (13-144)	CUR A (n = 30): 28/30 sero(+)→(-) in 6 mo; 2 no sero-conversion: 1 recurrence CUR B (n = 5): 2 sero(+)→(-) no recurrence. 3 no sero-conversion, 2 had metastases CUR C: No sero-conversion Correlation between post-operative negative conversion and operative curability
Takeda *et al*[98]	17 mucosal/submucosal, ELISA (anti-*p53* EIA, Pharmacell, France)	Up to 2 years	94%, 16/17 sero(+)→(-) within 3 wk post-surgery No recurrences as early stage tumours and hence not able to comment on anti-*p53* and recurrence rates
Polge *et al*[128]	10, ELISA (Dianova, Hamburg, Germany)	Up to 6 mo	8 followed-up: 5/8 remained sero(+) post-operatively. All developed metastases 3/8 decreased anti-*p53* titres. No metastases or recurrence. Anti-*p53* titres decreased within 1 mo of surgery/chemotherapy but no sero-conversion to anti-*p53*(-)
Angelopoulou *et al*[81]	6, "In house" immunofluorometric assay	Up to 17 mo	Anti *p53* decreases with surgery/chemotherapy but persists at low levels Anti-*p53* increases with recurrence Anti-*p53* reflects tumour load more sensitively than CEA (n = 5) and in non-CEA producing tumour (n = 1)
Hammel *et al*[175]	12, "In house" ELISA	Up to 20 mo	Anti-*p53* in 5/8 patients decrease by > 25% within 1 mo. At 1 year, 3 with normal anti-*p53* levels and 3 with substantial decrease in anti-*p53* remain disease-free 2 patients with post-operative increased anti-*p53*: 1 developed recurrence and 1 developed metastases Anti-*p53* decreased again following surgery in both patients. CEA and CA19-9 were normal in both cases

Sero(-): Sero-negative; Sero(+): Sero-positive; CUR A: No residual tumour macroscopically; CUR B: No residual tumour but not as evaluable as CUR A; CUR C: Definite residual tumour; CEA: Carcino-embryonic antigen.

to 60 mo and 5-year survival > 50%[89,123,130]. Remarkably, anti-*p53* auto-antibody prognostic significance was even weaker than CA19-9, a pancreatic tumour marker considered unsuitable for pancreatic cancer screening by ASCO[112,122]. The results of this study are hard to credit. As such, anti-*p53* auto-antibody has no independent prognostic value.

CONCLUSION

The anti-*p53* auto-antibody response is the end-point of a complex multi-factorial humoral response to the accumulation of p53 protein which is a product mainly of *p53* gene mutation, but also mutation of *p53* regulators and non-mutative pathways. Anti-*p53* auto-antibody has low (13%-32%) sensitivity in CRC but is nearly 100% specific for malignancy. The auto-antibody frequency may increase with early local invasion or late nodal progression but is not sufficiently consistent to form a separate stage classification. There may be a promising future role of anti-*p53* auto-antibody in screening and monitoring for disease recurrence. The characteristics of the immunoglobulin and the benefits of serum testing provide a promising role in guiding the radiological and endoscopic screening of high risk populations in conjunction with other current tumour markers. The most promising future focus of anti-*p53* auto-antibody lies in being part of a bio-molecular panel of tumour markers to guide endoscopic and radiological screening in general population and high-risk population screening; and in post-operative

Table 6 Anti-p53 auto-antibody and prognosis in colorectal cancer

Ref.	*n* (%)	Follow-up	Findings
Suppiah *et al*[130]	20/92 (21.7)	Median 97 mo	No difference in overall survival (62 mo *vs* 60 mo) or disease-free survival (73 mo *vs* 82 mo)
Müller *et al*[123]	70/243 (28.8)	5-year trial protocol	No survival difference with anti-*p53* in CRC and other cancers. Trend towards decreased survival in anti-*p53* positive patients with HCC and breast carcinoma
Tang *et al*[89]	130/998 (13)	Recruitment 1995-2000	Anti-*p53* associated with decreased survival in univariate analysis but not multivariate analysis. Anti-*p53* associated with advanced nodal disease (Stage N2→N3) and metastases (M1)
Chang *et al*[85]	147/167 (28)	Median 36.3 mo (22-85)	*p53* mutation associated with poor differentiation and advanced stage. Multivariate analysis shows *p53* mutation most significant survival predictor, followed by CRC stage. No prognostic significance of p53 protein expression or anti-*p53*
Shiota *et al*[112]	18/71 (25)	Not stated	Anti-*p53* associated with shorter overall survival (20 mo *vs* 56 mo) but highly significant association with metastases (M1). Cox regression showed prognostic significance with liver metastases, TNM stage, Dukes stage, Ca19-9 and anti-*p53* (in that order)
Kressner *et al*[131]	59/184 (32.1)	Median 6 years	Anti-*p53* associated with decreased survival in univariate, but not multivariate analysis. Anti-*p53* is independent prognostic indicator in Dukes' A-C with curative surgery (*i.e.*, when metastases excluded)
Houbiers *et al*[132]	65/255 (25.5)	36 mo	Anti-*p53* associated with reduced overall (75% *vs* 88%) and disease-free survival (56% *vs* 64%) at 3 years in subgroup analysis of Dukes' A and B1. No difference in overall survival (61% *vs* 68%) or disease-free survival (51% *vs* 58%) when all stages included

CRC: Colorectal cancer; HCC: Hepatocellular carcinoma.

cancer surveillance to guide earlier detection of cancer and cancer-recurrence; and finally with more significant impact on cost-efficacy and survival.

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REVIEW

Update on small intestinal stem cells

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Abstract

Among somatic stem cells, those residing in the intestine represent a fascinating and poorly explored research field. Particularly, somatic stem cells reside in the small intestine at the level of the crypt base, in a constant balance between self-renewal and differentiation. Aim of the present review is to delve into the mechanisms that regulate the delicate equilibrium through which intestinal stem cells orchestrate intestinal architecture. To this aim, special focus will be addressed to identify the integrating signals from the surrounding niche, supporting a model whereby distinct cell populations facilitate homeostatic *vs* injury-induced regeneration.

Key words: Intestinal stem cells; Organoids; Intestinal regeneration; Lgr5; Niche

Core tip: Among somatic stem cells, those residing in the intestine represent a fascinating and poorly explored research field. Aim of the present review is to delve into the mechanisms that regulate the delicate equilibrium through which intestinal stem cells orchestrate intestinal architecture, integrating signals from the surrounding niche and supports a model whereby distinct cell populations facilitate homeostatic *vs* injury-induced regeneration.

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INTRODUCTION

In an adult organism, stem cells are characterized by their ability to generate multiple differentiated cell types while maintaining their capacity for long-term self-renewal[1,2]. These are generally known as "adult" or "somatic" stem cells, including all stem cells residing in adult organs, regardless of the age of the individual. These include mesenchymal stem cells[3-7], residing in the connective stroma of most organs, and haematopoietic stem cells[8,9] among the best known and characterized, that are already being tested in clinical trials[10-14].

The amazing renewal capacity of the intestinal epithelium[1] has made this organ an attractive site to study stem-cell regulation. The intestinal tract is anatomically subdivided into the small intestine and large intestine. The inner mucosal surface, composed by an absorptive and secretory epithelium, is folded into repeated units comprising finger-like invaginations (called crypts of Lieberkühn) associated with numerous protrusions (villi), which increase the surface area, allowing efficient absorption of nutrients from the bowel lumen[2].

In normal homeostasis, the specialized differentiated cell types that orchestrate the uptake of nutrients into the body, are routinely and rapidly turned over. In fact, the intestinal epithelium is the most rapidly self-renewing tissue in the human body, with a 3-5 d turnover rate[2]. It is widely accepted that this complex process is regulated, *via* a highly regulated process of self-renewal, by a population of multipotent stem cells, residing within the bottom of the crypt namely the intestinal stem cells (ISCs)[15-19].

The number and location of these cells are still debated. Clonal analysis has demonstrated the existence of multiple stem cells in each crypt[20], with an estimated number in the 4-6 cells per crypt range[21]. ISCs have the properties of self-renewing and generating rapidly dividing transit-amplifying (TA) daughter cells, *via* asymmetric cell division[22]. TA cells undergo rapid cell division and migrate upwards into the villus. During their migration, TA cells start differentiating and finally localize at the surface of the villus epithelium as either mature absorptive enterocytes, which represent the main cell type, or mucous secreting goblet cells, or hormone-producing enteroendocrine cells[22]. Upon completing their life cycle, TA die and are discarded into the lumen[23,24].

A distinct cell type, the Paneth cell, evades this upward migration program, completing the differentiation at the base of the crypt, where it start producing lysozyme, maintaining the sterile environment of the crypt, and regulating the stem cell compartment[25-27].

Converging evidence suggests the existence of two distinct populations of intestinal stem cells: one that remains quiescent for a long time and one that actively proliferate[28]. The actively dividing ISCs provide to the baseline regeneration, whereas quiescent stem cells represent a reserve subpopulation that copes to injuries. These two subpopulations are located in adjacent sites within the crypt and are probably maintained by specific signals from the surrounding niche. Nonetheless, the precise identity of the ISCs is still a matter of debate. Two alternative models are currently proposed in the literature: the label-retaining cells (LRC) + 4 model, which identifies the quiescent stem cells, and the crypt base columnar (CBC) cells model, which identifies the actively cycling stem cells.

According to the LRC+4 model, the ISCs should be located specifically at the +4 position from the bottom of the intestinal crypt region, precisely at the origin of the migratory epithelial cell column[29]. This prediction was supported by Potten *et al*[30], who showed that cells most commonly found in this position, are characteristically label-retaining and extremely sensitive to X- and γ-radiation, two features ascribed to stem cells. Furthermore, the expression of Bmi1, a gene thought to be involved in stem cell maintenance, was shown to be elevated in the +4 cells[31]. Alternatively, the CBC cell model is based on a series of electron microscopy studies on the crypts of the small intestine, showing slender, immature, cycling cells interspersed between Paneth cells at positions 1-4, hence termed crypt base columnar cells. To support the hypothesis of CBC as the ISCs, mutagenesis studies demonstrated that 90% of the crypts, that contained a mixed population of mutant cells of different epithelial lineages, also contained mutant CBC cells, indicating the CBC cells as the common source of these different lineages[32]. Further studies, based on targeted lineage tracing strategies, have definitively identified the CBC as the intestinal stem cells, and have revealed the strategy by which the balance between proliferation and differentiation is maintained[33]. Taken together, these studies suggested that ISCs operate within a complex and dynamic environment, in which stochastic cell loss is compensated by the proliferation of neighboring stem cells.

INTESTINAL STEM CELLS MARKERS

The crypt stem cells responsible for the renewal capacity of the intestinal epithelium, represent a minority of the whole intestinal population, therefore, their identification is extremely troublesome[14]. Indeed, until relatively recently, ISCs could be identified only by indirect measurements. The recent discovery of specific ISC markers has allowed their isolation and paved the way towards a clearer understanding of their biology and role in tissue homeostasis, repair, and cancer[14].

Among the various ISCs markers, the best characterized one is the leucine rich-repeat containing G-protein coupled receptor (Lgr5), a Wnt-target gene that expressed by the cycling crypt base columnar cells, interspersed between Paneth cells[22]. *Lgr5* encodes an orphan G-protein-coupled receptor, characterized by a large leucine-rich extracellular domain[34].

Barker and co-workers demonstrated that CBC cells are capable of long-term maintenance and support the epithelium self-renewal, using the lineage tracing technique (*i.e.*, introducing permanent genetic marker into candidate stem cell genes *in situ*, thus allowing the visualization of the modified stem cells and their progeny over time)[34]. One of the major advantages of *in vivo* lineage tracing, compared to transplantation-based methods, is the lack of a physical manipulation of the candidate stem cell, so that ISCs are studied in their physiological niche[34]. In particular, the visualization and isolation of putative ISCs was obtained by targeting the *Lgr5/Gpr49* gene locus by knock-in of a dual expression cassette encoding enhanced green fluorescent protein (EGFP) and CreERT2. This resulted in the tamoxifen-induced expression of EGFP in the Lgr5+ fraction, which allowed the *in vivo* lineage tracing when combined with inducible reporter strains such as R26RLacZ[34]. This study hence showed that Lgr5 expression was confined to CBCs, and that these cells give rise to the variety of epithelial cells present in crypts, proving that CBCs function as ISCs as well[34].

In addition, it has been demonstrated that Lgr5+ cells form self-renewing epithelial organoids in *ex vivo* culture assays, resembling the *in vivo* structure and composition

of crypt/villus epithelial units. It is also worth noticing that cells expressing low, if any, Lgr5 were unable to produce organoids[35]. Gene expression analysis of purified Lgr5+ stem cells, indicated that they express additional specific markers, such as *Olfm4* and the Achaete scutelike 2 (*Ascl2*)[36]. Ascl2 is a basic helix-loop-helix transcription factor[19]; its expression in the intestinal epithelium is regulated by the Wnt pathway and is restricted to Lgr5+ stem cells. Ascl2 deletion results in the complete loss of Lgr5+ ISCs, whereas transgenic *Ascl2* expression induces crypt hyperplasia[36].

Moreover, several lines of evidence have demonstrated the existence of another putative intestinal stem cells marker, the Polycomb family member Bmi1[31]. The Polycomb family plays a key role in the development and in the regulation of progenitor self-renewal in several tissues, including the nervous system[37], the retina[38] and hematopoietic organs[39]. *In vivo* lineage tracing technique, showed that Bmi1+ cells are mainly located at the +4 position in the crypts of the small intestine, contributing to the long-term maintenance of all its epithelial cell types[31]. In addition, the selective ablation of the Bmi1+ population led to a disorganization of the intestinal mucosa, resulting in the absence of the crypts[31]. Unexpectedly, Bmi1 transgenic expression was restricted only to a minority of the crypts in the proximal small intestine, while being completely absent in the distal tract[31]. This could be possibly due to the existence of Bmi1-negative ISC populations in other regions of the intestine, or, perhaps, to an inaccurate reporting of endogenous Bmi1 expression, as a result of the variegated transgene activity, frequently observed in the intestine. Interestingly, microarray analysis showed that sorted Lgr5+ cells express Bmi1, raising the question whether the two markers really characterize independent stem cells populations. Yan *et al*[40] clarified this issue by demonstrating that Bmi1 and Lgr5 mark two functionally distinct ISCs *in vivo*. Lgr5 identifies actively cycling ISCs that are sensitive to Wnt modulation, involved in homeostatic regeneration and markedly ablated by irradiation, *i.e.*, the CBC cells. Conversely, Bmi1 is expressed by quiescent ISCs insensitive to Wnt modulations, that contribute partly to homeostatic regeneration, and are resistant to radiation injury, namely the LRC stem cells[40].

Another interesting molecule in the scenario of ISCs putative markers, is the CD133/Prominin 1(Prom1), originally discovered as novel glycoprotein expressed on neural[41] and hematopoietic stem cells[42,43]. More recently, CD133 has been described as marker of epithelial stem/progenitor cells in human kidney tubules[44] and in the prostate[45]. In the study by Zhu *et al*[46], a knock-in allele was used that integrated a CreERT2-IRES-nLacZ cassette at the first ATG codon of Prom1 (Prom1C-L). This allowed demonstrating a wide expression pattern for Prom1 in the colon; on the other hand, the expression in the small intestine, appeared to be restricted to the crypt base, overlapping with the Lgr5+ CBC cells[46] The Prom1+ cells were self-renewing, multipotent adult stem cells. By contrast with these findings, Snippert *et al*[47] reported that Prom 1 expression occurred in Lgr5+ stem cells as well as in their TA progenitors. A possible explanation for this discrepancy may reside in the different sensitivity of detection methods used in the two studies.

The RNA-binding protein Musashi 1 (Msi1), a regulator of asymmetric cell division[48], is also involved in stem cell maintenance[49,50]. Particularly, in neural stem cells Msi1 is able to maintain stemness properties through Notch pathway activation[51]. Independent immunoistochemical and *in situ* hybridization analyses, demonstrated that, Msi1 is expressed in the CBC cells immediately above the Paneth cells[52-54].

Moreover, Msi1 overexpression in the intestine increases both Wnt and Notch pathways, and induces the upregulation of Lgr5 and Bmi1[55]. Interestingly, although Msi1 is expressed in putative ISCs, in knockout mice lacking this marker, no defects in the development of the intestine are detected[56]. Taken together, these observations demonstrated that Msi1 is not a specific ISCs marker, but is expressed in both ISCs and in their early progeny[57].

To sum up, different markers point to distinct stem cells within the crypt: the marker Lgr5 points to the crypt base columnar cells located in between the Paneth cells at the crypt bottom[22], whereas the markers BMI1 identify the +4 position in the crypt, just above the Paneth cells[31]. The existence and interdependency of these different types of ISCs remain a matter of debate.

STEM CELL NICHE: HOMEOSTASIS AND MORPHOGENESIS SIGNALS

A key role in the dynamics of ISCs is ascribed to the niche, a complex and dynamic setting, that adapts in response to environmental stimuli and provides the cells essential signals, including the morphogenetic pathways, such as Wnt, Notch, bone morphogenetic proteins (BMPs) and Hedgehog[21,58-63]. The microenvironment of the niche surrounding ISCs features extracellular matrix, neural cells, lymphocytes, macrophages, endothelial cells, fibroblasts, smooth muscle cells, and myofibroblasts, that generate signals able to regulate stem cells properties and behavior[64-67].

A wide range of evidence indicates that the Wnt pathway has a crucial role in intestinal proliferation and ISC maintenance[68-74].

The Wnt pathway molecules are evolutionary conserved intracellular signaling molecules which regulate cellular fate in the crypt-villus axis in normal gut epithelium, and are implicated in stem cells self-renewal[75]. Indeed, loss of Wnt signaling *in vivo* effectively blocks cell proliferation in the intestinal crypts, destroying the epithelium[76]. Moreover, when the Wnt secretion inhibitor (IWP1) was added to organoids, the LacZ signal derived from Lgr5+ cells, was completely lost, and the proliferation was inhibited; this inhibition could be overcome administrating exogenous Wnt3A[76]. Recent evidences have

also demonstrated that Paneth cells residing next to ISCs are crucial for their maintenance and serve as the stem cell niche[77]. Paneth cells are known to secrete a variety of bactericidal products, such as cryptidins/defensins and lysozyme, epidermal growth factor (EGF), transforming growth factor β (TGF-β), and represent the main sources of Wnt3a. Indeed, the ablation of Paneth cells, decreases the number of ISCs in the crypt[78] confirming that an active Wnt signal is crucial for ISC maintenance[79].

A Wnt signaling gradient exists along the crypt-villus axis. When cells migrate away from the Wnt source at the base of the crypt, they progressively lose their proliferative capacity and differentiate. The activity of the Wnt pathway, in conjunction with other pathways such as Notch and bone morphogenetic protein (BMP), is vital for the proper organization of the colic epithelium.

In the small intestine, Notch activity determines lineage differentiation between enterocytes and secretory cell differentiation; indeed, Notch inhibition results in an increase of goblet cells, while its activation results in goblet cells depletion[78]. Recent data support the idea that Notch promotes proliferation when Wnt activity is high, while induces enterocyte differentiation when Wnt activity decreases at the top of the crypt[29]. Given that Notch receptors are membrane-bound, it would appear that only the neighboring Paneth cells can maintain active Notch signaling in Lgr5 stem cells.

BMP belongs to a family of ligands which comprises BMP and TGF-β family members, acting through the SMAD intracellular signaling cascade[80-82].

In the intestine, BMP2 and BMP3, are expressed by mesenchymal cells and are able to arrest proliferation at the crypt-villus edge, rather than promoting differentiation[29].

In fact, both mice lacking the BMP receptor (Bmpr1a), and mice overexpressing the BMP inhibitor noggin, present hyperproliferation and crypt fission[80].

Under physiological conditions, the amount of stem cells within the niche remains constant, thus these processes need to be highly regulated, probably through negative feedback mechanisms[40]. In fact, stem cells may divide: (1) asymmetrically, giving rise to another stem cell, which remains in the niche, and to a daughter cell which form a progenitor cell, that migrates upwards in the crypt and differentiate into a mature element; and (2) symmetrically, giving rise to two daughter stem cells, or two daughter non-stem progenitor cells, the latter phenomenon leading to stem cells exhaustion[75].

Overall, the current scenario indicates a niche organized into a complex network of morphogenetic signals, each crucial for ISCs and crypt maintenance[28].

STEM CELLS DERIVED ORGANOIDS: THERAPEUTIC APPLICATIONS FOR GASTROINTESTINAL DISEASES

Rapid progress in the field of intestinal stem cell biology now lends promise to the application of adult stem cell therapy in gastroenterology. The apparently unlimited scale at which these stem cells can be expanded *in vitro* offers particularly exciting therapeutic possibilities[83-85].

In particular, organoids, derived from *in vitro* expansion of a single adult colonic stem cell, can be used to repair damaged colon tissue.

Indeed, as discussed above, intestinal organoid cultures, with a gut like structure, and containing all epithelial cell types, can be derived from single Lgr5+ sorted stem cells[86]; the resulting organoids can be expanded efficiently and over long periods of time, without losing tissue identity. To date, protocols for the efficient generation of organoids from stomach, human small bowel and colon have been developed[87-90].

Interestingly, the growth factors used to supplement the culture medium are the natural growth factors to which the stem cells are exposed *in vivo*, suggesting a high clinical-grade biocompatibility of this approach[87]. Moreover, no genetic manipulation required, making the entire procedure extremely safe.

As a first step toward the development of stem cell transplantation, it has been shown that, using the colonic organoids culture system, significant amounts of tissue can be grown *in vitro* from a single adult colon stem cell[91,92].

Colon organoids were reintroduced into superficially damaged recipient colons of immunocompromised ($rag2^{-/-}$) mice, pretreated with dextran sulphate sodium (DSS), which induces superficial mucosal lesions. The engrafted organoids RFP+, were able to readily integrate into the existing epithelium (RFP-), and generated histologically and functionally normal crypts containing all differentiated cell types, covering the area that lacked epithelium in recipient mice. At 4 wk after transplantation, the donor-derived cells constituted a single-layered epithelium, which formed self-renewing crypts that were functionally and histologically normal. In long term studies, carried out at 25 wk after transplantation, the grafts still contributed to the epithelium without any sign of adenomatous or dysplastic transformation[91,93].

Moreover, transplanted mice displayed a significant lower weight loss than control mice[91]. These data showed the feasibility of colon stem-cell therapy based on the *in vitro* expansion of a single adult colonic stem cell; graft rejection can be managed by standard approaches, *i.e.*, by leukocyte antigens matching of donor and acceptor and by immunosuppressive therapy, as currently used for organ transplantation.

Protocols have also been developed to expand human small intestine and colon organoids from small biopsies[94]. As a first application, Cleavers and his collaborators have transplanted the organoid-derived small intestinal epithelium into the bowel of patients affected by the microvillus inclusion disease[48]. This is a rare hereditary defect of the enterocyte brush border resulting in insufficient nutrients' assimilation[95-97], requiring colon transplantation as the unique therapeutic strategy. Intes-

tinal organoids technology may allow a novel venue into gene therapy approaches, that involves the introduction of DNA sequences into the genomes of cells of the pertinent patient. The achievement of a safe gene transfer represents the major hurdle, which has largely hampered the introduction of gene therapy into the clinic despite three decades of intensive efforts. On this regard, retrovirus- and lentivirus-mediated gene transfer has already been proven to be feasible in organoids systems[98,99].

These viral vectors are though associated to documented risk for insertional mutagenesis. As organoids can be grown from single sorted stem cells, one could envisage an approach in which individual stem cells are analyzed after integration of the recombinant DNA sequences. Only stem cells with safe integrations could then be expanded clonally for subsequent transplantation.

Overall, adult stem-cell therapy holds promise for the treatment of gastrointestinal diseases, using tissues "harvested" from a single living donor, overcoming the difficulties of the organ transplantation, that is still limited by the availability of donor.

Clinical application of this protocol still waits the translation of the technical procedures to the good clinical practice standards, to generate the adequate amounts of tissue to treat human subjects, and the development of efficient transplantation approaches.

CONCLUSION

ISCs could be reasonably considered the key players that orchestrate the high-rate regenerative capacity of the intestinal epithelium. The understanding of the interplay between the ISCs and their niche, led by a complex molecular network, will pave the way for the future development ISCs based therapy especially to the application in gastroenterology.

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ORIGINAL ARTICLE

Hepatic arterial infusion chemotherapy in hepatocellular carcinoma with portal vein tumor thrombosis

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Abstract

AIM: To evaluate the prognostic factors and efficacy of hepatic arterial infusion chemotherapy in hepatocellular carcinoma with portal vein tumor thrombosis.

METHODS: Fifty hepatocellular carcinoma (HCC) patients with portal vein tumor thrombosis (PVTT) were treated using hepatic arterial infusion chemotherapy (HAIC) *via* a subcutaneously implanted port. The epirubicin-cisplatin-5-fluorouracil (ECF) chemotherapeutic regimen consisted of 35 mg/m^2 epirubicin on day 1, 60 mg/m^2 cisplatin for 2 h on day 2, and 500 mg/m^2 5-fluorouracil for 5 h on days 1-3. The treatments were repeated every 3 or 4 wk.

RESULTS: Three (6%) of the 50 patients achieved a complete response (CR), 13 (26%) showed partial responses (PR), and 22 (44%) had stable disease (SD). The median survival and time to progression were 7 and 2 mo, respectively. After 2 cycles of HAIC, CR was achieved in 1 patient (2%), PR in 10 patients (20%) and SD in 26 patients (52%). Significant pre-treatment prognostic factors were a tumor volume of < 400 cm^3 (P = 0.01) and normal levels of protein induced by vitamin K absence or antagonist (PIVKA)-Ⅱ (P = 0.022). After 2 cycles of treatment, disease control (CR + PR + SD) (P = 0.001), PVTT response (P = 0.003) and α-fetoprotein reduction of over 50% (P = 0.02) were independent factors for survival. Objective response (CR + PR), disease control, PVTT response, and combination therapy during the HAIC were also significant prognostic factors. Adverse events were tolerable and successfully managed.

CONCLUSION: HAIC may be an effective treatment modality for advanced HCC with PVTT in patients with tumors < 400 cm^3 and good prognostic factors.

Key words: Hepatocellular carcinoma; Hepatic arterial infusion chemotherapy; Portal vein tumor thrombosis

Core tip: The aim of this study was to investigate the prognostic factors of hepatic arterial infusion chemotherapy in advanced hepatocellular carcinoma patients with portal vein tumor thrombosis. The primary findings of this study were as follows: (1) The median survival and time to progression were 7 and 2 mo, respectively; (2) A tumor volume of < 400 cm^3 and protein induced by vitamin K absence or antagonist-Ⅱ were independent pre-treatment prognostic factors; (3) Disease control and ⩾ 50% tumor marker reduction were significant prognostic factors after the second cycle of hepatic arterial infusion chemotherapy (HAIC); and (4) Objective tumor response, disease control and portal vein tumor thrombosis response were independent post-treatment prognostic factors at the end of the HAIC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer globally and the third most common cause of cancer mortality[1]. Surveillance of high-risk patients facilitates the early diagnosis of HCC[2]. However, because many patients are diagnosed at intermediate or advanced stages, only 30% of patients benefit from curative therapies such as resection, transplantation, or percutaneous ablation[3]. For patients with vascular invasion and/or extrahepatic spread, *i.e.,* whose tumors were classified as advanced stage according to the Barcelona Clinic Liver Cancer (BCLC) staging system, the multi-kinase inhibitor sorafenib is recommended[4]. In recent randomized controlled trials, sorafenib significantly increased patient survival[5,6]. However, contrary to our expectations, the survival and therapeutic advantages of sorafenib are modest, and the current cost of the drug precludes sorafenib from becoming a more generalized treatment tool for advanced HCC[7]. Systemic chemotherapy also has limited utility in treating HCC due to frequent toxicity and is not associated with improved survival[8,9]. Therefore, alternatives to sorafenib and systemic chemotherapy are often required for the treatment of advanced HCC, and hepatic arterial infusion chemotherapy (HAIC) could be an alternative modality.

HAIC using an implantable port system is theoretically more effective against HCC than systemic chemotherapy. HAIC enables anti-cancer agents to be delivered locally at high concentrations to hypervascular tumors, thereby keeping systemic concentrations of chemotherapeutic agents low due to the first-pass effect[10]. Many studies using HAIC have reported that it is a useful modality for patients with advanced HCC[10-14]. However, there are limited data defining the clinical factors predicting its efficacy. In this study, we investigated the efficacy and predictive factors of HAIC in patients with advanced HCC with portal vein tumor thrombus (PVTT) using the HAIC regimen, which was composed of epirubicin, cisplatin, and 5-fluorouracil.

MATERIALS AND METHODS

Patients

Between March 2009 and January 2012, 68 consecutive patients with advanced HCC underwent HAIC *via* an implantable port system with epirubicin, cisplatin and 5-fluorouracil (5-FU) in Seoul St. Mary Hospital, Seoul, South Korea. The patients were refractory to previous treatments or not amenable to surgery or locoregional therapies such as ethanol injection, radiofrequency ablation, or transcatheter arterial chemoembolization due to metastasis or PVTT. HCC was diagnosed either histologically or using typical radiologic findings of HCC on two dynamic imaging examinations or one dynamic technique with an elevated serum α-fetoprotein (AFP) level ≥ 200 ng/mL[15,16]. Among these 68 patients, 50 patients who had PVTT and received more than two cycles of HAIC were enrolled in this study. All tumor thromboses were radiologically confirmed in the main trunk or in the first or second branch of the portal vein. Additional inclusion criteria were a white blood cell count ≥ 3000 cells/mm^3 or an absolute neutrophil count ≥ 1000 cells/mm^3 and a platelet count ≥ 50000 cells/mm^3. Other eligibility criteria included the following: ages 18-75 years, Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 1, and a Child-Pugh score ≤ 7. Patients with extrahepatic metastasis were also included in this study because extrahepatic metastasis is common in HCC patients with a large tumor and PVTT due to high AFP levels and large tumor volumes. Exclusion criteria included another concurrent malignancy or other underlying serious medical condition such as renal or cardiopulmonary insufficiency. HCC was staged using the BCLC, modified Union for International Cancer Control (UICC) and American Joint Committee on Cancer (AJCC) staging systems. Tumor volume was measured by a single radiologist using commercially available imaging software (Pinnacle^3 with AcQSim^3 v. 8.0, Philips, Fitchburg, WI, United States) and the summation-of-areas technique with a 5-mm reconstruction thickness.

Implantation of the arterial port system

After skin preparation and local anesthetic injection, the right common femoral artery was punctured using the Seldinger technique. The superior mesenteric and celiac arteries were selected under fluoroscopic guidance. After the selective angiographies were performed, the right gastric and gastroduodenal arteries were embolized with multiple microcoils to prevent reflux of the cytotoxic drug to the stomach and duodenum. After performing a follow-up celiac arteriography, a catheter was inserted and localized to the proper hepatic artery. The skin and right inguinal region was incised, and the subcutaneous pocket was prepared *via* dissection. The peripheral end of the catheter was connected to the infusion port, and the port device was implanted in a subcutaneous pocket in the right or left iliac fossa. To prevent the occlusion of the catheter, 10 mL of saline mixed with 10000 units of heparin were locked into the port after each cycle of chemotherapy. Hepatic angiography *via* the port system was performed every two cycles of treatment.

Chemotherapeutic regimen and additional therapy

The Epirubicin-Cisplatin-5-fluorouracil (ECF) chemotherapeutic regimen included 35 mg/m^2 epirubicin on day 1, 60 mg/m^2 cisplatin for 2 h on day 2, and 500 mg/m^2 5-FU for 5 h on days 1-3. Intravenous hydration was

performed prior to cisplatin infusion to prevent nephrotoxicity, and all patients were given prophylactic antiemetic treatment comprised of 5-hydroxytryptamine-3 antagonists. The treatment cycles were repeated every 3 or 4 wk until disease progression, unacceptable toxicity, or patient refusal to continue. The doses of chemotherapeutic agents or treatment intervals were adjusted at every treatment cycle depending on hepatic dysfunction or significant toxicity. The dose of subsequent treatment was reduced by 25% when repeated grade 2 or grade 3/4 toxicity occurred during the preceding cycle[14].

During or after the HAIC treatment, additional therapies were performed as necessary, depending on the tumor responses to HAIC, performance status, and hepatic function. Additional treatment included targeted therapy with sorafenib, external radiation therapy, transarterial chemolipiodolization (TACL), systemic chemotherapy, local therapies such as radiofrequency ablation (RFA) or percutaneous ethanol injection (PEI), or surgical treatment.

Study assessment

The primary endpoints were an objective response rate [complete response (CR) + partial response (PR)] and disease control rate [objective response rate + stable disease (SD)]. Response evaluations were performed after two cycles and at the end of the HAIC treatment. The overall survival (OS) and time to progression (TTP) were evaluated secondarily. The treatment response was classified according to the modified Response Evaluation Criteria In Solid Tumors (mRECIST). The pretreatment evaluation included medical history, physical examination, laboratory tests [complete blood count, blood chemistry, virologic marker, serum AFP and proteins induced by vitamin K absence or antagonist (PIVKA)-Ⅱ], and imaging studies such as a computed tomography (CT) scan, magnetic resonance imaging (MRI) or positron emission tomography (PET) scan. During the treatment, toxicity assessment, laboratory tests, as well as chest and abdominal X-rays were repeated prior to each treatment cycle. CT scans were performed every two cycles or as needed to evaluate the tumor response or to confirm the disease progression. OS was defined as the time from the first treatment to death or the last follow-up visit, and TTP was the time from the first treatment to the radiologic progression. The patient's liver function was classified according to the scheme of Child-Pugh. AFP and PIVKA-Ⅱ reduction were calculated according to the formula [(baseline level-level after the second cycle)/(baseline level) × 100] in patients whose AFP was elevated above 20 ng/mL[17] and PIVKA-Ⅱ was elevated above 40 mAU/mL[18]. The treatment toxicity was assessed using the Common Terminology Criteria for Adverse Events (CTCAE) v. 4.0[19]. The PVTT response was evaluated using dynamic imaging. Response was defined as complete disappearance or at least a 30% decrease in the diameter of PVTT, and non-response was defined as any case that did not qualify for response.

Statistical analysis

The Kaplan-Meier methods and log-rank tests were used in the analysis of time-to-event variables, and a 95%CI for the median time to event were computed. Cox-proportional hazard regression models were used to determine the hazard ratios (HRs) of pre-treatment and post-treatment prognostic factors for survival. The variables with P values < 0.05 at univariate analysis were used as input variables in the multivariate model using the enter methods. In the multivariate analysis of post-treatment prognostic factors, hazard ratios were adjusted for the significant variables in the multivariate analysis of pre-treatment variables. For each covariate, the proportional hazard assumption was verified using a log minus log survival plot, and Cox-Snell residuals were used to evaluate the fit of the model. A plot of the estimated cumulative hazard rate versus Cox-Snell residuals followed a 45° line.

The χ^2-test or Fisher's exact test were used for the analysis of clinical characteristics and prognostic factors between the disease control group (CR + PR + SD) and the disease progression group [progressive disease (PD)]. Statistical significance was defined as a P value < 0.05. All data were analyzed using the SPSS v. 14.0 software (SPSS, Chicago, IL, United States).

RESULTS

Patients characteristics

The characteristics of the 50 study patients are shown in Table 1. The median age was 54 years (range, 37-74), and 78% of the patients were male. The most common etiology of underlying liver disease was chronic hepatitis B (78%), and 84% had a Child-Pugh classification of A. All patients were BCLC stage C due to PVTT and extrahepatic metastasis, and 30 patients (60%) had main PVTT (Vp4). Sixteen patients (32%) had extrahepatic metastasis at the initiation of HAIC. Twenty-four patients (48%) received previous treatment, and the most common previous treatment was TACL.

Treatment efficacy

The patients received a total of 289 cycles of HAIC with a median of five cycles (range 2-25 cycles). The response rates are shown in Table 2. After two cycles of HAIC, 11 patients (22%) showed an objective response, and 37 patients (74%) achieved successful disease control. Based on the best response during HAIC, the objective response rate was 32% and disease control rate was 76%.

In total, the median OS was 7 mo (95%CI: 5.5-8.5) and TTP was 2 mo (95%CI: 1.3-2.7), as shown in Figure 1. We assessed the OS according to the presence of objective response and disease control (Figure 2). The treatment responses were evaluated after the second cycle of HAIC and at the end of the HAIC treatment. Based on the best response during HAIC, the median OS was 24 mo (95%CI: 12.9-35.1) in the objective responder group, 5 mo (95%CI: 3.6-6.4) in the non-responder group (P < 0.001), 8 mo (95%CI: 2.6-13.4) in the disease control

Table 1 Baseline patient characteristics

Patient characteristics	Statistic
Age (yr)	54 (37-74)
Gender (male/female)	39/11
Etiology	
HBV/HCV/non-viral	39/6/5
Child-Pugh classification	
A5/A6/B7	17/25/8
Staging	
BCLC staging C	50
Modified UICC (Ⅲ/Ⅳa/Ⅳb)	9/31/10
Tumor type	
Nodular/massive/infiltrative	3/4/43
Portal vein thrombosis	
Vp2/Vp3/Vp4	7/13/30
Maximal tumor size (cm)	
< 10/≥ 10	26/24
Tumor volume (cm^3)[1]	492.7 (26.1-2746.6)
Extrahepatic metastases	16
Previous treatment	
TACL/TACL + RFA/TACL + ERT	19/3/2
Total bilirubin (mg/dL)[1]	0.87 (0.34-1.99)
PT (INR)[1]	1.14 (0.94-1.38)
ALT (IU/L)[1]	37 (15-345)
Platelet count (× 10^3/mL)[1]	133 (50-326)
AFP (ng/mL)[1]	3084.15 (7.94-426100)
PIVKA-Ⅱ (mAU/mL)[1]	1190 (16-12000)

[1]Expressed as the median (range). AFP: Alpha-fetoprotein; AJCC: American Joint Committee on Cancer; ALT: Alanine aminotransferase; BCLC: Barcelona Clinic Liver Cancer; ERT: External radiation therapy; HBV: Hepatitis B virus; HCV: Hepatitis C virus; PIVKA: Protein induced by vitamin K absence or antagonist; PT: Prothrombin time; RFA: Radiofrequency ablation; TACL: Transarterial chemolipiodolization; UICC: Union for International Cancer Control.

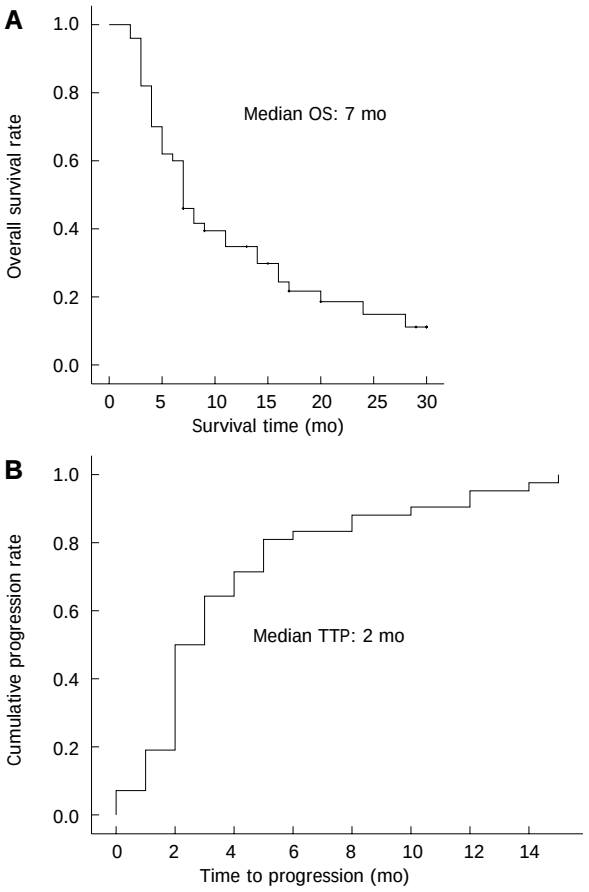

Figure 1 Overall survival rate (A) and time to disease progression (B) of the patients. OS: Overall survival; TTP: Time to disease progression.

Table 2 Tumor responses to hepatic arterial infusion chemotherapy treatment

	Response after two cycles	Best response during HAIC	Overall response after HAIC	Intra-hepatic tumor response
CR	2%	6%	6%	6%
PR	20%	26%	10%	26%
SD	52%	44%	12%	52%
PD	26%	24%	72%	16%
Objective response	22%	32%	16%	32%
Disease control rate	74%	76%	28%	84%

HAIC: Hepatic arterial infusion chemotherapy; CR: Complete response; PD: Progressive disease; PR: Partial response; SD: Stable disease.

group and 4 mo (95%CI: 2.9-5.1) in the progressive disease group ($P < 0.001$). Based on response after the second cycle of HAIC, the median OS was 17 mo (95%CI: 14.6-19.4) in the responder group and 7 mo (95%CI: 5.0-9.0) in the non-responder group ($P = 0.034$), 11 mo (95%CI: 6.7-15.3) in the disease control group and 5 mo (95%CI: 2.9-5.1) in the progressive disease group ($P < 0.001$).

Prognostic factors of survival

The prognostic factors affecting patient survival were analyzed by examining the pre-treatment and post-treatment parameters shown in Tables 3 and 4. Univariate analysis revealed that four pre-treatment prognostic factors were significantly associated with survival: tumor volume (< 400 cm^3), Child-Pugh score, pre-treatment PIVKA-Ⅱ level, and AJCC stage. Based on multivariate analysis, a tumor volume of < 400 cm^3 [$P = 0.01$, HR = 2.520 (95%CI: 1.252-5.072)] and PIVKA-Ⅱ [$P = 0.022$, HR = 3.121 (95%CI: 1.177-8.274)] were independent prognostic factors among the pre-treatment parameters. There was no significant difference in overall survival according to the presence of extrahepatic metastasis; the median OS was 8 mo (95%CI: 5.192-10.808) in the patients without extrahepatic metastasis and 5 mo (95%CI: 2.387-7.613) in the patients with extrahepatic metastasis ($P = 0.201$) (Figure 3).

The post-treatment parameters were analyzed after the second cycle and at the end of HAIC. Univariate analysis after the second cycle of HAIC determined that objective response, disease control, portal vein tumor thrombosis response, and ≥ 50% reduction of AFP and PIVKA-Ⅱ level were significant post-treatment prognostic factors. Multivariate analysis after the second cycle of HAIC determined that disease control [$P = 0.001$, HR = 3.850 (95%CI: 1.768-8.381)], PVTT response [$P = 0.003$, HR = 3.398 (95%CI: 1.529-7.552)] and ≥ 50% AFP reduction [$P = 0.02$, HR = 3.031 (95%CI: 1.194-7.691)] were significant predictors for longer survival. At the

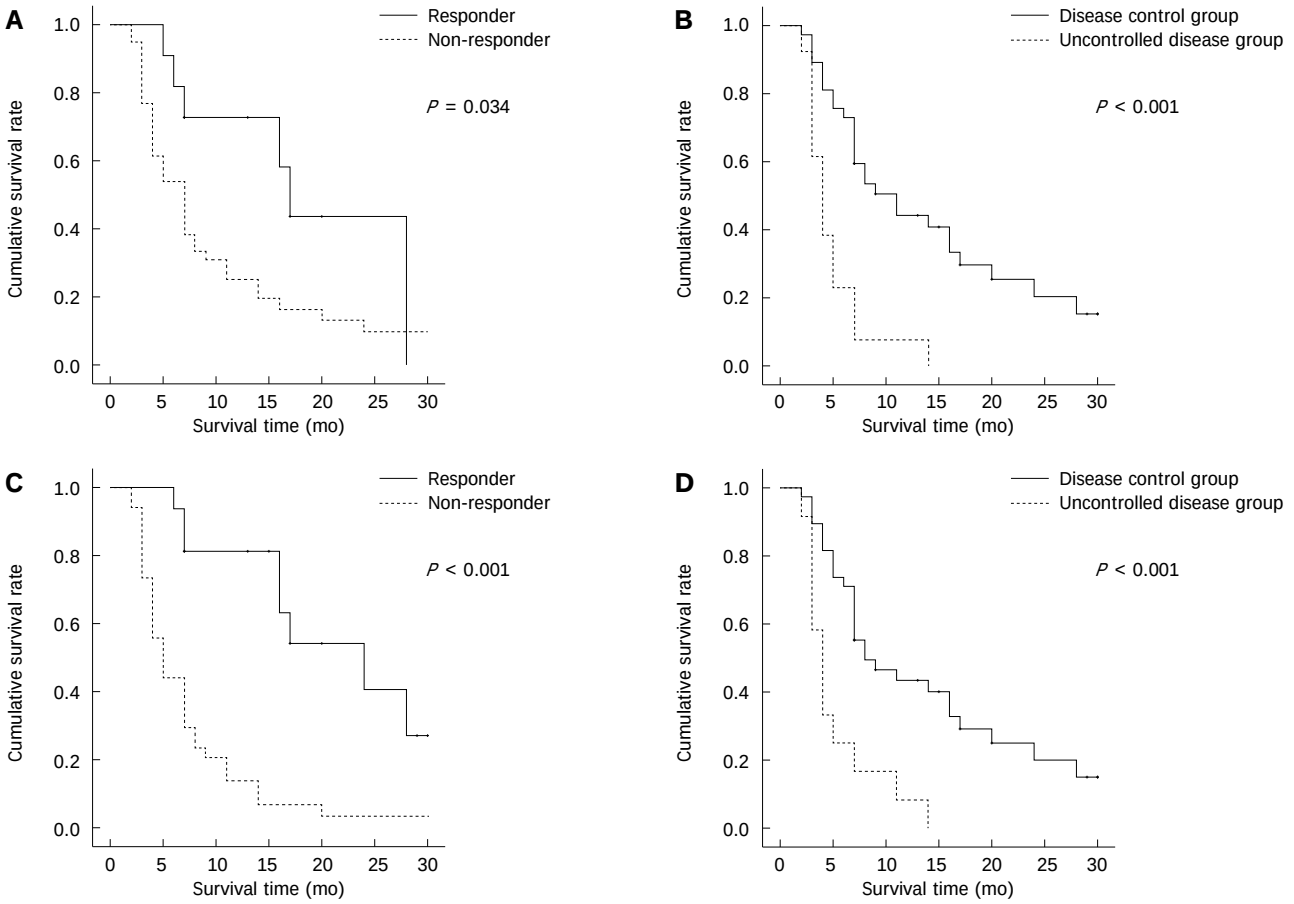

Figure 2 Overall survival of the objective response and disease control groups. A, B: After the second cycle of high-dose hepatic arterial infusion chemotherapy (HAIC); C, D: During HAIC.

Table 3 Pre-treatment prognostic factors for survival in hepatic arterial infusion chemotherapy treatment

Variables	Univariate HR (95%CI)	Multivariate[1] HR (95%CI)	*P*-value
Age (< 60/≥ 60 yr)	0.991 (0.514-1.912)		
Gender (male/female)	1.453 (0.687-3.076)		
Maximal tumor size (< 10/≥ 10 cm)	1.693 (0.894-3.204)		
Tumor volume (< 400/≥ 400 cm^3)	2.509 (1.289-4.885)	2.520 (1.252-5.072)	0.01
Child-Pugh score (5/> 5)	2.099 (1.038-4.244)	1.812 (0.878-3.738)	0.108
Stage			
mUICC stage (Ⅲ/Ⅳ)	1.198 (0.502-2.863)		
AJCC (Ⅲ/Ⅳ)	2.133 (1.085-4.193)	1.803 (0.895-3.634)	0.099
Extrahepatic metastases	1.508 (0.772-2.948)		
Pre-HAIC treatment	0.939 (0.503-1.755)		
Portal vein thrombosis (Vp4 *vs* non-Vp4)	1.603 (0.829-3.100)		
AFP level (< 200/≥ 200 ng/mL)	1.707 (0.845-3.448)		
PIVKA-II (< 40/≥ 40 mAU/mL)	2.860 (1.110-7.368)	3.121 (1.177-8.274)	0.022

[1]Those variables with $P < 0.05$ in univariate analysis were included. AFP: Alpha-fetoprotein; AJCC: American Joint Committee on Cancer; HAIC: Hepatic arterial infusion chemotherapy; PIVKA: Protein induced by vitamin K absence or antagonist; mUICC: Modified Union for International Cancer Control.

end of the HAIC treatment, univariate analysis was performed with combination therapy and factors related to tumor response. Six factors were significant: the overall tumor response (including objective response and disease control), intrahepatic tumor response (including objective response and disease control), PVTT response, and combination therapy. A multivariate analysis at the end of HAIC treatment determined that objective tumor response [$P = 0.001$, HR = 4.445 (95%CI: 1.893-10.439)], disease control [$P = 0.003$, HR = 3.137 (95%CI: 1.494-6.591)], objective intrahepatic tumor response [$P = 0.001$, HR = 4.445 (95%CI: 1.893-10.439)], intrahepatic tumor control [$P = 0.01$, HR = 3.009 (95%CI: 1.302-6.958)], PVTT response [$P = 0.001$, HR = 8.188 (95%CI: 2.403-27.898)], and combination therapy [$P = 0.029$, HR = 2.164 (95%CI: 1.082-4.328)] were independent predictors for longer survival.

Toxicity

The toxicities observed in this study are summarized in Table 5. No treatment-related mortality was detected. The most common toxicities were anemia and aspartate aminotransferase (AST) elevation, and all patients showed a toxicity grade of at least 1. The most common grade 3/4 toxicities were thrombocytopenia and AST elevation (22 patients, 44%). The toxicities were transient, tolerable,

Table 4 Post-treatment prognostic factors for survival in hepatic arterial infusion chemotherapy treatment

Variables		Univariate HR (95%CI)	Multivariate[1] Adjusted HR (95%CI)	*P*-value[2]
After 2nd HAIC cycle	Objective response			
	Responder	1		
	Non-responder	2.382 (0.995-5.704)		
	Disease control			
	Control group	1		
	Progressive group	3.708 (1.801-7.634)	3.850 (1.768-8.381)	0.001
	PVTT response			
	Response	1		
	Non-response	2.531 (1.164-5.505)	3.398 (1.529-7.552)	0.003
	AFP reduction			
	⩾ 50%	1		
	< 50%	3.242 (1.297-8.102)	3.031 (1.194-7.691)	0.02
	PIVKA-Ⅱ reduction			
	⩾ 50%	1		
	< 50%	3.164 (1.469-6.818)	2.254 (0.989-5.137)	0.053
Response during HAIC	Best tumor response			
	Objective response			
	Responder	1		
	Non-responder	4.747 (2.111-10.672)	4.445 (1.893-10.439)	0.001
	Tumor control			
	Control group	1		
	Progressive group	3.274 (1.594-6.724)	3.137 (1.494-6.591)	0.003
	Intra-hepatic tumor response			
	Objective response			
	Responder	1		
	Non-responder	4.747 (2.111-10.672)	4.445 (1.893-10.439)	0.001
	Tumor control			
	Control group	1		
	Progressive group	3.032 (1.348-6.821)	3.009 (1.302-6.958)	0.01
	PVTT response			
	Response	1		
	Non-response	9.587 (2.879-31.927)	8.188 (2.403-27.898)	0.001
	Combination therapy			
	Yes	1		
	No	2.367 (1.218-4.601)	2.164 (1.082-4.328)	0.029

[1]Adjusted for tumor volume and pre-treatment PIVKA-Ⅱ level; [2]*P* value for adjusted hazard ratio. AFP: Alpha-fetoprotein; HAIC: Hepatic arterial infusion chemotherapy; PIVKA: Protein induced by vitamin K absence or antagonist; PVTT: Portal vein tumor thrombosis.

and successfully managed using a conservative treatment; there were no discontinuation of the treatment due to toxicity. Hepatic arterial thrombosis developed in 4 of the 50 patients. However, thrombolysis by urokinase was effectively performed, and port removal was required in only 1 patient. Overall, 37 (74%) of the 50 patients in the treatment group died during the follow-up. The causes of death are listed in Table 6. The most common cause of death was tumor progression (57%), and six patients (16%) died from deteriorating hepatic function without any evidence of tumor progression, sepsis, or gastrointestinal bleeding. Five patients (14%) died from variceal bleeding, and three (8%) died from infection. Ten patients (20%) were still alive when the final analysis was performed, and three patients (6%) were lost to follow-up.

DISCUSSION

Portal vein tumor invasion is a common complication in HCC, reportedly observed in 64.7% of cases at autopsy[20]. PVTT often leads to extensive spreading of the tumor and can increase portal venous blood pressure, resulting in the fatal rupture of esophageal varices. PVTT can also decrease portal flow that may lead to ascites, jaundice, hepatic encephalopathy, or liver failure. Therefore, the presence of PVTT is one of the most significant prognostic factors of poor prognosis[21,22], and it has been reported that these patients survive only 2.7-4 mo if left untreated[22,23]. In advanced HCC patients with PVTT, standard treatments have not been established, especially in the Asia-Pacific region. Though the BCLC staging system recommends sorafenib in these patients, its efficacy is limited. Thus, HAIC is considered an alternative treatment modality, especially in Japan and South Korea.

In this study, we analyzed the response rate and overall survival of HAIC using the ECF regimen. In previous reports, the response rate and disease control rate of HAIC in advanced HCC patients with PVTT[11,24-26] were 33%-52% and 47%-77%, respectively. The median OS was 7-10 mo in those studies. The objective response

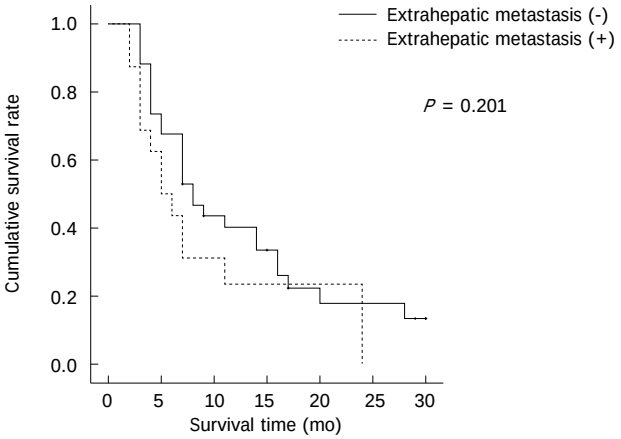

Figure 3 Cumulative survival rate according to the presence of extrahepatic metastasis.

Table 5 Adverse events related to treatment

	Grade 1	Grade 2	Grade 3	Grade 4
Hematological				
Leukopenia	26%	38%	8%	0%
Neutropenia	10%	26%	30%	12%
Anemia	26%	48%	26%	0%
Thrombocytopenia	30%	18%	44%	0%
Non-hematological				
Total bilirubin	30%	28%	10%	0%
AST	20%	36%	38%	6%
ALT	48%	12%	16%	2%

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

Table 6 Causes of death

	Patient number (n = 37)
Disease progression	21
Hepatic dysfunction	6
Variceal bleeding	5
Infection	3
Unknown	2

rate and disease control rate of our study, which are 32% and 76%, respectively, are in accordance with the above results. In addition, the median OS of 7 mo was similar to the results of previous reports. However, our study included patients with extrahepatic metastasis at the beginning of HAIC, whereas the previous studies excluded those patients. HAIC treatment is primarily used for the local control of liver tumors in patients with minimal extrahepatic spread[7]. Extrahepatic tumors would not respond well to HAIC. However, our data support the use of HAIC in HCC patients with extrahepatic metastasis because extrahepatic metastasis was not a significant factor in survival (Figure 3). The mortality in advanced HCC is related to intrahepatic tumors, and the leading cause of death in these patients is intrahepatic tumor progression[27,28]. In this study, all patients had advanced intrahepatic HCC with vascular invasion. Because the survival of these patients was influenced by intrahepatic tumor progression, extrahepatic metastasis may not influence overall survival. If the treatment response evaluation is confined to intrahepatic tumor lesions, the disease control rate was as high as 84%. These response and disease control rates are significantly higher than those with sorafenib treatment[5,29].

Several studies reported that the therapeutic effectiveness of HAIC was an important prognostic factor[10,11,30], which is consistent with our results. In the present study, the median OS of the disease control group after two cycles of HAIC was significantly longer than patients showing PD. In addition, patients with an objective response to HAIC treatment also had significantly longer survival than non-responders. These results indicate that the responses to HAIC were independent prognostic factors. In addition, this study showed that the response after the second cycle of HAIC also significantly influences survival; thus, patients with CR, PR or SD after the second cycle of HAIC could continue HAIC treatment and expect favorable results.

PIVKA-Ⅱ, also known as a des-gamma carboxy prothrombin (DCP), is an alternative tumor marker of AFP in diagnosing HCC. It is associated with aggressive features such as tumor size, vascular invasion, tumor stage and survival, and patients with high serum PIVKA-Ⅱ levels have a poor prognosis[31,32]. In the present study, the median OS in patients with a PIVKA-Ⅱ level ⩾ 40 and < 40 mAU/mL were 7 and 16 mo, respectively (Figure 4). Thus, patients with high serum PIVKA-Ⅱ levels prior to HAIC treatment would be expected to have a poorer prognosis. Some reports state that, independent of pre-treatment level, tumor marker response to treatment was associated with survival. Park *et al*[18] reported that AFP and DCP response were independent factors associated with survival. Personeni *et al*[17] reported that AFP response is an independent surrogate end point for survival in patients treated with sorafenib. Similarly, a ⩾ 50% decline in AFP and PIVKA-Ⅱ after the second cycle of HAIC treatment was associated with better outcomes among the patients with elevated AFP and PIVKA-Ⅱ levels prior to the initiation of HAIC treatment (though PIVKA-Ⅱ reduction is not statistically significant by multivariate analysis) (Figure 4). Therefore, tumor marker response (AFP and PIVKA-Ⅱ) after the second cycle may be a useful surrogate endpoint for good outcomes in those receiving HAIC treatment for large HCC with PVTT.

Many studies have shown that tumor size is a major determinant of survival[33,34]. However, few studies (with the exception of Hsu *et al*) have analyzed survival according to tumor volume[35]. In the current study, multivariate analysis of the pre-treatment parameters showed that a tumor volume < 400 cm^3 was an independent pre-treatment prognostic factor, while the maximal tumor diameter was not. Along with tumor volume, we evaluated the Child-Pugh score as a predictive factor of survival. Though not a significant multivariate variable, a Child-Pugh score of 5 was an important factor predicting good outcome (median OS of Child class A5, A6 and B were 16, 7 and 3 mo, respectively). This result is consistent

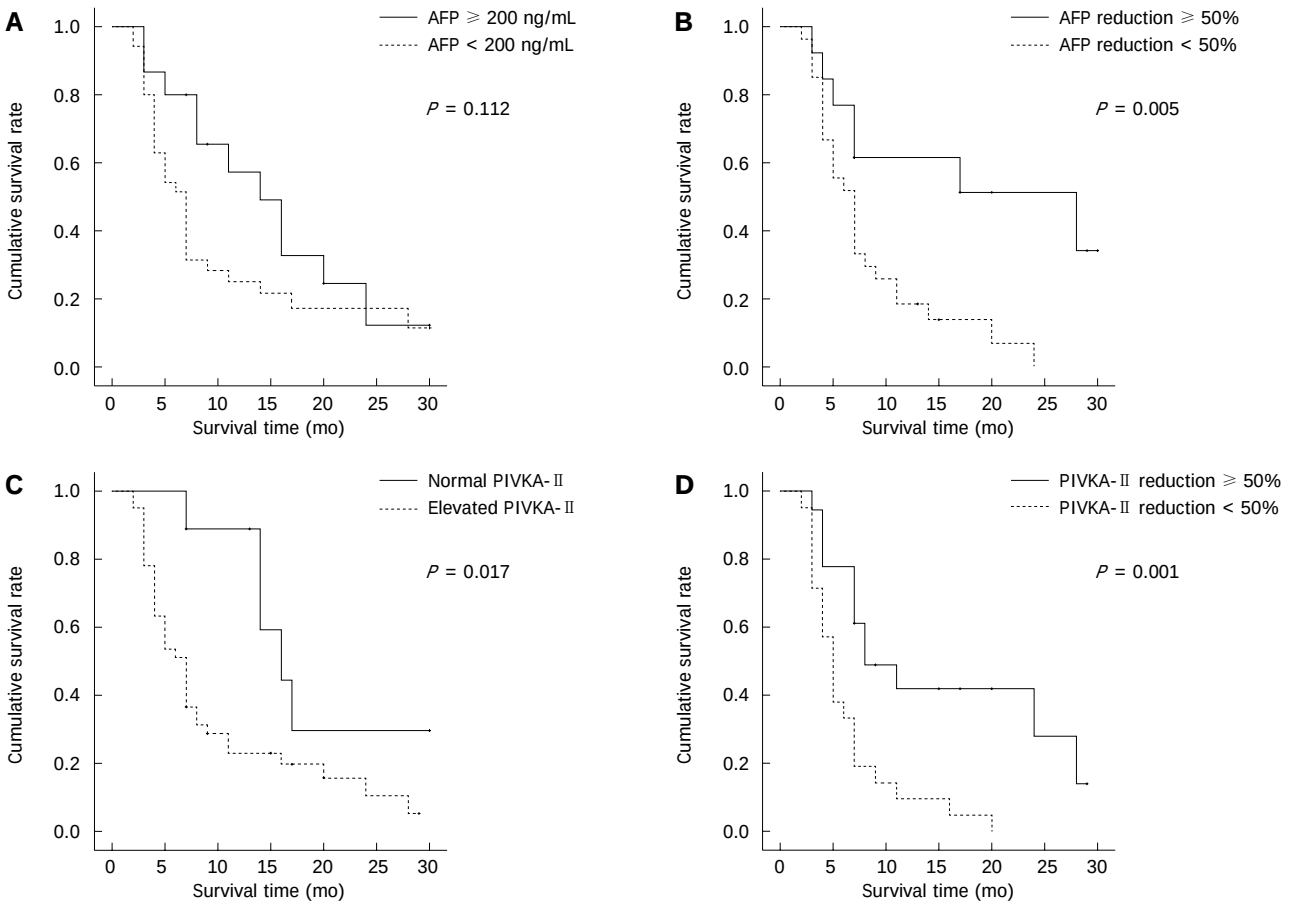

Figure 4 Cumulative survival rates. A: According to the pre-treatment α-fetoprotein (AFP) level; B: According to the AFP reduction after two cycles of hepatic arterial infusion chemotherapy (HAIC); C: According to the pre-treatment protein induced by vitamin K absence or antagonist (PIVKA)-Ⅱ level; D: According to the PIVKA-Ⅱ reduction after two cycles of HAIC.

with the results of HAIC studies showing that the Child-Pugh score was an independent survival factor[30,36]. Thus, HAIC using ECF could be indicated in selected patients with tumor volumes < 400 cm^3, good hepatic reserve function, and low PIVKA-Ⅱ levels.

Although many studies using HAIC have been performed over the last decade, the therapeutic regimen of this treatment has not been standardized. Ando *et al*[11] reported that HAIC using low-dose cisplatin and 5-FU demonstrated a good response rate and survival time in 48 patients with PVTT. Park *et al*[10] showed that repetitive HAIC with high-dose 5-FU and cisplatin given for 3 d was effective and safe. In Japan, interferon-combined HAIC is also commonly used. The ECF chemotherapeutic regimen in this study consisted of high dose cisplatin and 5-FU in combination with epirubicin. Woo *et al*[14] compared high-dose HAIC with low-dose HAIC and reported that high-dose HAIC was safe and achieved better tumor response compared with that of low-dose HAIC. The addition of epirubicin to the high-dose HAIC regimen resulted in more effective control of the intrahepatic tumor in our study.

However, the ECF regimen appears to be more toxic than in previous reports of high-dose HAIC[10,14]. While grade 3 hematologic or non-hematologic toxicities comprised less than 5% in previous studies, grade 3 leukopenia, anemia, and thrombocytopenia in this study comprised 8%, 26% and 44% of the toxicities, respectively, and hepatic toxicity was higher. In addition, hematologic toxicities tended to be more common in this study than in the trials using sorafenib such as the SHARP trial or an Asia-Pacific trial[5,29]. The more frequent grade 3/4 toxicities in this study may be due to our use of different inclusion criteria. The hematologic inclusion criteria were lower than those used in the previous study, and there were no inclusion criteria related to hepatic function such as AST, ALT and bilirubin. As a result, our study included more patients with lower blood cell counts or higher liver enzyme levels, which could lead to more toxic adverse events. However, all hematologic and hepatic toxicity returned to baseline levels within several days.

This study has some limitations. First, we included patients with extrahepatic metastasis even though HAIC is only considered effective for the treatment of intrahepatic tumors[7]. However, extrahepatic metastasis was not independently associated with survival, and the results were as good as those of previous studies despite the inclusion of patients with extrahepatic metastasis. Second, because most tumors were of the infiltrative type and margins were obscure, there was difficulty in accurately measuring tumor volume. Third, the retrospective nature of this study is underpowered due to a single arm regis-

try without a control group. In addition, this study may have inherent bias associated with a small sample size and heterogeneous treatments. Thus, large prospective studies are necessary to establish the efficacy of HAIC using the ECF regimen in patients with large tumors and PVTT.

In conclusion, HAIC with the ECF regimen may be a good option for advanced HCC with tumor volumes < 400 cm^3 and a normal PIVKA-Ⅱ level. In addition, this study suggests that response to chemotherapy after two cycles of HAIC, including radiologic tumor control and tumor marker reduction, is an independent predictor of longer survival. Therefore, HAIC treatment could be indicated in selected patients with favorable pre-treatment or post-treatment prognostic factors.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is a major health problem, accounting for more than 626000 new cases per year worldwide. Most HCC patients are diagnosed at an inoperable (intermediate or advanced) stage. The prognosis for advanced HCC is dismal. Sorafenib is the only approved drug for target therapy, and this drug is recommended for advanced HCC patients according to Barcelona Clinic Liver Cancer staging system. However, sorafenib shows only modest improvements in survival and tumor response.

Research frontiers

Hepatic arterial infusion chemotherapy (HAIC) has been used as a treatment modality for advanced HCC. Although sorafenib is regarded as the standard treatment of advanced HCC, HAIC can be an alternative treatment modality. The research hotspot in the area of HAIC treatment involves patients who will have good responses to HAIC.

Innovations and breakthroughs

Until now, the HAIC therapeutic regimen has not been standardized. This study is the first to use the epirubicin-cisplatin-5-fluorouracil (ECF) regimen, which consists of epirubicin, cisplatin, and 5-fluorouracil. HAIC treatment using the ECF regimen showed a good response, and its toxicity was tolerable. While previous studies on HAIC attempted to investigate the prognostic factors among the pre-treatment variables and the best response during treatment, this study revealed that the response after the second cycle of treatment, including radiologic response and tumor marker reduction, is also an important prognostic factor. The authors measured the actual tumor volume using imaging software and suggested a cut-off volume that could indicate a good response to HAIC.

Applications

Because the response after the second cycle is an important prognostic factor, those who showed a good response after the second cycle can continue the HAIC treatment and can expect favorable results.

Terminology

HAIC: HAIC is one type of arterial chemotherapy used in advanced hepatocellular carcinoma. HAIC requires an implantable port system for which a catheter is inserted and localized to the proper hepatic artery. HAIC enables the local delivery of high concentrations of anti-cancer agents to tumors, thereby maintaining a low systemic concentration of chemotherapeutic agent.

Peer review

This is a good article about the use of HAIC in the treatment of advanced HCC with portal vein tumor thrombosis. HAIC is shown to be an effective treatment modality.

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ORIGINAL ARTICLE

Garcinia Cambogia attenuates diet-induced adiposity but exacerbates hepatic collagen accumulation and inflammation

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Abstract

AIM: To investigate long-term effects of *Garcinia Cambogia* (GC), weight-loss supplement, on adiposity and non-alcoholic fatty liver disease in obese mice.

METHODS: Obesity-prone C57BL/6J mice were fed a high-fat diet (HFD, 45 kcal% fat) with or without GC (1%, w/w) for 16 wk. The HFD contained 45 kcal% fat, 20 kcal% protein and 35 kcal% carbohydrate. They were given free access to food and distilled water, and food consumption and body weight were measured daily and weekly, respectively. Data were expressed as the mean ± SE. Statistical analyses were performed using the statistical package for the social science software program. Student's *t* test was used to assess the differences between the groups. Statistical significance was considered at $P < 0.05$.

RESULTS: There were no significant changes in body weight and food intake between the groups. However, the supplementation of GC significantly lowered visceral fat accumulation and adipocyte size *via* inhibition of fatty acid synthase activity and its mRNA expression in visceral adipose tissue, along with enhanced enzymatic activity and gene expression involved in adipose fatty acid β-oxidation. Moreover, GC supplementation resulted in significant reductions in glucose intolerance and the plasma resistin level in the HFD-fed mice. However, we first demonstrated that it increased hepatic collagen accumulation, lipid peroxidation and mRNA levels of genes related to oxidative stress (superoxide dismutase and glutathione peroxidase) and inflammatory responses (tumor necrosis factor-α and monocyte chemoattractant protein-1) as well as plasma alanine transaminase and aspartate transaminase levels, although HFD-induced hepatic steatosis was not altered.

CONCLUSION: GC protects against HFD-induced obesity by modulating adipose fatty acid synthesis and β-oxidation but induces hepatic fibrosis, inflammation and oxidative stress.

Key words: *Garcinia Cambogia*; Anti-adiposity; Metabolic changes; Hepatic collagen accumulation; Hepatic inflammation; Hepatic oxidative stress

Core tip: *Garcinia Cambogia* (GC) is a popular dietary supplement for weight loss. However, little is known about the efficacy and hepatotoxicity of long-term GC supplementation in mice fed a high-fat diet (HFD). We observed that GC ameliorated HFD-induced adiposity by modulating enzymatic activity and gene expression involved in fatty acid metabolism. GC also reduced the plasma resistin level and glucose intolerance. However, GC caused hepatic collagen accumulation, inflammation and oxidative stress without affecting hepatic steatosis.

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INTRODUCTION

Obesity is one of the global public health problems commonly associated with metabolic diseases including insulin resistance, type 2 diabetes, non-alcoholic fatty liver disease (NAFLD) and dyslipidemia[1]. According to the World Health Organization global estimates from 2008, more than 1.4 billion adults are overweight and at least 500 million adults are obese[2]. Although the use of dietary supplements for weight loss becomes common[3], the optimal dose and safety profiles of many dietary supplements are poorly studied. The United States Food and Drug Administration (FDA) do not regulate dietary supplements in the same manner as pharmacological agents[4,5]. While pharmaceutical companies are required to obtain FDA approval, which involves assessing the risks and benefits prior to their entry into the market, dietary supplements are not subject to the same scientific scrutiny and are not required to demonstrate any scientific safety and efficacy pertaining to the claims made by manufacturers.

Several studies have shown that *Garcinia Cambogia* (GC), a fruit native to southeastern Asia and Western Africa, has beneficial effects on body weight and fat loss in both experimental animals and human[6-10]. Its main component hydroxycitric acid (HCA) not only inhibits ATP-citrate lyase, the enzyme response for *de novo* fatty acid synthesis, but also increases hepatic glycogen synthesis, reduces food intake by suppressing appetite and decreases body weight gain[6-9]. Although extensive experiments reported the weight loss and body fat-lowering effects of GC, some animal and clinical studies have shown controversial findings[6,10-13] and no studies have shown whether these effects persist beyond 13 wk of GC treatment. Furthermore, some studies have reported the potential for hepatotoxicity of hydroxycut, a formulation that contains GC among other ingredients[14,15].

The present study was therefore done to investigate the effect of long-term GC supplementation on adipogenesis and obesity-related metabolic changes, such as glucose intolerance and hepatic steatosis, in mice fed a high fat diet (HFD). We also examined the effect of GC on liver dysfunction, collagen accumulation, inflammation and oxidative stress.

MATERIALS AND METHODS

Animals and diets

Male C57BL/6J mice (4-wk-old) were purchased from Jackson Laboratories (Bar Harbor, ME, United States). The mice were individually housed in polycarbonate cages, which were kept in a room maintained at a constant temperature (24 ℃) with a 12-h light/dark cycle. The mice were fed a normal chow diet for acclimation for 1 wk after delivery. At 5 wk of age, they were randomly divided into two groups of 10 mice each and fed a HFD (D12451, Research Diets, New Brunswick, NJ, United States) with or without GC (1%, w/w, 60% hydroxyl citric acid; Newtree Inc., United States) for 16 wk. The HFD contained 45 kcal% fat, 20 kcal% protein and 35 kcal% carbohydrate. They were given free access to food and distilled water, and food consumption and body weight were measured daily and weekly, respectively. At the end of the experimental period, all the mice were anesthetized with isoflurane (5 mg/kg body weight, Baxter, United States) after a 12-h fast, and blood samples were collected from the inferior vena cava into heparin-coated tube for the measurement of plasma parameters. The blood was centrifuged at 1000 *g* for 15 min at 4 ℃, and the plasma was separated.

Fasting blood glucose, intraperitoneal glucose tolerance test and homeostatic index of insulin resistance

The blood glucose concentration was measured with whole blood obtained from the tail veins after withholding food for 12 h using a glucose analyzer (OneTouch Ultra, Lifescan Inc., United States) based on the glucose oxidase method. The intraperitoneal glucose tolerance test was performed on the 15th week. After a 12-h fast, the mice were injected intraperitoneally with glucose (0.5 g/kg body weight). The blood glucose level was measured from the tail vein at 0, 30, 60 and 120 min after glucose injection. Area under the curve (AUC) was calculated for all glucose levels as an index of glucose tolerance. Homeostatic index of insulin resistance (HOMA-IR) was calculated according to the homeostasis of the assessment as follows: HOMA-IR = [fasting glucose (mmol/L) × fasting insulin (μL U/mL)]/22.51.

Plasma biomarkers

Plasma adipokines were measured with a multiplex detection kit (171-F7001M, Bio-Rad, Hercules, CA, United States). Capture antibodies directed against the adipokines (resistin, leptin) were covalently coupled to the beads, and the coupled beads reacted with plasma. After a series of washes to remove unbound protein,

a biotinylated detection antibody was added to create a sandwich complex. The final detection complex was formed with the addition of streptavidin-phycoerythrin conjugate. Phycoerythrin served as a fluorescent indicator, or reporter. All samples were assayed in duplicate and analyzed with a Luminex 200 Labmap system (Luminex, Austin, TX, United States). Data analyses were done with Bio-Plex Manager software version 4.1.1 (Bio-Rad, Hercules, CA, United States).

Plasma cytokines were measured with a multiplex detection kit (M60-009RDPD, Bio-Rad, Hercules, CA, United States). Capture antibodies directed against the cytokines [insulin, tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1)] were covalently coupled to the beads, and the same procedure for plasma adipokine analysis as described above was used to determine the plasma cytokines levels.

Plasma lipid and apolipoprotein concentrations were determined with commercially available kits: Plasma free fatty acid (01120301.HE98), phospholipid (01120251), apolipoprotein A (14535014) and apolipoprotein B (14537014) levels were measured using the Nittobo enzymatic kit (Nittobo medical Co., Tokyo, Japan), and triglyceride (AM157S-K), total cholesterol (AM202-K) and high-density lipoprotein (HDL)-cholesterol (AM203-K) levels were determined using Asan enzymatic kits (Asan, Seoul, South Korea).

Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined using enzymatic kits (AM101-K, Asan, Seoul, South Korea).

Hepatic lipids contents

Hepatic lipids were extracted[16], and then the dried lipid residues were dissolved in 1 mL of ethanol for triglyceride and cholesterol assays. Triton X-100 and a sodium cholate solution in distilled water were added to 200 μL of the dissolved lipid solution for emulsification. The hepatic triglyceride and cholesterol contents were analyzed with the same enzymatic kit used for the plasma analysis.

Lipid-regulating enzyme activity

To measure the lipid-regulating enzymes activities in the epididymal white adipose tissue (WAT) and liver, samples were prepared and analyzed as previously described[17]. Briefly, fatty acid synthase (FAS) activity was determined with a spectrophotometric assay according to the method by Carl *et al*[18]; one unit of FAS activity represented the oxidation of 1 nmol of NADPH per minute at 30 ℃. Carnitine palmitoyltransferase (CPT) activity was determined according to the method by Markwell *et al*[19] and the results were expressed as nmol/min per milligram protein. Fatty acid β-oxidation was measured spectrophotometrically by monitoring the reduction of NAD to NADH in the presence of palmitoyl-CoA as described by Lazarow[20], with slight modification. Protein concentration was measured by the Bradford method using BSA as the standard[21].

Lipid peroxidation assay

The hepatic thiobarbituric acid-reactive substances (TBARS) concentration, as a marker of lipid peroxide production, was measured spectrophotometrically by the method of Ohkawa *et al*[22]. Hepatic homogenates containing 8.1% sodium dodecyl sulfate were mixed with 20% (w/v) acetic acid (pH 3.5), distilled water and 0.8% (w/v) TBA. The reaction mixture was heated at 95 ℃ for 60 min. After cooling the mixture, n-butanol: pyridine (15:1, v/v) was added and centrifuged at 3000 rpm for 15 min. The resulting colored layer was measured at 535 nm.

Analysis of gene expression

Epididymal WAT and liver were homogenized in TRIzol reagent (15596-026, Invitrogen Life Technologies, Grand Island, NY, United States) and total RNA was isolated according to the manufacturer's instructions. The total RNA was converted to cDNA using the QuantiTect Reverse Transcription Kit (205313, QIAGEN Gmbh, Hilden, Germany). The RNA expression was quantified by quantitative real-time PCR using the QuantiTect SYBR green PCR kit (204143, QIAGEN Gmbh, Hilden, Germany) and the SDS7000 sequence-detection system (Applied Biosystems, CA, United States). Each cDNA sample was amplified using primers labeled with SYBR Green dye for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The amplification was performed as follows: 10 min at 90 ℃, 15 s at 95 ℃ and 60 s at 60 ℃ for a total of 40 cycles. The cycle threshold values obtained were those cycles at which a statistically significant increase in the SYBR green emission intensity occurred. Ct data were normalized using GAPDH, which was stably expressed in mice. Relative gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method[23]. The following gene-specific primers were used: for catalase (CAT), 5'-GCGTCCGTCCCTGCTGTC-3' (forward), 5'-TGCTCCTTCCACTGCTTCATCTG-3' (reverse); for cell death-inducing DNA fragmentation factor-α-like effector A (CIDEA), 5'-TTTCAAACCATGACCGAAGTAGCC-3' (forward), 5'-CCTCCAGCACCAGCGTAACC-3' (reverse); for CPT, 5'-ATCTGGATGGCTATGGTCAAGGTC-3' (forward), 5'-GTGCTGTCATGCGTTGGAAGTC-3' (reverse); for FAS, 5'-CGCTCCTCGCTTGTCGTCTG-3' (forward), 5'-AGCCTTCCATCTCCTGTCATCATC-3' (reverse); for fatty acid translocase/cluster of differentiation 36 (FAT/CD36), 5'-ATTGGTCAAGCCAGCT-3' (forward), 5'-TGTAGGCTCATCCACTAC-3' (reverse); for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-ACAATGAATACGGCTACAGCAACAG-3' (forward), 5'-GGTGGTCCAGGGTTTCTTACTCC-3' (reverse); for glutathione peroxidase (GHS-Px), 5'-TCGCAATGAGCCAAAACTGACG-3' (forward), 5'-GCCAGGATTCGTAAACCACACTC-3' (reverse); for MCP-1, 5'-TTCCTCCACCACCATGCAG-3' (forward), 5'-CCAGCCGGCAACTGTGA-3' (reverse); for peroxisome proliferator-activated receptors (PPAR)α, 5'-CCTGAACATCGAGTGTCGAATAT (forward), 5'-GGTCTTCTTCTGAATCTTGCAGCT-3'

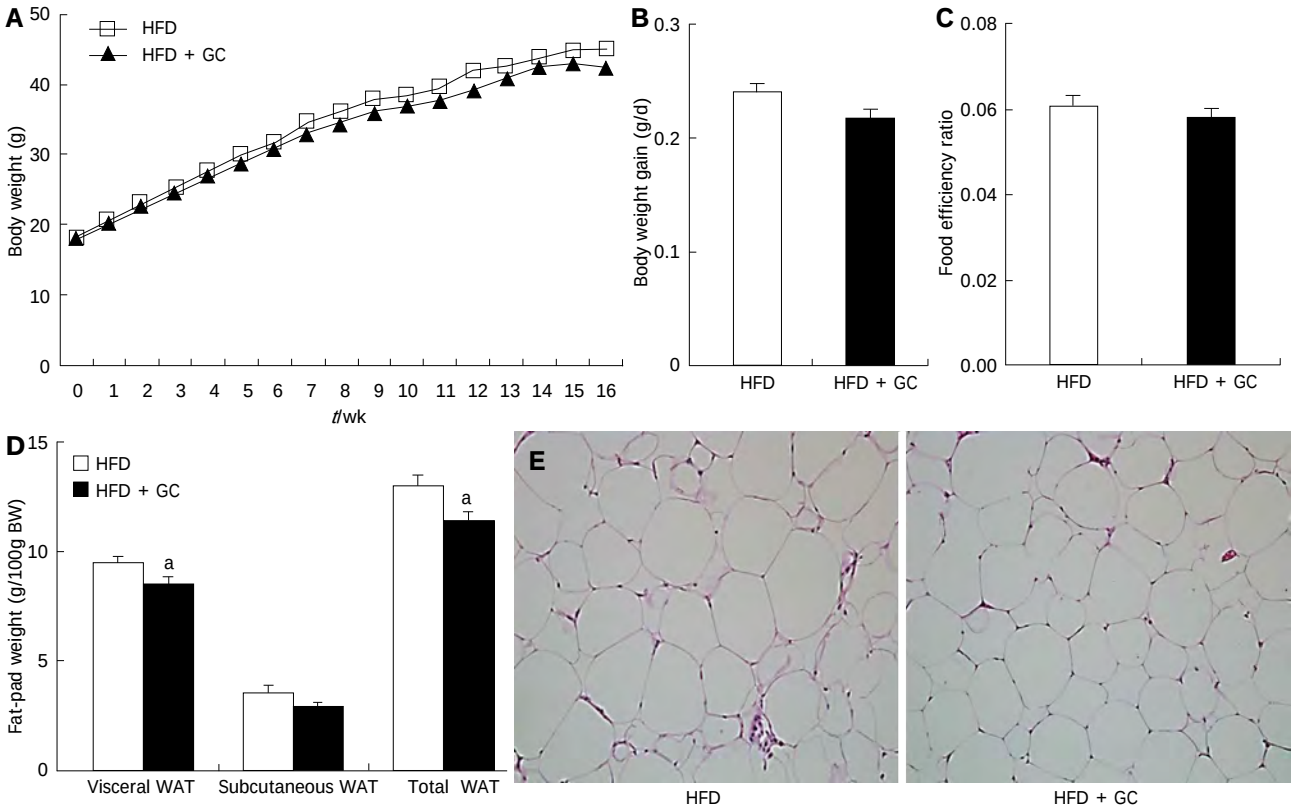


Figure 1 Effects of *Garcinia Cambogia* supplementation on body weight gain, food intake, fat-pad weight and adipocyte size in mice fed a high-fat diet for 16 wk. A-D: Data are expressed as the mean ± SE (n = 10); E: HE staining is shown. Representative photographs of epididymal white adipose tissue (WAT) (original magnification × 200). High-fat diet (HFD), mice fed a high-fat diet alone; HFD + *Garcinia Cambogia* (GC), mice fed a high-fat diet containing GC (1%, w/w). $^{a}P < 0.05$ *vs* control group.

(reverse); for TNF-α, 5'-GCAGGTCTACTTTAGAGTCATTGC-3' (forward), 5'-TCCCTTTGCAGAACTCAGGAATGG-3' (reverse); for stearoyl-CoA desaturase (SCD1), 5'-CCCCTGCGGATCTTCCTTAT-3' (forward), 5'-AGGGTCGGCGTGTGTTTCT-3' (reverse); for superoxide dismutase (SOD), 5'-TGGTTGAGAAGATAGGCGACA-3' (forward), 5'-CATCTCGGCAGCATCCACCTC-3' (reverse); and for sterol-regulatory-element-binding protein 1c (SREBP1c), 5'-GGAGCCATGGATTGCACATT-3' (forward), 5'-CCTGTCTCACCCCCAGCATA-3' (reverse).

Histological analysis

Epididymal WAT and liver were fixed in a buffer solution of 10% formalin and embedded in paraffin for staining with hematoxylin and eosin (HE) and Masson's trichrome**.** Stained areas were viewed using an optical microscope (Nikon, Tokyo, Japan) with a magnifying power of × 200.

Ethics

After blood collection, epididymal WAT, perirenal WAT, retroperitoneal WAT, mesentery WAT, subcutaneous WAT and liver were promptly removed, rinsed with physiological saline and weighed. Among them, epididymal WAT and liver were snap-frozen in liquid nitrogen and stored at -70 ℃ until enzyme activity and RNA analyses. All experimental procedures were performed in accordance with the protocols for animal studies approved by the Kyungpook National University Ethics Committee (Approval No. KNU-2011-49).

Statistical analysis

Data were expressed as the mean ± SE. Statistical analyses were performed using the statistical package for the social science software (SPSS) program. Student's t test was used to assess the differences between the groups. Statistical significance was considered at $P < 0.05$.

RESULTS

Long-term GC supplementation did not alter body weight but significantly lowered body fat weight in HFD-induced obese mice

To investigate the effects of long-term GC supplementation in diet-induced obese mice, we provided 5-wk-old male C57BL/6J mice with HFD or 1% (w/w) GC supplemented HFD for 16 wk. During the experimental period, there was no significant difference in daily food intake between the groups (HFD, 3.96 ± 0.14 g; GC, 3.87 ± 0.07 g). The body weight gain was slightly lower in the GC-supplemented mice compared to the HFD control mice but the effects of GC were not significant (Figure 1A and B). Thus, the food efficiency ratio was not significantly different between the groups (Figure 1C). However, the weight of the visceral WAT including

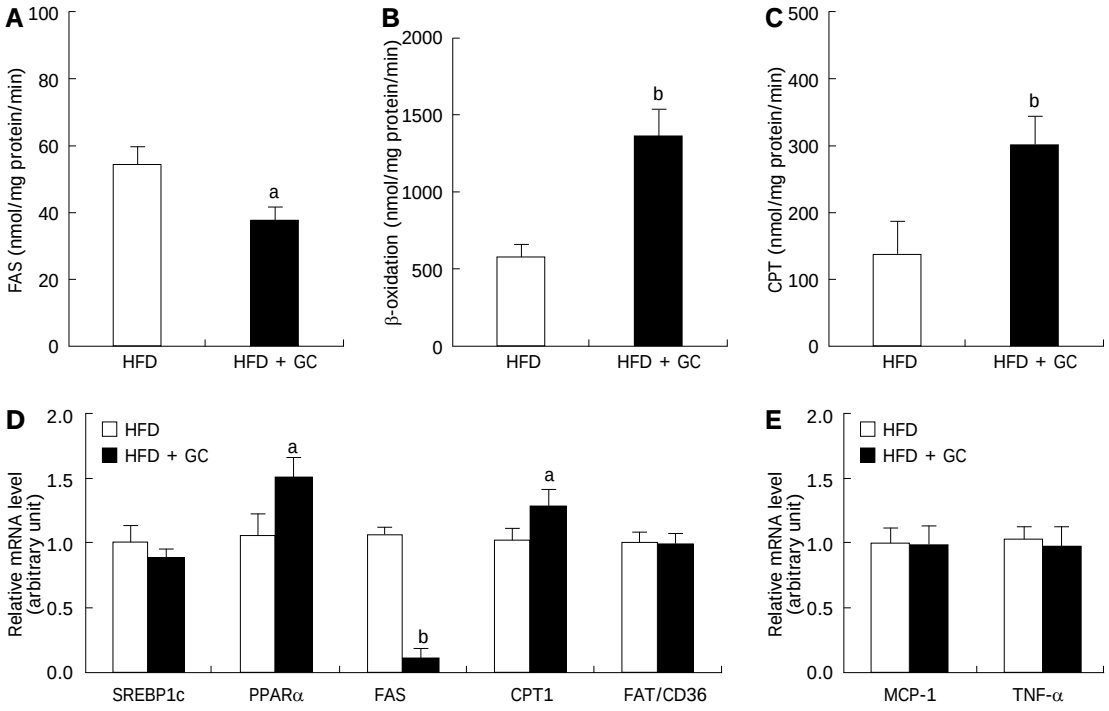


Figure 2 Effects of *Garcinia Cambogia* supplementation on fatty acid-regulating enzyme activity and gene expression in epididymal white adipose tissue of mice fed a high-fat diet for 16 wk. Data are expressed as the mean ± SE (n = 10). A: Fatty acid synthase (FAS); B: β-oxidation; C: Carnitine palmitoyltransferase (CPT); D, E: Relative mRNA level. High-fat diet (HFD), mice fed a high-fat diet alone; HFD + *Garcinia Cambogia* (GC), mice fed a high-fat diet containing GC (1%, w/w). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group. WAT: White adipose tissue; PPARα: Peroxisome proliferator-activated receptor α.

the epididymal, perirenal, retroperitoneal and mesentery WAT was significantly lower in the GC-supplemented mice than in the HFD control mice (Figure 1D). The GC supplementation also tended to lower the subcutaneous WAT weight compared to the HFD control group by 17% although it was not significantly different. Hence, the weight of the total WAT (visceral and subcutaneous WAT) was significantly lower in mice fed a GC supplemented HFD. Morphological observations also indicated the epididymal adipocyte size was smaller in the GC-supplemented mice than in the HFD control mice (Figure 1E). However, GC supplementation did not alter the extent and degree of fibrosis in the epididymal WAT of HFD-fed mice (data not shown).

Long-term GC supplementation alters the activity of enzymes and expression of genes related to fatty acid synthesis and fatty acid oxidation in visceral WAT

To examine the mechanism through which GC supplementation reduces the visceral WAT weight, we measured the activity of enzymes that regulate lipid accumulation in visceral WAT. The GC supplementation resulted in a significant decrease in the activity of FAS in the epididymal WAT of mice fed a HFD (Figure 2A). Furthermore, GC-supplemented mice showed a significant increase in the activity of CPT and β-oxidation in the epididymal WAT (Figure 2B and C).

We also examined the expression of genes that regulate adipogenesis and inflammation. Consistent with the activity of adipose enzymes, GC supplementation significantly down-regulated FAS mRNA expression, whereas it markedly up-regulated CPT mRNA expression in the epididymal WAT of HFD-fed mice (Figure 2D). Moreover, GC-supplemented mice showed a significant increase in the mRNA expression of transcription factor PPARα in the epididymal WAT compared to the control mice. However, there were no significant differences in the mRNA expression of SREBP1c, FAT/CD36, MCP-1 and TNF-α between the two groups (Figure 2D and E).

Long-term GC supplementation improved HFD-induced glucose intolerance but did not alter plasma lipid, apolipoprotein and pro-inflammatory cytokine levels

We next determined whether GC influenced HFD-induced glucose intolerance. The fasting blood glucose, plasma insulin and HOMA-IR levels were not significantly altered by GC supplementation (data not shown). However, GC supplementation significantly lowered the blood glucose level compared to the control group at 120 min after glucose loading (Figure 3A). The level of AUC was also markedly decreased in the GC-supplemented mice compared to the control obese mice.

No significant differences were observed in the levels of plasma lipids (triglycerides, total cholesterol, HDL-cholesterol, phospholipids and free fatty acids) and apolipoproteins (apolipoproteins A and B) between the two groups (Table 1). GC supplementation also did not affect the plasma leptin, TNF-α and MCP-1 levels in the HFD-fed mice; however, it significantly lowered the plasma resistin level (Figure 3B and C).

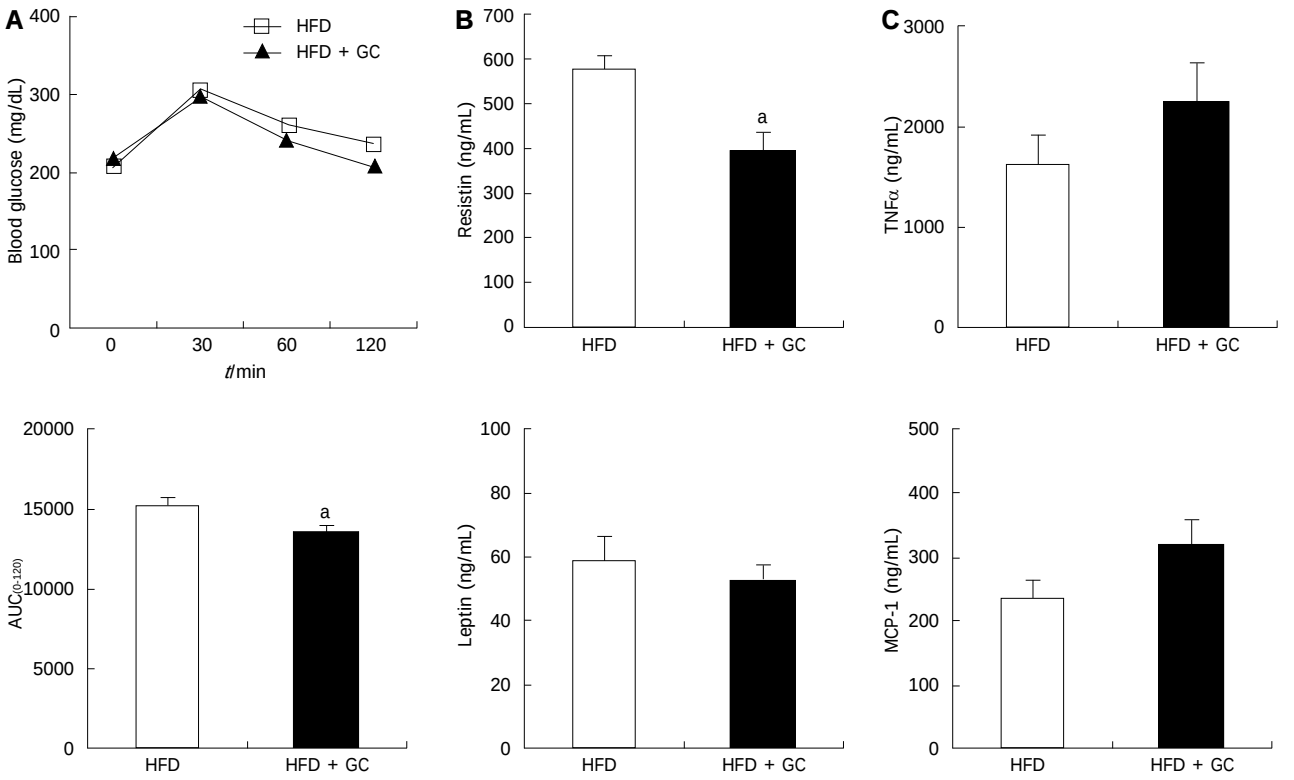


Figure 3 Effects of *Garcinia Cambogia* supplementation on glucose tolerance and plasma adipocytokine levels in mice fed a high-fat diet for 16 wk. Data are expressed as the mean ± SE (n = 10). A: The intraperitoneal glucose tolerance test was performed on the 15th week of *Garcinia Cambogia* (GC) supplementation in high-fat diet (HFD)-fed mice. Following a 12-h fast, the mice were injected intraperitoneally with glucose (0.5 g/kg body weight). Blood glucose was then measured *via* the tail vein at the indicated time [above: Blood glucose values; below: Areas under the curves (AUC)]; B, C: Plasma levels of leptin, resistin, monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) were assayed after 16 wk of GC supplementation in HFD-fed mice. HFD, mice fed a high-fat diet alone; HFD + GC, mice fed a high-fat diet containing GC (1%, w/w). $^aP < 0.05$ *vs* control group.

Table 1 Effects of *Garcinia Cambogia* supplementation on plasma lipids and apolipoproteins levels in mice fed a high-fat diet for 16 wk

	HFD	HFD + GC
Triglyceride (mg/dL)	97.47 ± 5.62	83.56 ± 4.51
Total cholesterol (mg/dL)	162.65 ± 10.16	167.73 ± 14.40
HDL-cholesterol (mg/dL)	76.27 ± 6.05	76.06 ± 6.37
Phospholipid (mmol/L)	2.35 ± 0.15	2.05 ± 0.15
Free fatty acid (mmol/L)	0.95 ± 0.08	1.02 ± 0.17
Apolipoprotein B (mmol/L)	5.32 ± 0.59	5.97 ± 0.39
Apolipoprotein A (mmol/L)	50.87 ± 1.24	45.22 ± 1.35

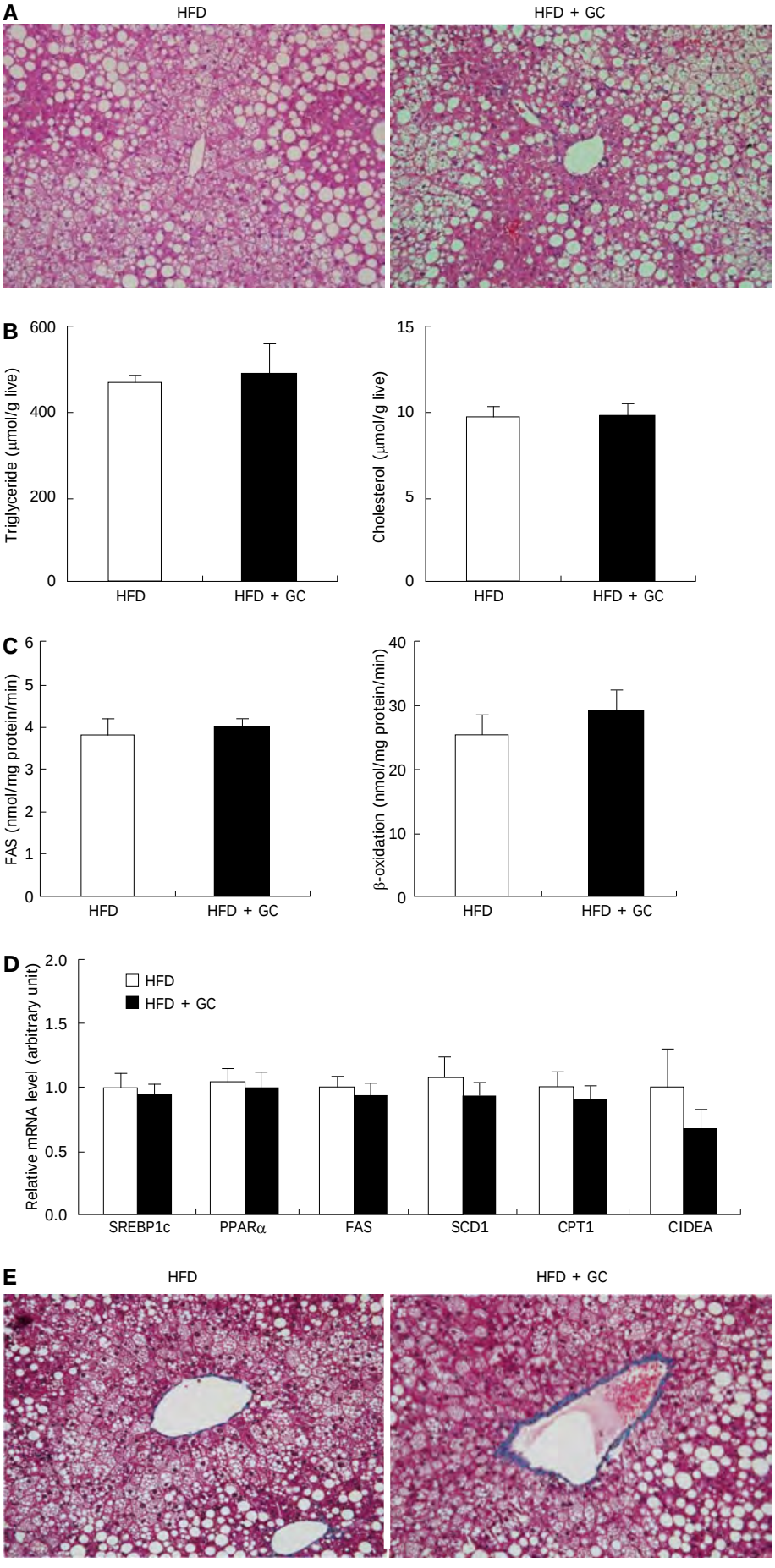
Data are expressed as the mean ± SE (n = 10). HFD: High-fat diet; GC: *Garcinia Cambogia*; HDL: High-density lipoprotein.

Long-term GC supplementation did not affect HFD-induced hepatic steatosis but increased hepatic collagen accumulation, inflammation and oxidative stress

Next, we examined the effect of GC supplementation on NAFLD induced by HFD. GC supplementation did not alter the hepatic triglyceride and cholesterol contents as well as the accumulation of hepatic lipid droplets in HFD-fed mice (Figure 4A and B). There were also no significant changes in the activities of hepatic FAS and β-oxidation and in the mRNA levels of the genes involved in lipogenesis and fatty acid oxidation, including FAS, SCD1, CPT, CIDEA, SREBP1c and PPARα, between the two groups (Figure 4C and D). However, trichrome staining of the liver revealed GC supplementation increased collagen deposition (blue staining) compared to the control mice (Figure 4E). Furthermore, Plasma ALT and AST levels were significantly increased in the GC group compared to the control mice (Figure 4F). The mRNA levels of TNF-α and MCP-1, pro-inflammatory markers, were significantly increased in the liver of GC-supplemented mice compared to the control mice (Figure 4G). GC supplementation also caused significant increases of hepatic SOD and glutathione peroxidase (GSH-Px) mRNA levels as well as TBARS level compared to the control mice, although there was no significant difference in hepatic CAT mRNA level between the two groups (Figure 4H and I).

DISCUSSION

Since unhealthy eating habits combined with limited activity are a major contributor to obesity and its related metabolic disease, lifestyle changes may present a cost-effective first-line of intervention for obesity[24-26]. Dietary supplements seemed to be an inefficient agent for dietary intervention in obese subjects[26]. Such supplements are not recommended by the position papers/guidelines for management of NAFLD[27-32], and have been associated


A
HFD
HFD + GC
B
Triglyceride (μmol/g live)
600
400
200
0
HFD
HFD + GC
Cholesterol (μmol/g live)
15
10
5
0
HFD
HFD + GC
C
FAS (nmol/mg protein/min)
6
5
4
3
2
1
0
HFD
HFD + GC
β-oxidation (nmol/mg protein/min)
40
30
20
10
0
HFD
HFD + GC
D
Relative mRNA level (arbitrary unit)
2.0
1.5
1.0
0.5
0.0
HFD
HFD + GC
SREBP1c
PPARα
FAS
SCD1
CPT1
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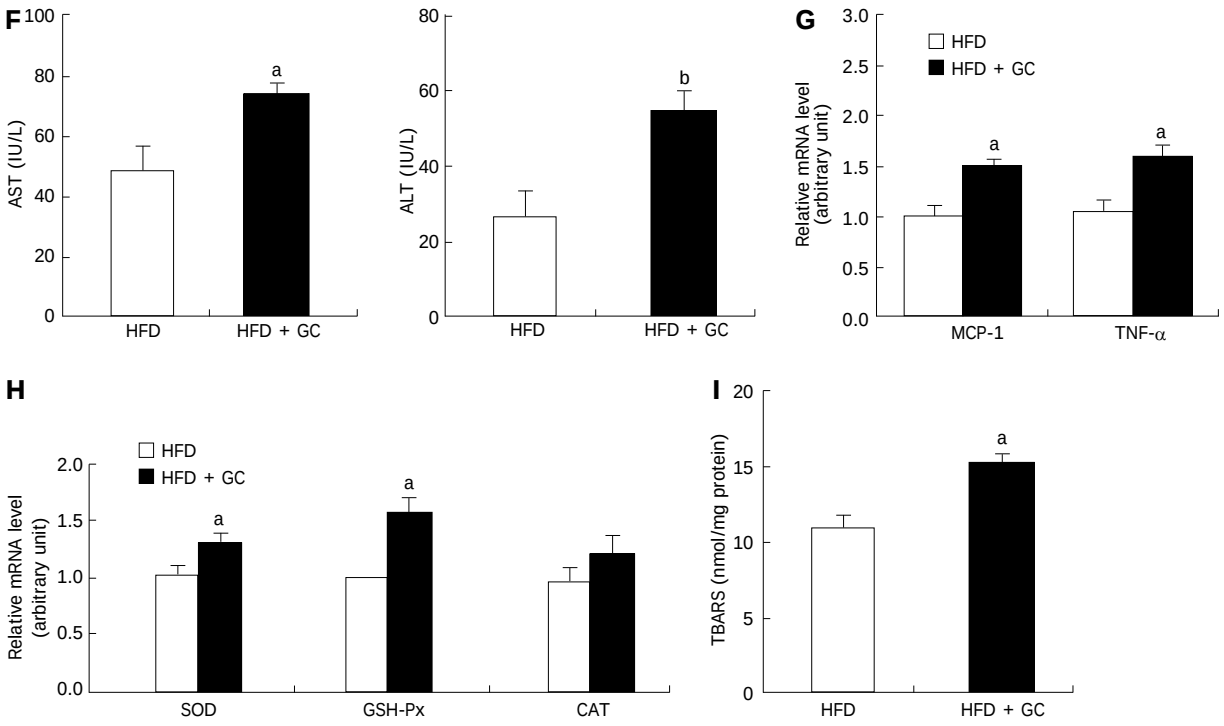

Figure 4 Long-term *Garcinia Cambogia* supplementation did not affect high-fat diet-induced hepatic steatosis but increased hepatic collagen accumulation, inflammation and oxidative stress. A: The liver tissue sections were stained with hematoxylin and eosin; B: Triglyceride and cholesterol level; C: Fatty acid synthase (FAS) and β-oxidation level; D: Relative mRNA level; E: The liver tissue sections were stained with Masson's trichrome; F: Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) level; G, H: Relative mRNA level; I: thiobarbituric acid-reactive substances (TBARS) concentration. Representative images are shown (original magnification × 200). Data are expressed as the mean ± SE (n = 10). High-fat diet (HFD), mice fed a high-fat diet alone; HFD + *Garcinia Cambogia* (GC), mice fed a high-fat diet containing GC (1%, w/w). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group. CAT: Catalase; CIDEA: Cell death-inducing DNA fragmentation factor-α-like effector A; CPT: Carnitine palmitoyltransferase; FAS: Fatty acid synthase; GSH-Px: Glutathione peroxidase; MCP-1: Monocyte chemoattractant protein-1; PPARα: Peroxisome proliferator-activated receptor α; SCD1: Stearoyl-CoA desaturase; SOD: Superoxide dismutase; SREBP1c: Sterol-regulatory-element-binding protein 1c; TNF-α: Tumor necrosis factor-α.

with increased mortality in some studies[33]. However, there are a number of natural dietary supplements for weight management, including GC, guar gum and chitosan[34]. Among them, HCA-containing GC has been shown to be efficacious in lowering body weight and body fat[6,10-13]. But, some clinical trials and animal studies have shown conflicting results[6,12], and a case series on hepatotoxicity has been reported in patients taking GC-containing hydroxycut, although the individual chemical component underling liver injury remains poorly understood[14,15]. Therefore, the aim of this study was first to determine the effects from long-term GC supplementation on obesity and related metabolic diseases as well as the hepatotoxicity in mice fed a HFD.

In the present study, GC supplementation (1%, w/w) in a HFD for 16 wk did not lead to significant changes in body weight and food intake in mice. However, it resulted in significant decreases in visceral WAT weight and adipocyte size in HFD-induced obese mice. The anti-adiposity effect of GC was partly associated with marked decreases in FAS activity and its gene expression in the epididymal WAT. FAS is a key enzyme involved in *de novo* fatty acid synthesis and WAT is a major site of fatty acid synthesis and storage. We also found that the activity of CPT as well as fatty acid oxidation in epididymal WAT was elevated by GC supplementation. Furthermore, the enhanced adipose fatty acid oxidation in GC-supplemented mice was accompanied by the up-regulated mRNA expression of genes involved in fatty acid oxidation such as CPT and PPARα in the epididymal WAT. The CPT is a major rate-limiting enzyme for fatty acid oxidation, and its gene expression is regulated by PPARα in adipocytes[35]. The PPARα mRNA expression was decreased in the WAT of both genetic and HFD-induced obese mice, and the down-regulation of PPARα in obese WAT was involved in obesity-induced mitochondrial dysfunction and metabolic disorders[36]. Taken together, our findings suggest that in HFD-fed mice, a significant reduction in visceral fat accumulation by GC supplementation could be partly due to decreased fatty acid synthesis as well as increased fatty acid oxidation in adipose tissue. The results of this study are supported by the findings of a previous study which suggested GC supplementation significantly lowered body fat mass, but not body weight and food intake, by inhibiting adipose ATP-citrate lyase (ACL) activity in Zucker obese rats[12]. The ACL is another lipogenic enzyme catalyzing the cleavage of citrate to oxaloacetate and acetyl-CoA for *de novo* fatty acid synthesis[37]. The inhibitory action of HCA on ACL reduces the acetyl-CoA pool, which can lead to a decreased concentration of malonyl-CoA, a physiological inhibitor of CPT[38], and thus results in the suppression of body

fat accumulation through stimulation of fatty acid oxidation[39]. Kim *et al*[11] also reported that HCA-containing GC (1%, w/w) supplementation in HFD-fed mice for 12 wk significantly lowered body fat accumulation by modulating multiple genes associated with adipogenesis in mice fed a HFD.

Along with the anti-obesity effects of HCA, previous studies have reported on the beneficial effects of HCA on insulin resistance[40,41]. HCA increases the cellular pool of citrate by inhibiting ACL, which in turn can increase glycogen production[42]. Recently, HCA supplementation enhanced the glycogen synthesis rate in skeletal muscles and improved post-meal insulin sensitivity[41]. Although we did not measure the level of glycogen in the liver, HCA-containing GC supplementation improved glucose tolerance in HFD-induced obese mice. Furthermore, the plasma resistin level was significantly lowered by GC supplementation in the current study. Resistin is one of the adipokines proposed to link obesity with insulin resistance. Circulating resistin level was elevated in obesity and insulin resistance[43], and HFD significantly increased plasma resistin levels in mice compared to a standard low-fat/high-carbohydrate diet[44]. Resistin deficiency ameliorated glucose homeostasis in mice[45], whereas administration of resistin impaired glucose tolerance and insulin action[43,46]. Thus, the beneficial effect of GC on glucose intolerance might be associated with the decreased resistin level in plasma. Another mechanism in which GC could contribute to improve glucose tolerance is the lowered body fat mass because excess adiposity, especially in visceral WAT, is considered to promote insulin resistance[47]. However, there was no significant difference in HOMA-IR which estimates insulin sensitivity from fasting glucose and insulin concentrations. Tripathy *et al*[48] reported a significant relationship between hepatic insulin sensitivity and HOMA-IR regardless of the stage of glucose tolerance, suggesting that the HOMA-IR is dependent upon hepatic insulin sensitivity rather than peripheral insulin sensitivity. Another clinical study also demonstrated that HOMA-IR did not accurately predict insulin sensitivity[49].

Increased adiposity with the consequences of inflammation and insulin resistance has been linked to the development of NAFLD, which refers to a wide spectrum of liver damage, ranging from simple steatosis to steatohepatitis and cirrhosis. Steatosis represents the accumulation of fat within the liver through multiple mechanisms including an altered balance in fatty acid uptake and triglycerides secretion, increased *de novo* lipogenesis, and decreased fatty acid oxidation[50,51]. Steatohepatitis is the combination of steatosis with hepatic inflammation and fibrosis. Liver fibrosis is excess synthesis and deposition of extracellular matrix proteins including collagen[52], and pro-inflammatory factors including MCP-1 and TNF-α that contribute to the second hit in the pathogenesis of steatohepatitis. Among the inflammatory mediators, MCP-1 is a pro-inflammatory chemokine which coordinates leukocyte recruitment to the liver by activation of the CC chemokine receptor 2 (CCR2) on inflammatory cells including monocytes and macrophages promoting the inflammatory response[53]. Several studies indicate that MCP-1 is an important mediator of liver fibrosis[54-56]. MCP-1 mRNA expression was markedly increased in the livers of patients with steatohepatitis and in murine models of steatohepatitis such as mice fed a HFD or methionin-cholin deficient diet[57-63]. CCR2 inhibitor suppressed the early and late features of steatohepatitis including fibrosis[64], and chronic exposure of HFD induced hepatic MCP-1 mRNA expression in mice before induction of other pro-inflammatory cytokine mRNAs including TNF-α and prior to the onset of steatohepatitis, suggesting that MCP-1 plays a major role in initiating the inflammatory process in steatohepatitis[65]. Moreover, MCP-1 deficiency reduced liver fibrosis (collagen deposition) and pro-fibrogenic gene expression in mice fed a methionine-choline deficient diet although it did not affect liver steatosis in this model[66].

Similarly, we found that GC supplementation did not affect hepatic lipogenesis and lipid droplet formation, but it markedly increased collagen deposition as well as pro-inflammatory MCP-1 and TNF-α mRNA expression in the liver of HFD-fed mice. Furthermore, GC-supplemented mice exhibited impaired liver function indicated by the elevations of plasma ALT and AST, suggesting that GC possibly promotes liver injury in HFD-fed mice. Several case reports have suggested HCA-containing hydroxycut has potential hepatotoxicity but the underlying mechanism remains unknown[14,15,67,68]. Our experiments provide new information regarding hepatotoxicity after long-term GC supplementation in HFD-induced obese mice. Accordingly, GC supplementation contributes to steatohepatitis by increasing hepatic collagen accumulation and hepatic MCP-1 and TNF-α expression in mice fed a HFD, which are independent of its effects on HFD-induced hepatic steatosis.

On the other hand, oxidative stress is considered to play an important role in progression of nonalcoholic steatohepatitis and hepatocellular injury[69]. Reactive oxygen species (ROS) can damage DNA, lipids and proteins, induce necrosis and apoptosis of hepatocytes and amplifie the inflammatory response. The ROS also stimulate the production of pro-fibrogenic mediators from Kupffer cells and inflammatory cells and directly induce hepatic stellate cells proliferation, resulting in the initiation of fibrosis[70]. Antioxidant enzymes such as SOD and GSH-Px ameliorate the damaging effects of ROS. SOD converts superoxide radicals into hydrogen peroxide, which is then further metabolized by GSH-Px, where it catalyzes the destruction of hydrogen peroxide and lipid hydroperoxide. We observed that the supplementation of GC significantly up-regulated hepatic SOD and GSH-Px mRNA expression with concomitant increase in lipid peroxidation in the liver, suggesting that the increases in antioxidant gene expression by GC seems to be a compensatory response of the liver to cope with oxidative stress.

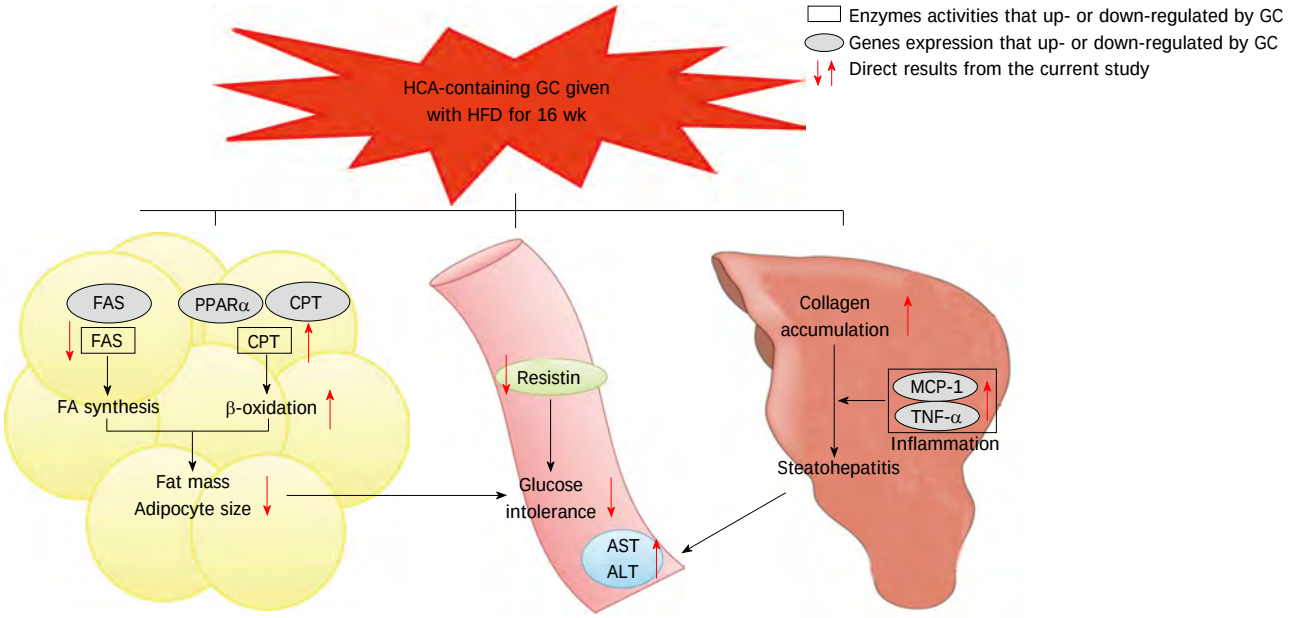

Figure 5 Summary of the long-term *Garcinia Cambogia* supplementation effects on adiposity, glucose tolerance and steatohepatitis in high-fat diet-induced obese mice. The *Garcinia Cambogia* (GC) supplementation decreased fatty acid synthase (FAS) mRNA expression and its activity, while peroxisome proliferator-activated receptor α (PPARα) and carnitine palmitoyltransferase (CPT) mRNA expression along with the activities of CPT and β-oxidation were increased in the visceral adipose tissue, indicating that these changes may be potential mechanisms for reducing body fat accumulation and glucose intolerance induced by high-fat diet (HFD). Furthermore, GC supplementation decreased the plasma resistin level, which may be also related to improved glucose tolerance. There were no significant changes in hepatic lipid accumulation as well as in hepatic gene expression and enzymatic activity involved in fatty acid synthesis, oxidation and storage. However, GC increased pro-inflammatory monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) mRNA expression, lipid peroxidation and collagen accumulation in the liver. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were also increased by GC supplementation in HFD-induced obese mice, thus suggesting that GC may negatively affect liver function by increasing hepatic fibrosis, inflammation and oxidative stress without affecting hepatic fat accumulation. FA: Fatty acid; HCA: Hydroxycitric acid. Open square means enzymes activities that up- or down-regulated by GC. Solid circle means genes expression that up- or down-regulated by GC.

In conclusion, this study demonstrated that long-term GC supplementation ameliorated adipogenesis in mice fed a HFD by promoting fatty acid oxidation with a simultaneous decrease in fatty acid synthesis in visceral WAT. Furthermore, GC exhibited a protective role against glucose intolerance induced by HFD. Moreover, this study provides the first evidence that long-term GC supplementation significantly increased hepatic collagen accumulation, lipid peroxidation and MCP-1 and TNF-α mRNA expression as well as plasma AST and ALT levels, thereby contributing partly to the exacerbation of steatohepatitis in HFD-induced obese mice at the doses given. The observations described above are summarized in Figure 5. Although further research is required to elucidate the efficacy and safety of long-term use of GC in humans, caution is needed when using GC supplements for weight management.

COMMENTS

Background

Garcinia Cambogia (GC) is a popular dietary supplement for weight loss, but the efficacy and hepatotoxicity of long-term GC supplementation remain poorly understood. Thus, authors investigated the long-term supplementation effects of GC on adiposity and non-alcoholic fatty liver disease (NAFLD) in mice fed a high-fat diet (HFD).

Research frontiers

A number of experiments reported GC has beneficial effects on weight loss and body fat in some animals and human. However, there are controversial findings and little studies have reported whether these effects persist beyond 13 wk of GC supplementation.

Innovations and breakthroughs

This is first report which shows the supplementation of GC increased hepatic collagen accumulation, inflammatory genes expression and lipid peroxidation as well as plasma alanine aminotransferase and aspartate aminotransferase levels, although HFD-induced hepatic steatosis did not change. Thus their findings suggest that long-term supplementation of GC induces hepatic fibrosis and inflammation, although it protects against HFD-induced adiposity and glucose intolerance in mice fed a high fat diet.

Applications

Extensive dietary supplements are popular and widely used for weight management. However, the optimal dose and safety profiles of many dietary supplements are poorly studied and they are not regulated by the Food and Drug Administration in a manner observed for pharmacological agents. The authors suggest that caution is needed when using GC supplements for weight management, although further research is required to elucidate the efficacy and safety of long-term use of GC in humans.

Peer review

The authors investigated long-term supplementation effects of GC on adiposity and NAFLD in diet-induced obese mice. They reported that long-term GC supplementation improved adipogenesis by promoting fatty acid oxidation along with a decrease in fatty acid synthesis in visceral white adipose tissue. They also reported a protective role of GC against glucose intolerance induced by HFD. However, they also showed that long-term GC supplementation increased hepatic collagen accumulation and cytokine expression, thereby exacerbating steatohepatitis. The manuscript has no structural flaws. The hypothesis is relevant and methods to test the hypothesis were uptodate.

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ORIGINAL ARTICLE

Mesenchymal stem cells alleviate TNBS-induced colitis by modulating inflammatory and autoimmune responses

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Abstract

AIM: To investigate the potential therapeutic effects of mesenchymal stem cells (MSCs) in inflammatory bowel disease (IBD), we transplanted MSCs into an experimental model of IBD.

METHODS: A rectal enema of trinitrobenzene sulfonic acid (TNBS) (100 mg/kg body weight) was administered to female BALB/c mice. Bone marrow mesenchymal stem cells (BMSCs) were derived from male green fluorescent protein (GFP) transgenic mice and were transplanted intravenously into the experimental animals after disease onset. Clinical activity scores and histological changes were evaluated. GFP and Sex determining region Y gene (*SRY*) expression were used for cell tracking. Ki67 positive cells and Lgr5-expressing cells were determined to measure proliferative activity. Inflammatory response was determined by measuring the levels of different inflammatory mediators in the colon and serum. The inflammatory cytokines included tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-2 (IL-2), IL-6, IL-17, IL-4, IL-10, and transforming growth factor (TGF-β). Master regulators of Th1 cells (T-box expressed in T cells, T-bet), Th17 cells (retinoid related orphan receptor gamma(t), RORγt), Th2 cells (GATA family of transcription factors 3, GATA3) and regulatory T cells (forkhead box P3, Foxp3) were also determined.

RESULTS: Systemic infusion of GFP-BMSCs ameliorated the clinical and histopathologic severity of colitis, including body weight loss, diarrhea and inflammation, and increased survival ($P < 0.05$). The cell tracking study showed that MSCs homed to the injured colon. MSCs promoted proliferation of intestinal epithelial cells and differentiation of intestinal stem cells ($P < 0.01$). This therapeutic effect was mainly mediated by down-regulation of both Th1-Th17-driven autoimmune and inflammatory responses (IL-2, TNF-α, IFN-γ, T-bet; IL-6, IL-17, RORγt), and by up-regulation of Th2 activities (IL-4, IL-10, GATA-3) ($P < 0.05$). MSCs also induced activated $CD4^+CD25^+Foxp3^+$ regulatory T cells (TGF-β, IL-10, Foxp3) with a suppressive capacity on Th1-Th17 effecter responses and promoted Th2 differentiation *in vivo* ($P < 0.05$).

CONCLUSION: MSCs are key regulators of immune and inflammatory responses and may be an attractive candidate for cell-based therapy of IBD.

Key words: Mesenchymal stem cells; Transplantation; Inflammatory bowel disease; Inflammatory response; Immunomodulation; Trinitrobenzene sulfonic acid colitis; Therapy

Core tip: In this study, the following factors were identi-

fied: (1) The differentiation of intestinal stem cells in injured gut was determined by detecting Lgr5+ cells; (2) Th1-Th2-Th17-Tregs-related inflammatory and immune cytokine expressions in serum and local intestinal tissues were determined; (3) A Th2 shift and correction of the imbalanced Th17/Tregs were found; (4) Master regulators of Th1, Th2, Th17 and Tregs were detected in bone marrow mesenchymal stem cells -treated trinitrobenzene sulfonic acid-induced colitis; and (5) The passways of Th1-T-bet, Th2-GATA family of transcription factors 3, Th17-retinoid related orphan receptor gamma(t) and Tregs-Foxp3 which serve as important immunoregulators in the correction of immune disorders and enhance the healing of injured intestinal mucosa were identified.

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INTRODUCTION

Inflammatory bowel diseases (IBD) are comprised of Crohn's disease (CD), ulcerative colitis (UC) and indeterminate colitis, and are found mainly in Western countries[1]. Recent studies indicated that enhanced abnormalities of the immune system, normal gut flora and environmental influences may play central roles in these diseases[2,3]. Conventional therapy for IBD includes corticosteroids, 5-aminosalicylates, 6-mercaptopurine, sulfasalazine, antimicrobial therapy, immunosuppressive agents and monoclonal antibodies (mAbs)[4]. Surgery is indicated for complications and failure of medical treatment[5]. Although the above therapies are effective for maintaining remission, they also have many side effects[6,7]. Several studies have reported that the transplantation of mesenchymal stem cells (MSCs) could temper IBD both in clinical trials and in animal models, which indicated that MSCs may be a promising therapeutic option for IBD[8-12]. However, the molecular mechanisms of this effect are still unclear.

Trinitrobenzene sulfonic acid (TNBS)-induced colitis (CD-like) is a well-established animal model for studying IBD. The model is well characterized by inflammatory cell infiltration accompanied by heightened Th1-Th17 response both systematically and locally in the gut mucosa[13]. T-lymphocytes secrete cytokines (cytokines can be both regulators and effectors) which can be divided into Th1 (characterized by secretion of [tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-2 (IL-2)], Th17 (characterized by secretion of IL-17), Th2 (characterized by secretion of IL-4 and IL-10), and regulatory T cells [Tregs, characterized by expression of forkhead box P3 (Foxp3) and CD25][14]. These T cells can be identified by the expression of specific transcription factors, T-bet for Th1, GATA family of transcription factors 3 (GATA3) for Th2, FoxP3 for Tregs, and retinoid related orphan receptor gamma(t) (RORγt) for Th17 cells, respectively[15]. In this study, we used the TNBS-induced colitis animal model to investigate the therapeutic role of MSCs in CD-like diseases.

Many recent studies have demonstrated that bone marrow mesenchymal stem cells (BMSCs) were seen in injured intestinal mucosa. These cells may repopulate and differentiate into intestinal stem cells (ISCs)[16]. ISCs are the progenitors of various functional intestinal cell types, including intestinal epithelial cells (IECs), interstitial cells, endothelial cells, vascular smooth muscle cells, and ill-defined inflammatory cells[17,18]. *Lgr5* gene product can be used as a specific marker for identifying these ISCs[19,20]. However, it is not clear which factors from the transplanted BMSCs promote the repopulation of cells in recipient intestinal tissues. Several experiments have shown that MSCs can release soluble factors (cytokines, chemokines, and growth factors) that result in cell cycle arrest in pro-inflammatory lymphocytes and induce T cell apoptosis[21]. Reports also indicated that the regenerative, immunomodulatory, and anti-inflammatory effects of MSCs may provide a potential remedy for autoimmune diseases, such as focal cerebral ischemia, multiple sclerosis (MS), systemic sclerosis (SSc), type I diabetes and juvenile idiopathic arthritis[22-24]. However, there have been few reports regarding the roles of MSCs in IBD as well as the molecular mechanisms of MSCs in alleviating IBD.

In this study, we focused on the trafficking of transplanted GFP-BMSCs into diseased colon tissues and on their immune modulating effects in TNBS-induced colitis. We investigated the inhibitory effect of BMSCs on Th1 and Th17 mediated inflammatory response. We also examined the enhancement of anti-inflammatory immune activities of Th2 and Tregs by BMSCs in an animal model of TNBS-induced colitis.

MATERIALS AND METHODS

Animals

Male Green fluorescent protein (GFP) transgenic mice [TgN (β-act-EGFP) Osb; 2-3 wk] on a C57BL/6 background were kindly provided by the Molecular Biology Laboratory of Chinese PLA General Hospital. Female BABL/c mice (6-8 wk) were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences of China (Beijing). All studies were performed under approval of the Ethics Committee of the Animal Facility of Chinese PLA General Hospital and were in agreement with the Guidance suggestion of caring for laboratory animals[25]. Mice were group-housed under controlled temperature (25 ℃) and a 12-h light/dark cycle, fed standard mouse chow and tap water and maintained for 2 wk in our animal facilities before the start of the experiments.

Establishment of TNBS model

Colitis was induced in female BABL/c mice (6-8 wk,

18-22 g) using 2,4,6-trinitrobenzene sulfonic acid (2,4,6-TNBS; Sigma-Aldrich, Deisenhofen, Germany) in 50% ethanol[26]. Then 0.1 mL of TNBS (100 mg/kg body weight) was administered *via* a vinyl catheter positioned 2.5 cm from the anus. To ensure distribution of TNBS within the entire colon and rectum, mice were held in a vertical position for 2 min after instillation of the TNBS enema. The control group was administered 0.1 mL of 50% ethanol.

Culture and identification of putative BMSCs

Isolation, culture, and expansion of BMSCs: Male GFP-mice (2-3 wk) were sacrificed by cervical dislocation and their femurs and tibia were carefully flushed with Dulbecco's modified Eagle's medium-low glucose (DMEM-LG; HyClone Lab, Inc. Logan, UT) using a 0.45-mm syringe needle until the bones become pale. The released cells were discarded and the bones were dissected into fragments of 1-3 mm^3 and digested with collagenase Ⅱ (Sigma) for 1-2 h in a shaking incubator at 37 ℃ and a shaking speed of 200 rpm. The collagenase was removed by dilution with DMEM-LG containing 10% heat inactivated fetal bovine serum (FBS; Gibco-Invitrogen, Carlsbad, CA). In addition, the digested bone fragments were washed by centrifuging twice for 5 min at 1000 rpm and then cultivated in a 60 mm dish (Corning International, Tokyo, Japan) with DMEM-LG containing 10% FBS and penicillin/streptomycin (100 U/mL and 100 g/mL; Sigma, St. Louis, MO) at 37 ℃ in a 5% CO_2 humidified incubator. To isolate putative MSCs, after 72 h of culture, nonadherent cells and tissue debris were removed with phosphate-buffered saline (PBS, HyClone), and adherent cells were maintained. On reaching 70%-80% confluence, these adherent cells were replanted using 0.25% (wt/v) trypsin/0.02% (wt/v) EDTA (Gibco) for 2-3 min. The medium was changed every 2-3 d[27].

Flow cytometric analysis: The cultured MSCs were retrieved by trypsin-EDTA digestion. Cell aliquots (1×10^6) were washed with cold PBS and resuspended in 100 μL of PBS per EP tube and stained with FITC-conjugated anti-mouse CD45 (Becton Dickinson), or PE-conjugated anti-mouse CD11b (eBioScience, San Diego, CA) and CD90.2 (eBioScience) at a concentration of 2 μg/mL at 4 ℃ for 30 min. One tube of unstained cells was prepared as a control for the antibodies. Cells were examined using a Becton Dickinson Fluorescence Activated Cell Sorting (BD FACScalibur) cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed using a FACS Vantage cytometer and CellQuest software (BD).

Differentiation of BMSCs: The potential of BMSCs to differentiate into osteogenic and adipogenic lineages has been examined[27,28]. For osteogenesis, the isolated cells (passage 3) were plated in osteogenic conditioned medium supplemented with 10 mmol/L β-glycerol phosphate (Sigma), 50 μg/mL ascorbate-2-phosphate (Sigma) and 10 nmol/L dexamethasone (Sigma). The culture medium was changed every 3 d for 3 wk. Alizarin red staining was used to examine the culture mineralization. For staining, the cultures were first fixed using 4% paraformaldehyde for 20 min and then subjected to alizarin red solution at 37 ℃ for 30 min.

For adipogenesis, the cultured cells (passage 3) were incubated in adipogenic medium supplemented with 100 μmol/L indomethacin (Sigma), 1 μmol/L dexamethasone (Sigma), and 0.5 mmol/L 1-methyl-3-isobutylxanth (Sigma). The culture medium was changed every 3 d for 8 d. At the end of this period, the cells were then fixed in 4%paraformaldehyde for 1 h and stained with Oil Red (Sigma) for 1-2 h at room temperature.

Identification of fluorescence distribution in intestinal mucosa

GFP-BMSCs were harvested from male GFP-mice as described above. Then 1×10^6 cells in 100 μL PBS were transplanted on the indicated day *via* the tail vein on the second day after TNBS administration, while control mice received 100 μL PBS without BMSCs. In addition, the mice were killed on day 1, 2, 3, 5, 7 and 9 and their colons were washed with PBS. The colonic tissues were embedded in Tissue-Tek OCT compound (Sakura Finechemical Co., Tokyo, Japan) and circumferential sections 3 μm thick were prepared for fluorescent microscopy examination (Olympus, Tokyo, Japan). Paraffin-embedded tissue slides were stained with hematoxylin and eosin (HE) to assess the intensity of colitis. Additional specimens were frozen in liquid nitrogen for further use.

Isolation of DNA and detection of Y chromosomal DNA by polymerase chain reaction: DNA was isolated from snap-frozen colon specimens using the QIAamp DNA mini kit (Qiagen, CA) and Wizard DNA purification resin (Promega, Madison, WI). Polymerase chain reaction (PCR) was used to investigate the transplanted male donor cells using primers specific for the sex-determining region of the mouse Y-chromosome (Sry). The male group acts as a positive control, while the female group acted as a negative control. PCR amplification was performed as described below. The reaction mixture included 2 μL of genomic DNA, 2 μL Taq DNA polymerase (Takara, Japan), 5 μL of each oligonucleotide primer, 1 μL of a 20 mmol/L solution of each dNTP, 5 μL of 10 × PCR buffer, and 5 μL of 25 mmol/L $MgCl_2$ in a final volume of 25 μL. The primer sequences used were as follows: mouse *Sry* gene, 5'-GGTGTG-GTCCCGTGGTGAGAG-3' and 5'-ATGGCATGT-GGGTTCCTGTCC-3'. PCR was performed in a thermal cycler (PerkinElmer Cetus) for 35 cycles of denaturation (9 ℃, 20 s), annealing (64 ℃, 20 s), and extension (72 ℃, 20 s). The product was analyzed by electrophoresis on a 2% agarose gel followed by ethidium bromide staining.

Assessment of inflammation and colitis severity

Clinical activity score of colitis: Mice were observed daily for weight, water/food consumption, morbidity, stool consistency, piloerection, and the presence of rectal bleeding. The clinical activity score of colitis was calculat-

Table 1 Sequence of gene-specific primers used for real-time polymerase chain reaction analysis

mRNA target	Forward primer sequences 5′→3′	Reverse primer sequences 5′→3′	bp
Actin	CGTTGACATCCGTAAAGACC	CTAGGAGCCAGAGCAGTAATC	111
IL-2	TGAGTGCCAATTCGATGATGAG	TTATTGAGGGCTTGTTGAGATGAT	91
IL-4	CAGCAACGAAGAACACCACAG	CGAAAAGCCCGAAAGAGTC	148
IL-6	AATTTCCTCTGGTCTTCTGG	ACTCTGGCTTTGTCTTTCTTGTT	93
IL-10	AGTGGAGCAGGTGAAGAGTGATTT	CTATGCAGTTGATGAAGATGTC	92
IL-17A	GAGAGCTGCCCCTTCACTTTCA	GGCTGCCTGGCGGACAAT	87
IFN-γ	TGGTGACATGAAAATCCTG	TTGCTGTTGCTGAAGAAG	141
TNF-α	AGTTCCCAAATGGCCTCCCT	ACTTGGTGGTTTGCTACGAC	115
GATA3	AGGTGCATGACGCGCTGGAG	GGAGTGGCTGAAGGGAGAG	107
Foxp3	GAAGAATGCCATCCGCCACAA	TGCTCCCTTCTCGCTCTCCA	70
TGF-β	TGCCCTCTACAACCAACACAAC	GCAGGAGCGCACAATCAT	142
T-bet	ACCTCTTCTATCCAACCAGTATCC	GAGGTGTCCCCAGCCAGTA	104
RORγt	GAAGGCAAATACGGTGGTGTGG	GCTGAGGAAGTGGGAAAAGTC	90

IL: Interleukin; IFN: Interferon; TNF: Tumor necrosis factor; GATA3: GATA family of transcription factors 3; Foxp3: Forkhead box P3; TGF: Transforming growth factor; T-bet: T-box expressed in T cells; RORγt: Retinoid related orphan receptor gamma(t).

ed independently by two blinded investigators using the disease activity index (DAI)[13]. The length of the colon, which was measured from the cecum to the anus, served as an indirect marker of the intensity of inflammation.

Histological scoring of the colon: The sections were HE stained and were used to assess colonic damage microscopically. The criteria were graded as follows: 0 point = no ulcer, no inflammation; 1 point = no ulcer, local hyperemia; 2 points = ulceration without hyperemia; 3 points = ulceration and inflammation at one site only; 4 points = two or more sites of ulceration and inflammation; and 5 points = ulceration extending more than 2 cm.

Immunohistochemistry for Ki67 and Lgr5: The slides of 4 μm colon sections were incubated with a rabbit anti-mouse primary antibody to Ki67 (1:500, ab66155, Abcam,) or a rabbit anti-human primary antibody to Lgr5 (1:400, LS-A1236, MBL International Co., Woburn, MA) in PBS at 4 ℃ overnight. After 3 washes in PBS, the sections were incubated with a goat anti-rabbit secondary antibody (1:1000, Beijing Zhongshan Biotechnology, Beijing, China) at 37 ℃ for 30 min. Finally, the sections were visualized by incubating in 3,3'-diaminobenzidine tetrahydrochloride with 0.05% H_2O_2 (Liquid DAB + Substrate Chromogen System; Dako) for 3 min to induce a color reaction. The expression and localization of Ki67 and Lgr5 were examined under a light microscope (Olympus, Japan), and a brown color indicated a positive result.

Images were acquired using a digital camera connected to a light microscope (both from Olympus, Tokyo, Japan). Five immunostained sections (1280 × 960 pixels/per section) were selected from each image (× 200, 3 mice per group) for analysis. Ki-67 positivity and mean optical density of Lgr5 (mean optical density = total optical density/area) that best discriminated staining from the background was obtained using the NIH Image J 1.36b imaging software (NIH, Bethesda, MD). For immunohistochemistry analysis, immunostained sections were evaluated by two investigators following the principle of "blinded".

Quantification of Th1/Th2/Th17 cytokines by FACS: The BD Cytometric Bead Array Mouse Th1/Th2/Th17 Cytokine Kit (Becton Dickinson, San Jose, CA) was used to quantify TNF-α, IFN-γ, IL-2, IL-4, IL-6, IL-10 and IL-17A secretions in murine peripheral blood and colon proteins according to the manufacturer's instructions using the BD FACScalibur cytometer (Becton Dickinson). Fifty microliters of supernatant were collected at each of the time points indicated above. Data were acquired on a FACS ARIA and samples were analyzed using FCAP Array Software (BD Biosciences).

RNA extraction and real-time PCR: RNA was extracted from colon (100 mg) using the Total RNA Kit Ⅱ (Qiagen) following the manufacturer's instructions. The quantity of extracted RNA was evaluated using the Nanodrop ND1000 (Thermofisher Scientific). Complementary DNA (cDNA) was created using DNase I /RNase-free (EN0521, Fermentas). Single intra-exon gene-specific primers listed in Table 1 were generated using Primer Express Software (Perkin Elmer Applied Biosystems). Applied Biosystems PRISM® 7300 (Applied Biosystems) and SYBR green fluorescent dye were used to detect amplification under the following amplification conditions: (1) 1 warm-up cycle for 2 min at 50 ℃; (2) 1 pre-amplification cycle for 10 min at 95 ℃, 40 amplification cycles for 30 s at 95 ℃ and for 31 s at 60 ℃; and (3) end-amplification cycle for 15 s at 95 ℃, 30 s at 60 ℃ and 15 s at 95 ℃. Quantitative PCR crossing threshold (Ct) values were obtained during the exponential amplification phase using SDS 2.3 Software (Applied Biosystems).

Western blotting analysis for Foxp3, T-bet, GATA-3, TGF-β3 (III) and ROR gamma (t)

Total proteins (100 μg) were added to lysis buffer containing protease inhibitors (Sigma; St. Louis, MO) and boiled at 95 ℃ for 5 min. The proteins were then routinely processed for Western blotting. Briefly, the proteins were respectively separated on 10% SDS polyacrylamide gels and blotted onto nitrocellulose membranes which

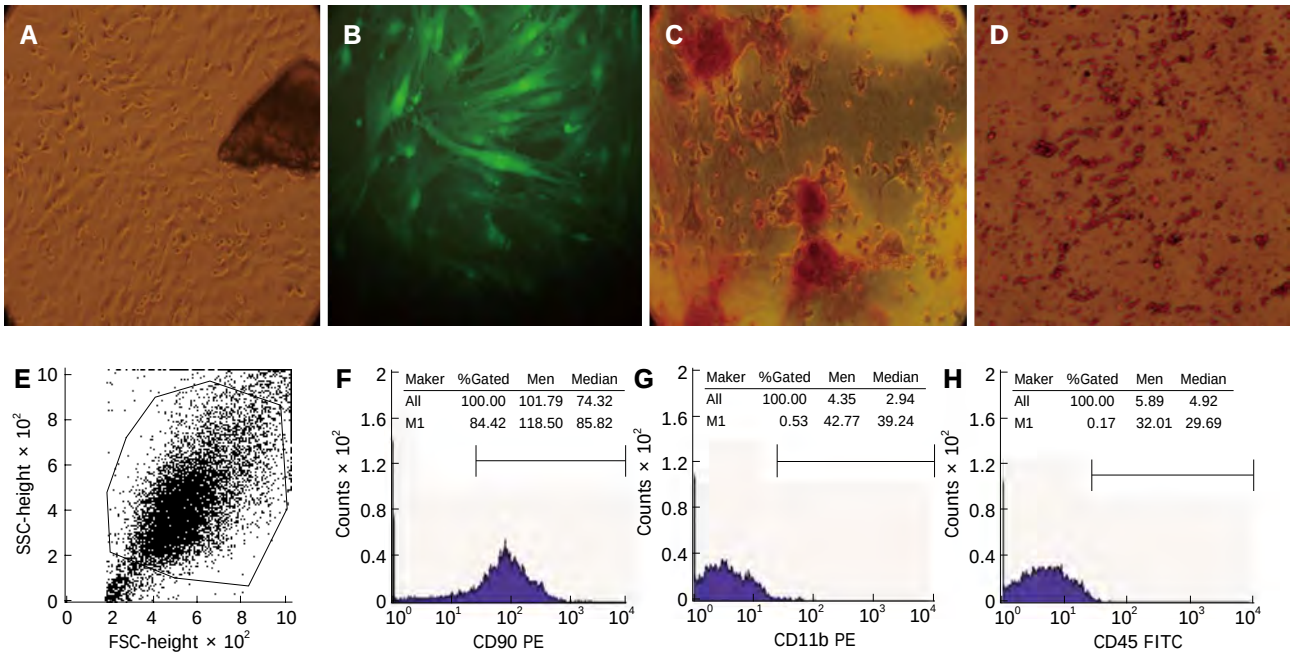

Figure 1 Morphological features, immunophenotypic characterization and differentiation assays of compact bone-derived mouse mesenchymal stem cells. A: At 72 h after initial culture, the cells migrate out from the enzyme-digested bone chips (× 100); B: Green fluorescent protein -bone marrow mesenchymal stem cells (BMSCs) arranged in a bunched or radiated shape without weakening of the green fluorescence after passages (× 400); C: Osteoplastic differentiation of BMSCs (× 400); D: Apoplastic differentiation of BMSCs (× 400); E-H: BMSCs' phenotypes detected by flow cytometry.

were incubated in PBST-milk (PBS buffer containing 0.1% Tween-20 and 5% milk), followed by primary antibodies (1 h) for mouse FoxP3 (1:400 dilution, PM024S, MBL International Co., Woburn, MA); T-bet (1:250 dilution, 14-5825, eBioscience, San Diego, CA); GATA-3 (1:250 dilution, 14-9966, eBioscience); transforming growth factor (TGF-β3) (III) (1:1000 dilution, sc-83, Biotechnology, Santa Cruz, CA); ROR gamma(t) (1:250 dilution, 14-6981, eBioscience). Loading controls were obtained using a goat anti-rabbit antibody (1:3000, CoWin Biotech Co., Beijing, China). Blots were then washed with PBST 3 times (10 min each) and subsequently incubated (1 h) with actin antibody (1:3000, Biotechnology, Santa Cruz, CA) diluted in PBST milk. Each Western blot was repeated at least twice. Bands were detected after exposure to Hyperfilm-MP (1:10000, Amersham International PLC, Buckinghamshire, United Kingdom). The bands were then detected with scanning densitometry using a Desaga Cab UVIS scanner and Desaga ProViDoc software (Desaga, Wiesloch, Germany).

Statistical analysis

Data are represented as mean ± SD. Statistical comparisons between experimental groups were performed with one-way ANOVA using SPSS 17.0 software. $P < 0.05$ was considered statistically significant.

RESULTS

Characterization of the putative GFP-BMSCs

The bone marrow-derived GFP-BMSCs were obtained by cultivation of collagenase Ⅱ digested bone fragments. At 72 h after initial culture, fibroblast-like cells were observed to migrate out from the bone fragments and adhere to the dish (Figure 1A). Some fibroblast colonies were observed after removing nonadherent cells and tissue debris when changing the culture medium. After 5 consecutive passages, the cell population started to demonstrate clustering and a radial pattern maintaining strong GFP expression (Figure 1B). The differentiation assays showed that these cells could differentiate into osteoblasts and adipocytes. For osteogenic differentiation, alizarin red staining was used after 21 d of culture and mineralized nodules were formed after induction (Figure 1C). For adipogenic differentiation, fat red particles were seen following Oil-red-O-staining after 8 d of culture (Figure 1D). Flow cytometry showed that these cells were homogenously positive for the mesenchymal marker CD90, but negative for hematopoietic markers CD11b and CD45 (Figure 1E-H).

Transplanted GFP-BMSCs migrated into the inflamed colon in TNBS-induced colitis

The transplanted GFP-BMSCs were found to be localized in the inflamed colon. Green fluorescence was mainly observed in the lamina propria, near the bottom of crypts 48 h after GFP-BMSCs transplantation (Figure 2A). No GFP-labeled MSCs were observed in non-inflamed tissues. *Sry* gene was detected in the TNBS-MSC-female group and the male-control group, but not in the TNBS-PBS-female group on day 9 (Figure 2B).

Clinical and mucosa healing of TNBS-induced colitis

TNBS instillation in 50% ethanol led to a substantial wasting disease caused by severe diarrhea, weight loss, decreased water/food consumption, piloerection and the

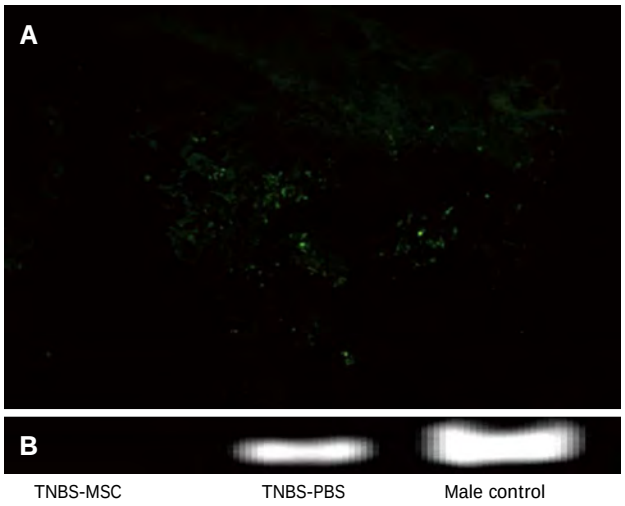

Figure 2 Localization of transplanted green fluorescent protein-bone marrow mesenchymal stem cells. A: Fluorescence in the lamina propria of colon using fluorescence microscopy (× 100); B: *Sry* gene expression (1188 bp)in the colons of 3 groups by polymerase chain reaction. TNBS-MSC: Trinitrobenzene sulfonic acid-mesenchymal stem cells; TNBS-PBS: Trinitrobenzene sulfonic acid-phosphate-buffered saline.

presence of rectal bleeding with about 55% mortality (Figure 3E). BMSCs significantly reduced colitis intensity in acute TNBS-induced colitis compared with the TNBS-PBS group assessed by DAI scores (Figure 3F). In the TNBS-MSC group, weight loss was improved from day 3 (-1.60 ± 0.45 g *vs* day 2 -4.71 ± 1.42 g, $P < 0.05$), but not in the TNBS-PBS group from day 5 (-4.40 ± 0.76 g *vs* day 3 -8.78 ± 1.08 g, $P < 0.01$) (Figure 3D). In the TNBS-MSC group, bloody stools decreased and became solid, while food and water intake also improved. We assessed the colon length in these 3 groups on day 0, 1, 2, 3, 5, 7 and 9. On days 3-7, macroscopic findings in the colon in the TNBS-PBS group included severe shortening, and wine-colored tissue with bloody stools compared to the TNBS-MSC group (Figure 3B). On day 3, a significant difference in colon length was observed between the TNBS-MSC group and TNBS-PBS group (11.05 ± 0.31 cm *vs* 7.43 ± 0.33 cm, $P < 0.01$) (Figure 3A and G). This significant difference was also seen in the TNBS-PBS group compared with the control group (7.43 ± 0.33 cm *vs* 11.68 ± 1.01 cm, $P < 0.01$) (Figure 3A and G).

In addition, histological changes in TNBS-induced colitis were mainly observed in the colon-rectum with severity progressively less towards the proximal site. In the TNBS-MSC group, the histological colitis score significantly decreased compared with the TNBS-PBS group on day 3 (3.00 ± 0.82 *vs* 8.75 ± 0.96, $P < 0.01$) (Figure 3C and H). BMSCs treatment reduced the extent of the inflamed area in the intestine. Compared with the TNBS-PBS group, TNBS-MSC reduced TNBS-induced crypt damage and infiltration of inflammatory cells composed mainly of neutrophils and macrophages (Figure 3H).

Expression of Ki67 and Lgr5 in colon tissues

To determine whether administration of BMSCs could promote proliferation and differentiation of intestinal epithelial cells into ISCs, the expression of Ki67 (one of the markers of cell proliferation) and Lgr5 (one of the markers of ISCs), were examined by immunohistochemistry. Four days after transplantation of BMSCs, the expression of Ki67 and Lgr5 in damaged colonic tissues increased significantly compared with TNBS-PBS (Ki67: 12.09% ± 3.95% *vs* 8.33% ± 3.1%, $P < 0.01$; Lgr5: 52.54 ± 14.77 × 10^3 *vs* 30.00 ± 8.08 × 10^3) (Figure 4).

BMSCs corrected the imbalance in T cell disorders in mice with TNBS-induced colitis. Since BMSCs are known to have *in vitro* immunosuppressive and anti-inflammatory properties[29], we investigated the potential therapeutic effects of BMSCs in an *in vivo* experimental model of IBD induced by TNBS. In TNBS-induced colitis, Th1-Th17 cells were activated to promote an exaggerated macrophage and neutrophil infiltration, giving rise to a prolonged severe transmural inflamed intestinal mucosa and immune response, characterized by the production of inflammatory cytokines and their transcription factors[30]. BMSCs can rapidly reduce inflammation by affecting the differentiation of T cells, such as promoting Th2 cells and enhancing regulatory T cell functions.

Transplanted MSCs affect immune responses systemically in IBD models

Cytokines are principal mediators of the systemic and local immune responses in immunological or inflammatory diseases. We examined the expression levels of 7 cytokines: IL-2, TNF-α, IFN-γ, IL-4, IL-10, IL-6, and IL-17. In acute TNBS-induced colitis, the level of proinflammatory cytokines (including IL-2, TNF-α, IFN-γ, IL-6, and IL-17) increased significantly ($P < 0.01$) when compared to the control group (Figure 5A-C, E and F). Following the transplantation of MSCs, the level of proinflammatory cytokines decreased significantly ($P < 0.01$), while the level of IL-4 and IL-10 increased ($P < 0.05$) when compared to the TNBS-PBS group (Figure 5A-G).

BMSCs inhibit the differentiation of Th1 cells in the gut in TNBS-induced colitis

The differentiation of Th1 lymphocytes is known to be associated with a specific transcription factor, T-bet, which is a key component regulating the expression of Th1 cytokines[31]. The up-regulation of Th1 activities in TNBS-induced colitis and the reduction of Th1 activities after BMSCs transplantation were further confirmed by the analysis of T-bet expression using both real-time PCR and Western blotting (Figure 6D and H). In addition, we analyzed the expression of IL-2, IFN-γ, and TNF-α by real-time PCR. There was an increase in Th1 activities in acute TNBS-induced colitis (*vs* the control group) and a decrease in Th1 activities after treatment with BMSCs (*vs* the TNBS-PBS group) (Figure 6A-C). FACS analysis of IL-2, IFN-γ and TNF-α proteins (days 1-9) also showed the same trend. In the TNBS-PBS group, IL-2, IFN-γ and TNF-α expressions were significantly up-regulated (*vs* the control group, IL-2: 4.37 ± 0.27 pg/mL *vs* 2.30 ± 0.03 pg/mL, $P < 0.01$; IFN-γ: 4.82 ± 0.11 pg/mL *vs* 3.64 ± 0.39 pg/mL, $P < 0.01$; TNF-α 32.45 ± 3.52 pg/mL *vs*

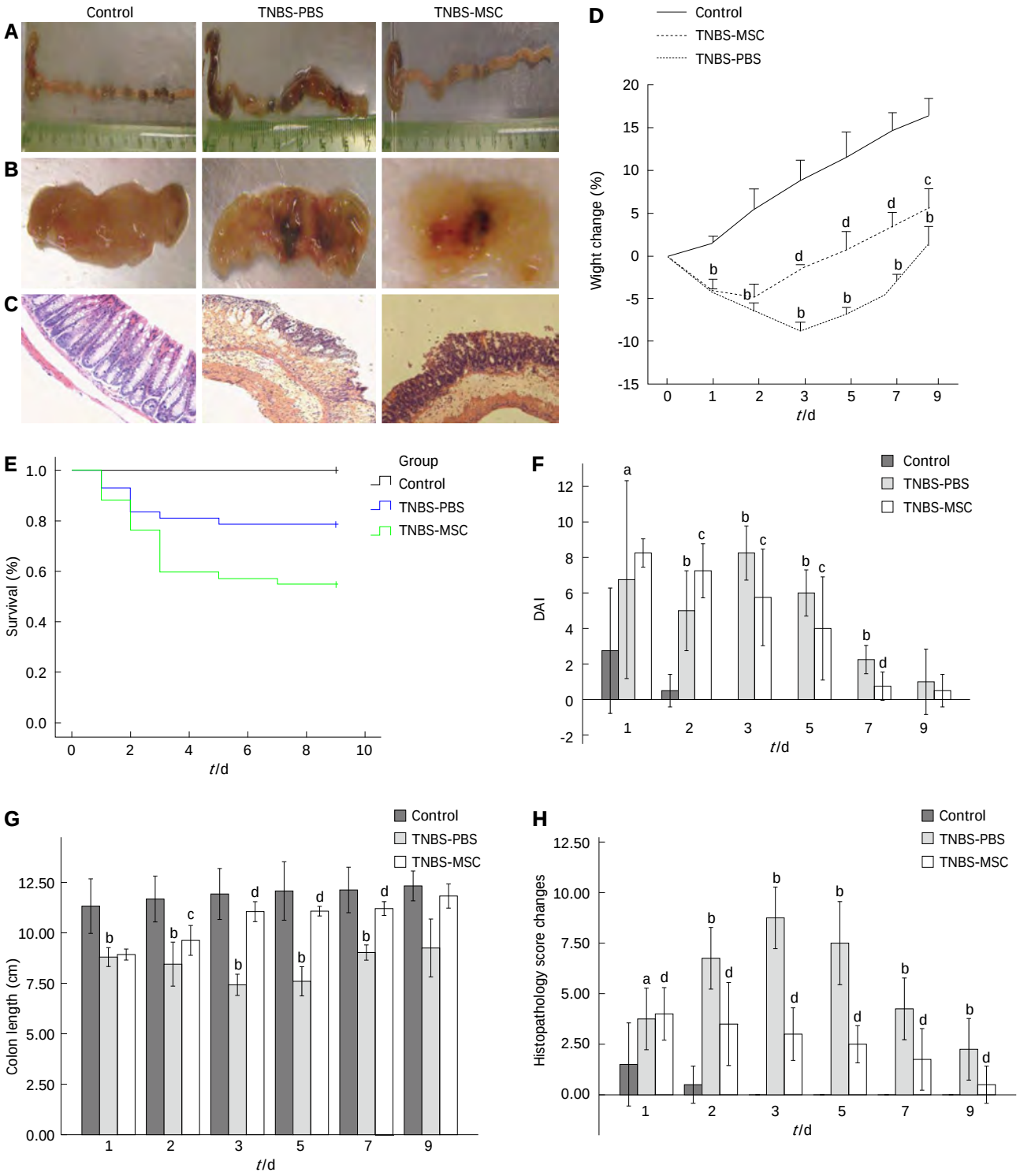

Figure 3 Therapeutic efficacy of bone marrow mesenchymal stem cells treatment. A: Colon length at day 3; B: Macroscopic colonic damage at day 3; C: Histopathologic colonic damage by hematoxylin and eosin staining at day 3 (× 100); D-F: Clinical evolutions were monitored by body weight changes, colitis score and survival; G) Colon length changes; H: Histopathology score changes. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* TNBS-PBS. TNBS-MSC: Trinitrobenzene sulfonic acid-mesenchymal stem cells; TNBS-PBS: Trinitrobenzene sulfonic acid-phosphate-buffered saline.

13.91 ± 0.94 pg/mL, $P < 0.01$). However, BMSCs treatment led to a distinct reduction in the above factors (*vs* the TNBS-PBS group, IL-2:2.37 ± 0.20 pg/mL *vs* 2.87 ± 0.25 pg/mL, $P < 0.05$; IFN-γ: 3.71 ± 0.17 pg/mL *vs* 4.44 ± 0.07 pg/mL, $P < 0.01$; TNF-α: 13.12 ± 1.76 pg/mL *vs* 19.45 ± 0.82 pg/mL, $P < 0.01$) (Figure 6E-G).

BMSCs promote Th2 differentiation in the gut in TNBS-induced colitis

A possible role for BMSCs in promoting the Th2 subset was investigated by analyzing IL-4 and IL-10 production as well as the Th2 lineage transcription factor GATA3. There was no significant change in GATA3 (day 3) ex-

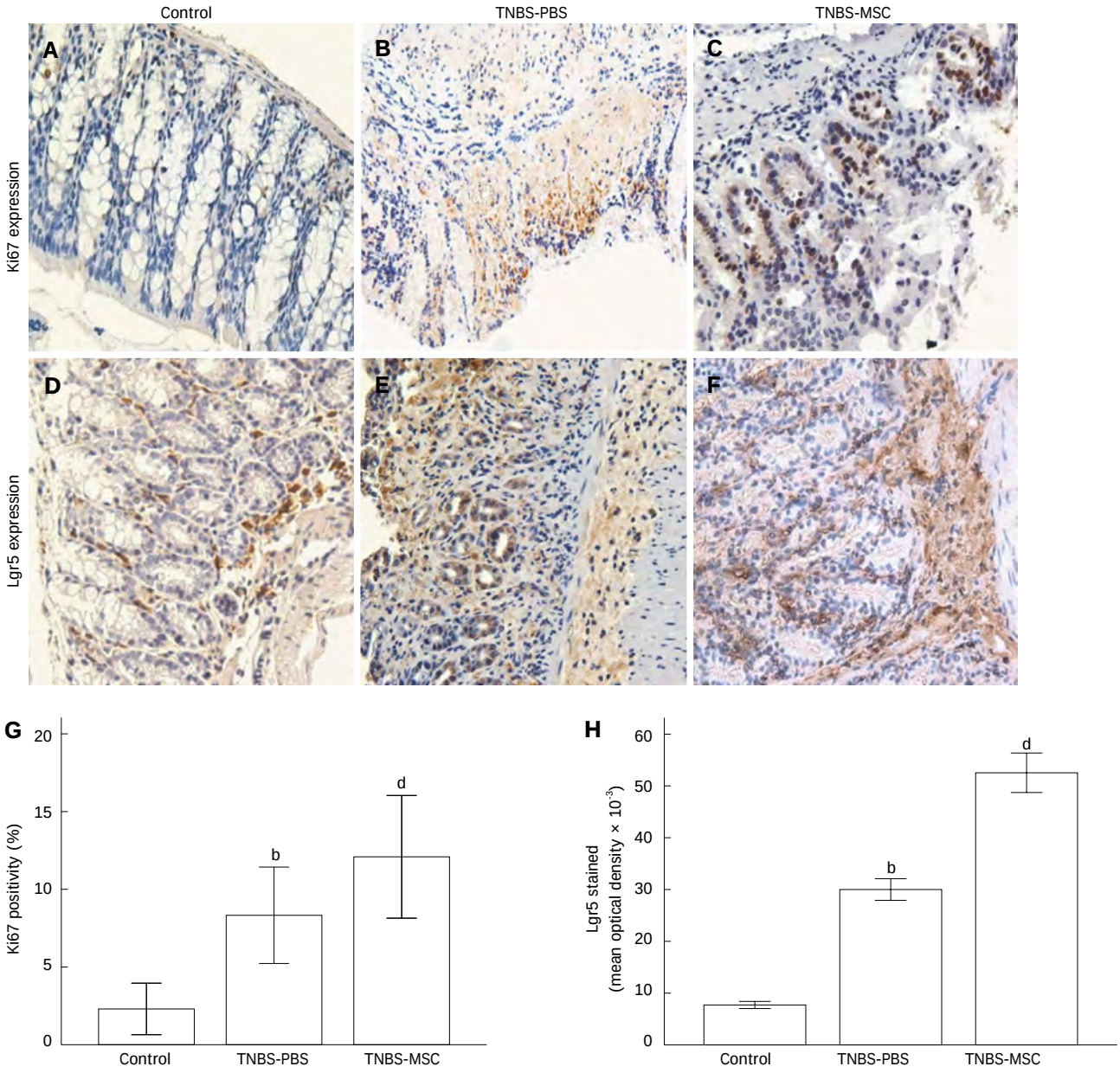

Figure 4 Immunohistochemistry of Ki67 and Lgr5 in colon tissues (× 200). A-C: Ki67; D-F: Lgr5; G, H: Five days after MSCs transplantation, Ki67 and Lgr5 expressions were significantly increased (*vs* control, $^{b}P < 0.01$; *vs* TNBS-PBS, $^{d}P < 0.01$). Ki67 staining was evaluated by positivity, and Lgr5 expression was represented by mean optical density. TNBS-MSC: Trinitrobenzene sulfonic acid-mesenchymal stem cells; TNBS-PBS: Trinitrobenzene sulfonic acid-phosphate-buffered

pression in the TNBS-PBS group, however, GATA3 expression was increased in the TNBS-MSC group confirmed by either real-time PCR or Western blotting (Figure 7C and F). IL-4 and IL-10 were also up-regulated at both the mRNA and protein levels in the TNBS-MSC group, while no significant changes were observed in TNBS-induced colitis (Figure 7A and B). The expression of IL-4 and IL-10 at the protein level (days 1-9) was further determined by FACS analysis and showed a similar pattern to that of the mRNAs. In the TNBS-MSC group, IL-4 and IL-10 were up-regulated (*vs* the TNBS-PBS group, IL-4: 2.15 ± 0.16 pg/mL *vs* 1.90 ± 0.15 pg/mL, $P < 0.05$; IL-10: 25.93 ± 0.63 pg/mL *vs* 19.09 ± 2.85 pg/mL $P < 0.01$). There were no significant changes in IL-4 and IL-10 in the TNBS-PBS group (*vs* the control group, $P > 0.05$) (Figure 7D and E).

BMSCs inhibit Th17 cell differentiation in the gut of mice with TNBS-induced colitis

Th17 cell differentiation requires at least two cytokine signals that transmit through the RORγt and Smad-dependent signaling pathways[32]. Real-time PCR and Western blot results of RORγt expression at day 7 revealed that BMSCs clearly inhibited the differentiation of pathogenic Th17 effector cells (Figure 8C and F). In this study, the expressions of IL-6 and IL-17 were significantly up-regulated in the TNBS-PBS group, but were down-regulated in the TNBS-MSC group shown by real-time PCR (Figure 8A and B). The protein expressions of IL-6 and IL-17 (days 1-9) were further determined by FACS and the re-

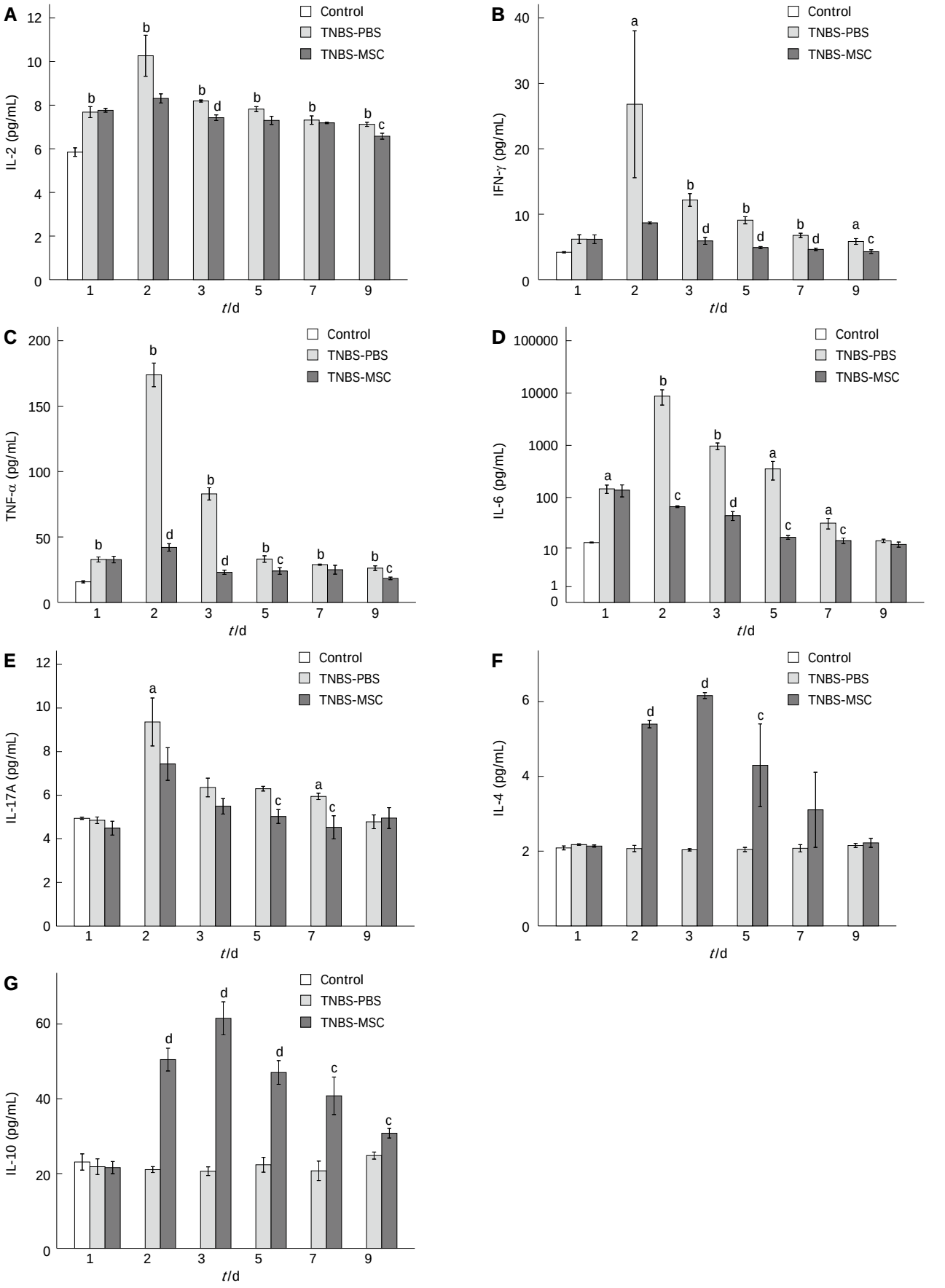

Figure 5 Levels of 7 cytokines were measured simultaneously from mouse serum using Fluorescence Activated Cell Sorting. A-E: TNBS-induced colitis exhibits heightened Th1-Th17 response of a complex, dynamic cytokine network [interleukin (IL)-2, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IL-6, IL-17], and MSCs maintain the balance of T-lymphocytes. F, G: In the TNBS-MSC group, transplanted MSCs promote T-lymphocytes to secrete cytokines (IL-4, IL-10) which regulate their functions. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* TNBS-PBS. TNBS-MSC: Trinitrobenzene sulfonic acid-mesenchymal stem cells; TNBS-PBS: Trinitrobenzene sulfonic acid-phosphate-buffered saline.

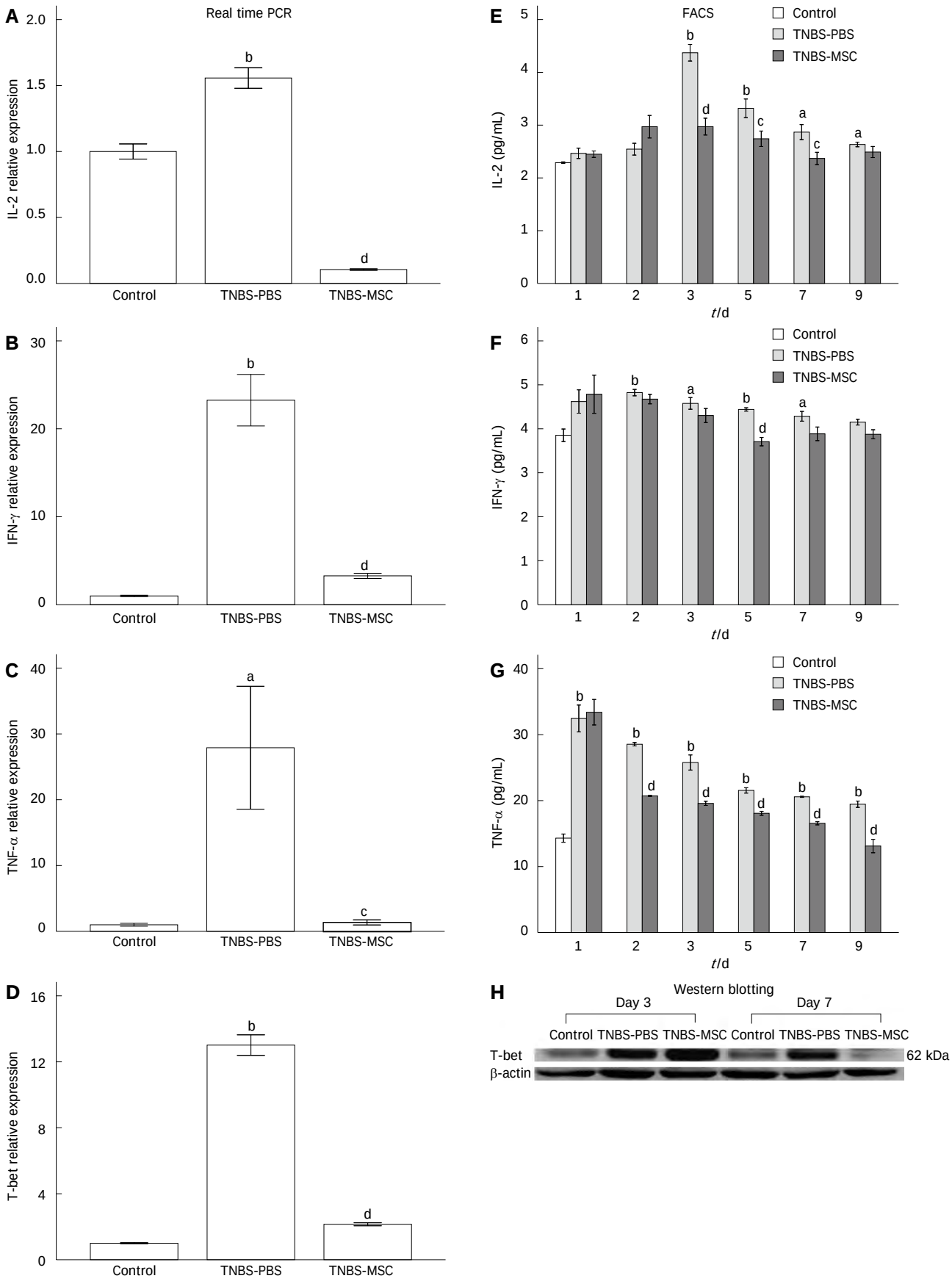

Figure 6 Changes in mRNA expression and colonic protein levels of Th1-related inflammatory mediators following treatment with bone marrow mesenchymal stem cells. A-D: Real-time polymerase chain reaction. Acute TNBS colitis demonstrated a cytotoxic and chemotactic profile with significantly elevated mRNA expressions of interleukin (IL)-2, interferon (IFN)-γ, tumor necrosis factor (TNF)-α and T-bet when compared to controls. Preventive treatment with BMSCs reduced the levels of the above cytokines; E-G: Flow cytometry. The changes in colonic protein levels of IL-2, IFN-γ and TNF-α followed the same trend; H: Western blotting. The changes in colonic protein levels of T-bet followed the same trend. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* TNBS-PBS. TNBS-MSC: Trinitrobenzene sulfonic acid-mesenchymal stem cells; TNBS-PBS: Trinitrobenzene sulfonic acid-phosphate-buffered saline.

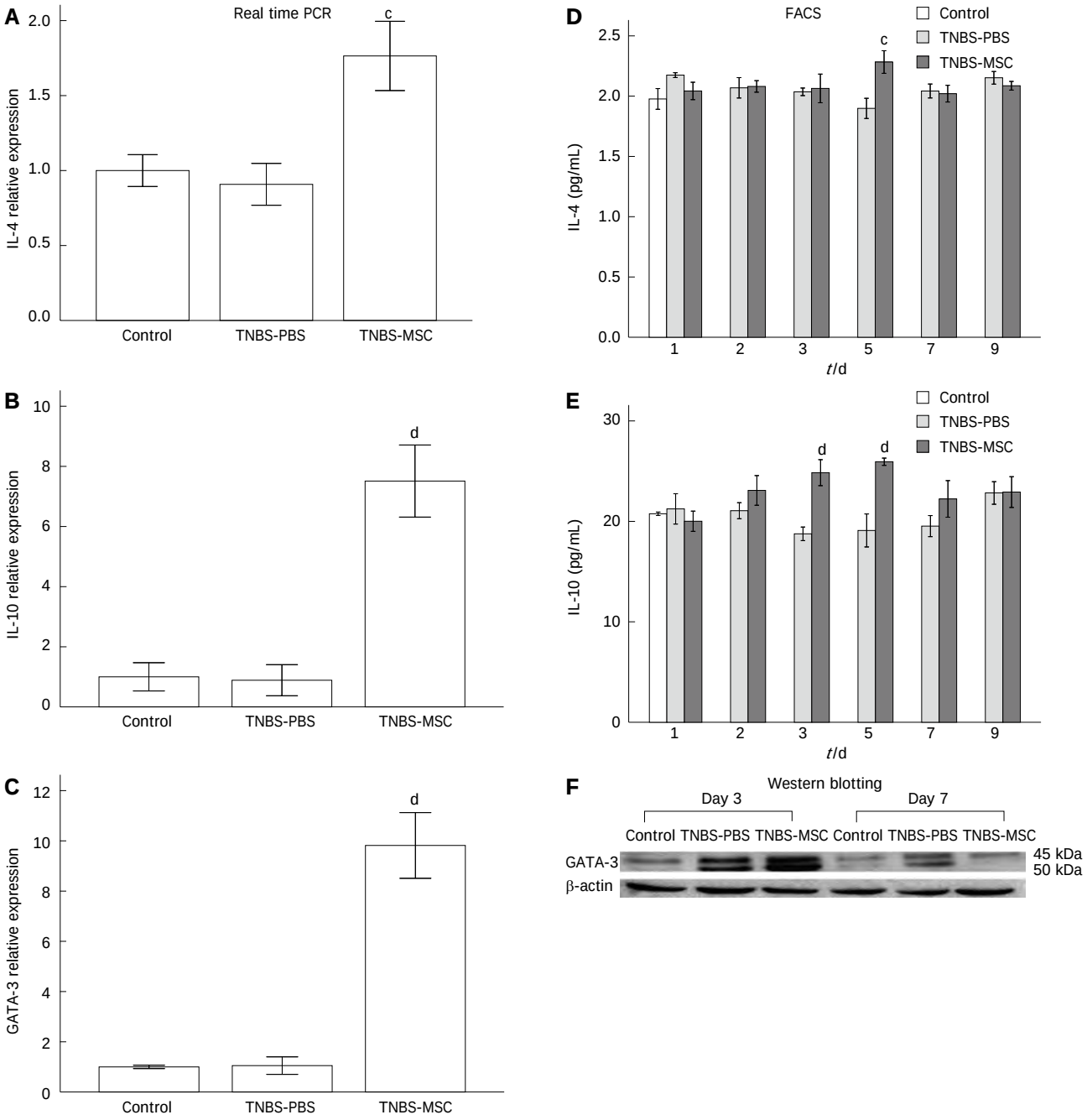

Figure 7 Changes in mRNA expression and colonic protein levels of Th2-related inflammatory mediators following treatment with bone marrow mesenchymal stem cells. A-C: Real-time polymerase chain reaction. No significant differences in mRNA expressions of interleukin (IL)-4, IL-10 and GATA family of transcription factors 3 (GATA-3) were observed when compared to controls ($P > 0.05$). After treatment with bone marrow mesenchymal stem cells, the mRNA expressions of IL-4, IL-10 and GATA-3 were elevated. D, E: Flow cytometry. Colonic protein levels of IL-4 and IL-10 followed the same trend; F: Western blotting. Colonic protein level of GATA-3 followed the same trend. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* TNBS-PBS. TNBS-MSC: Trinitrobenzene sulfonic acid-mesenchymal stem cells; TNBS-PBS: Trinitrobenzene sulfonic acid-phosphate-buffered saline.

sults showed the same trend as their mRNA expressions. In the TNBS-PBS group, IL-6 and IL-17A expressions were significantly up-regulated (*vs* the control group, IL-6: 14.52 ± 0.96 pg/mL *vs* 3.80 ± 0.52 pg/mL, $P < 0.01$; IL-17A: 4.97 ± 0.19 pg/mL *vs* 3.61 ± 0.29 pg/mL, $P < 0.01$). However, BMSCs treatment led to a distinct reduction in the above factors (*vs* the TNBS-PBS group, IL-6: 4.22 ± 0.40 pg/mL *vs* 7.50 ± 0.51 pg/mL, $P < 0.05$; IL-17A: 2.87 ± 0.21 pg/mL *vs* 4.54 ± 0.14 pg/mL, $P < 0.01$) (Figure 8D and E).

BMSCs enhance regulatory T cell functions

The suppressor cytokines IL-10 and TGF-β are also produced by $CD4^{+}Foxp3^{+}$ Tregs which are involved in the control of colitis as indicated in recent investigations[10,33]. BMSCs significantly up-regulated IL-10 as well as TGF-β levels ($P < 0.01$, Figure 7B, E, Figure 9A and C). The expression of Foxp3 also increased in the TNBS-MSC group when compared to the TNBS-PBS group ($P < 0.01$, Figure 9B and D). However, the production of IL-10, TGF-β and Foxp3 using real-time PCR and Western

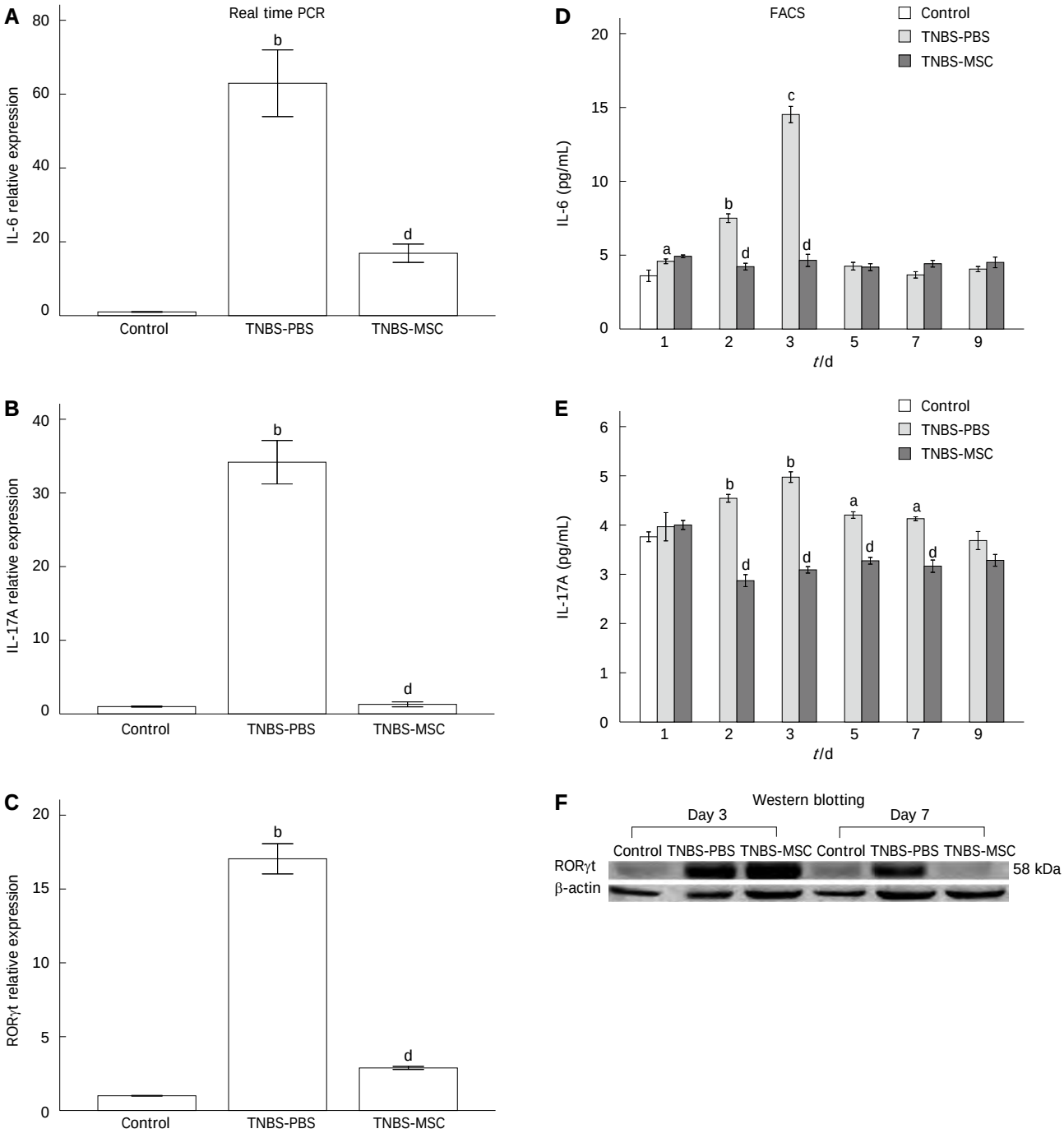

Figure 8 Changes in mRNA expression and colonic protein levels of Th17-related inflammatory mediators following treatment with bone marrow mesenchymal stem cells. A-C: Real-time polymerase chain reaction. The results showed a significant increase in mRNA expressions of interleukin (IL)-6, IL-17A and retinoid related orphan receptor gamma(t) (RORγt) when compared to controls ($P < 0.01$). After treatment with bone marrow mesenchymal stem cells, the levels of the above cytokines were reduced ($P < 0.01$); D, E: Flow cytometry. The same changes in colonic protein levels of IL-6 and IL-17A were further confirmed; F: Western blotting. The same changes in colonic protein levels of RORγt were further confirmed. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* TNBS-PBS. TNBS-MSC: Trinitrobenzene sulfonic acid-mesenchymal stem cells; TNBS-PBS: Trinitrobenzene sulfonic acid-phosphate-buffered saline.

blotting showed no changes in the TNBS-MSC group compared with the control group ($P > 0.05$, Figure 7B and E and Figure 9A-D).

DISCUSSION

Kashyap *et al*[34] reported a patient with non-Hodgkin lymphoma and CD who was maintained for more than 7 years in clinical remission after autologous hematopoietic stem cell transplantation. Since then, more and more studies have focused on the use of stem cell therapy in IBD. The results from our study were consistent with previous reports, which demonstrated that intravenously transplanted BMSCs may home to injured intestinal tissues, promote intestinal cell regeneration, ameliorate the inflammatory symptoms in an experimental IBD mouse model and increase survival[35,36]. However, our study also identified the following: (1) We showed the differentia-

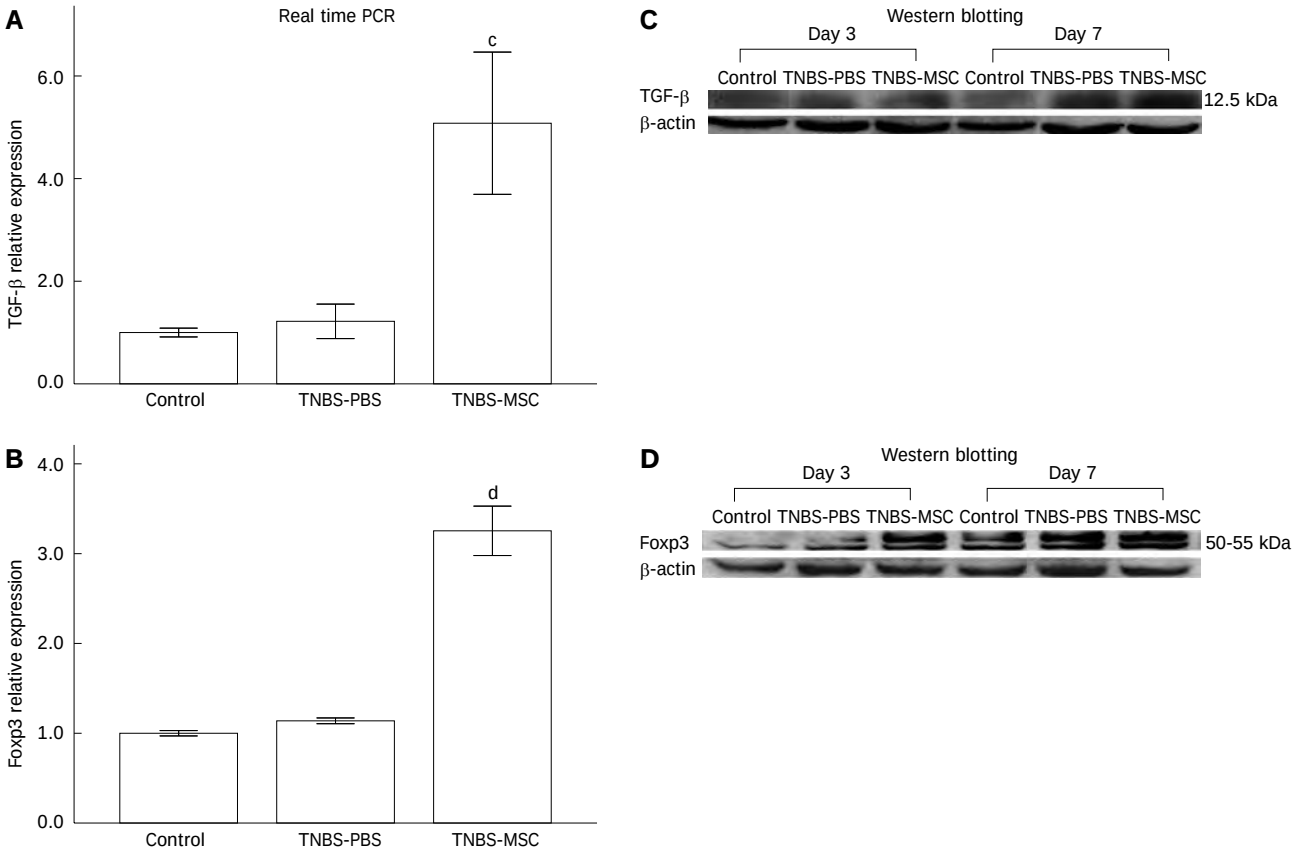

Figure 9 Changes in mRNA expression and colonic protein levels of Tregs-related inflammatory mediators following treatment with bone marrow mesenchymal stem cells. A, B: Real-time polymerase chain reaction. No significant changes in mRNA expressions of transforming growth factor (TGF)-β and Foxp3 were observed when compared to controls ($P > 0.05$). While the mRNA expressions of TGF-β and Foxp3 increased after BMSCs transplantation ($P < 0.01$); C, D: Western blotting. The colonic protein level of TGF-β and Foxp3 were confirmed to show the same results as real-time polymerase chain reaction. $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* TNBS-PBS. TNBS-MSC: Trinitrobenzene sulfonic acid-mesenchymal stem cells; TNBS-PBS: Trinitrobenzene sulfonic acid-phosphate-buffered saline.

tion of ISCs in injured gut by detecting Lgr5 positive cells; (2) We examined the expression levels of Th1-Th2-Th17-Tregs-related inflammatory and regulatory cytokines in peripheral blood and in local intestinal tissues; (3) We found a Th2 shift and subsequent correction of imbalanced Th17/Tregs; (4) Master regulators of Th1, Th2, Th17 and Tregs were detected in BMSCs-treated TNBS-induced colitis; and (5) We showed that the signaling pathways of Th1/T-bet, Th2/GATA-3, Th17/RORγt and Tregs/Foxp3 may serve as important immunoregulators in the correction of immune disorders and can enhance the healing of injured intestinal mucosa.

In our study, we established a mouse model of TNBS-induced colitis and transplanted BMSCs into the mice. On day 1, 2, 3, 5, 7 and 9 after transplantation, the mice were sacrificed. Moreover, 48 h after BMSCs transplantation, we found that a small proportion of the infused BMSCs had homed to the inflammatory or injured tissues by observing GFP and detecting *Sry* gene (only located on the Y chromosome). In addition, we investigated the role BMSCs played in TNBS-induced colitis at different time points by assessing the expression of factors, pathological changes, and clinical symptoms. We found that BMSCs had a strong therapeutic effect on TNBS-induced colitis. We also detected the expression of Ki67 (one of the markers of cell proliferation) and Lgr5 (an intestinal stem cell marker) using immunohistochemistry. The significantly increased expression of Ki67 and Lgr5 after BMSCs transplantation suggests that these BMSCs in recipient colonic tissues may have trans-differentiated into ISCs. Taken together, we showed that there are two possible therapeutic mechanisms of BMSCs treatment. First, some of these BMSCs could directly differentiate into pluripotent stem cells, such as ISCs. Second, MSCs could secrete cytokines or chemokines to influence the imbalanced intestinal micro-environment and stimulate the tissue-specific ISCs to proliferate. However, these experimental findings might be an indirect consequence of the general improvement in tissue regeneration of acute colitis mediated by BMSCs and the detailed mechanism involved requires further clarification.

The mechanism could be as follows: First, when BMSCs were intravenously transplanted into the experimental animal model, they first circulated around the whole body through blood circulation. During this process, BMSCs secrete related cytokines to activate the systemic immune system. Second, these BMSCs migrated, scatter implanted and survived within the injured gut mucosa and subsequently reversed the imbalance in Th1/Th2/Th17/Tregs, which is important in maintaining the intestinal mucosa microenvironment.

In IBD, the ratio of pro-inflammatory (IL-2, TNF-α,

IFN-γ, IL-6 and IL-17) and anti-inflammatory cytokines (IL-4 and IL-10) are imbalanced systematically. In our study, we found that intravenously transplanted MSCs were able to modulate the release and/or expression of pro-inflammatory cytokines in the serum by effectively inducing remission and promoting the release and/or expression of anti-inflammatory cytokines in the serum to balance inappropriate immune system disorders systemically. These appropriate responses would finally slow down and/or reverse the natural course of the disease and even prevent complications such as fistulae and colorectal cancer.

Little work has been carried out on how BMSCs display their immune regulatory and anti-inflammatory properties in damaged colonic tissues. According to previous investigations, BMSCs can efficiently suppress the proliferative response of T cells *in vitro*[37,38]. Our study showed that the complex immune-modulating effects of GFP-BMSCs may result from the differential down-regulation of proinflammatory signaling of Th1 and Th17 lymphocytes as assessed by analysis of IL-2, TNF-α, IFN-γ (Th1-related) and IL-6, IL-17 (Th17-related), and the up-regulation of anti-inflammatory signaling of Th2 activities with IL-4 and IL-10. This also led to a moderate induction of induced/activated regulatory T cells (IL-10, TGF-β), which may influence Th1 and Th17 cell function. Naive T helper cells (Th0) can be induced to differentiate into Th1, Th2, Th17 and regulatory (Treg) phenotypes based on the mode of stimulation, antigen concentration, co-stimulation and cytokine milieu[39]. These factors exert their functions by cross-regulating one another and are selectively expressed in the corresponding cell populations[31,40]. Our experiments showed that when MSCs migrated into the inflamed colon, they began to produce cytokines, chemokines, growth factors and adhesion molecules by themselves or promoted intestinal lymphocytes to regulate the inappropriate inflammatory responses.

In this study, we found significant inhibition of the production of Th1-cytokines such as IL-2, TNF-α, IFN-γ and the Th1-specific transcription factor, T-bet, using real-time PCR, FACs and/or Western blotting, suggesting that MSCs can alter the inflammatory process by down-regulating Th1-driven autoimmune and inflammatory responses. We also found increased expression of IL-4 and GATA-3 using the same methods. In conjunction with the changes in Th1-cytokines, this showed that MSCs may shift the pathways of differentiation towards Th1 and Th2 cells with IL-4 signaling. This is possibly due to the fact that T-bet expression inhibits GATA-3 activity and Th2 cytokines block the differentiation of Th1 cells[41,42]. In this study, the scenarios are very complex. (1) The transplanted MSCs had their own effect in secreting cytokines; (2) Endogenous MSCs can have similar or different effects; (3) The transplanted or endogenous MSCs may migrate to inflamed tissues and recruit Th1/Th2/Th17/Tregs; (4) The transplanted or endogenous MSCs may secrete cytokines/chemokines/growth factors and exert remote effects; (5) Transplanted or endogenous MSCs may affect local ISCs and change the local environment; and (6) Transplanted MSCs could recruit endogenous MSCs or ISCs to the local colon; and even more complex, the combination of all of them or some of them.

In addition to suppressive effects on Th1-activated immune and inflammatory responses, there is ample evidence from our experiments to suggest that MSCs also mediate the modulation of Th17-cytokines such as IL-17 and IL-6 to ameliorate TNBS-induced colitis. Our data showed high mRNA and protein levels of IL-17, RORγt and IL-6 in colonic tissue, but decreased levels in the MSCs-treated group. The reason for this may be that Th17 cells, characterized by expression of IL-17 (also known as IL-17A), differentiate from naive T helper cells in the presence of IL-6 and TGF-β[43]. RORγt is an essential factor for Th17 differentiation[43]. Previous experiments showed that TGF-β and IL-6 enhanced RORγt mRNA expression in naive T cells, which in turn induced IL-17A expression. We also demonstrated that the mRNA and protein levels of IL-10, TGF-β and Foxp3 were increased after administration of MSCs. This indicated that MSCs could restore the balance between Th17 cells and $CD4^+CD25^+Foxp3^+$ Treg in the intestinal tissues. Foxp3 directly interacts with RORγt to inhibit its function, resulting in decreased IL-17 expression[44,45]. IL-10 produced by regulatory subsets of T cells exerts a variety of anti-inflammatory and immunoregulatory functions linked with Foxp3 *in vivo*[46].

In conclusion, we have shown that BMSCs have a possible therapeutic effect in Th1- or Th17-driven IBD, including (1) homing to and surviving in the injured location; (2) exerting an immunoregulatory effect and controlling inflammation systematically; (3) accelerating colon mucosa regeneration; and (4) orchestrating a shift from Th1 and Th17 toward Th2 and the enhanced activities of Tregs to suppress local inflammation in colon tissues. Thus, exogenous MSCs transplantation is a novel therapeutic strategy for human IBD. However, for further clinical application, there are some unresolved questions owing to the complexity of human IBD. These include: (1) When is the best time for BMSCs treatment? (2) What dosage of BMSCs should be used to achieve optimal therapeutic results with least side effects? and (3) Should a combination of other drugs be used?

ACKNOWLEDGMENTS

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COMMENTS

Background

Inflammatory bowel disease (IBD) is a chronic disease characterized by severe

T-helper cell-driven inflammation and immune disorder. Mounting evidence suggests that mesenchymal stem cells (MSCs) have properties including low immunogenicity, immunomodulation and anti-inflammatory activity both *in vitro* and *in vivo*, and especially regulate T-cell responses. However, the molecular mechanisms involved in these effects are still unclear. Therefore, we transplanted MSCs into an experimental model of IBD to investigate their potential therapeutic effects *in vivo*.

Research frontiers

MSCs can release soluble factors (cytokines, chemokines, and growth factors) which result in cell cycle arrest in pro-inflammatory lymphocytes and induce T cell apoptosis. Many studies have shown that these cells are a potential treatment for autoimmune diseases. However, there have been few reports on the roles of MSCs in IBD and the molecular mechanisms of MSCs in alleviating IBD.

Innovations and breakthroughs

The results demonstrated changes in Th1-Th2-Th17-Tregs-related inflammatory and immune cytokine expressions and master regulators of these immune cells in serum and local intestinal tissues after MSCs transplantation. The authors found that MSCs resulted in a Th2 shift and correction of the imbalanced Th17/Tregs to enhance the healing of injured intestinal mucosa.

Applications

This data will contribute to future research on the immunomodulatory properties of MSCs and support a rationale for the clinical application of stem cell therapy in IBD.

Terminology

T-bet is a T-isolated box gene family of transcription factors, which is selectively expressed on Th1 cells. Retinoic acid-related orphan receptor γt (RORγt) is the orphan nuclear receptor that regulates the development of Th17 cells. GATA family of transcription factors 3 (GATA3) plays a central role in Th2 differentiation. Forkhead box P3 (Foxp3) acts as a master switch governing the development and function of $CD4^+$ regulatory T cells.

Peer review

In this manuscript, the authors studied the influence of bone marrow stem cells on colitis. This is an interesting study showing the mechanisms by which MSCs attenuate colitis. However, the direct contribution of transplanted MSCs in $Ki67^+$ proliferation assay and the data showing an increase in interleukin (IL)-4, IL-10, tumor growth factor-β and Foxp3 in inflamed tissue is are not expressed in transplanted MSCs, but in infiltrating immune cells.

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BRIEF ARTICLE

Probiotic supplementation decreases intestinal transit time: Meta-analysis of randomized controlled trials

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Abstract

AIM: To determine the efficacy of probiotic supplementation on intestinal transit time (ITT) and to identify factors that influence these outcomes.

METHODS: A systematic review of randomized controlled trials (RCTs) of probiotic supplementation that measured ITT in adults was conducted by searching MEDLINE and EMBASE using relevant key word combinations. Main search limits included RCTs of probiotic supplementation in healthy or constipated adults that measured ITT. Study quality was assessed using the Jadad scale. A random effects meta-analysis was performed with standardized mean difference (SMD) of ITT between probiotic and control groups as the primary outcome. Meta-regression and subgroup analyses were conducted to examine the impact of moderator variables on ITT SMD.

RESULTS: A total of 11 clinical trials with 13 treatment effects representing 464 subjects were included in this analysis. Probiotic supplementation was associated with decreased ITT in relation to controls, with an SMD of 0.40 (95%CI: 0.20-0.59, $P < 0.001$). Constipation ($r^2 = 39\%$, $P = 0.01$), higher mean age ($r^2 = 27\%$, $P = 0.03$), and higher percentage of female subjects ($r^2 = 23\%$, $P < 0.05$) were predictive of decreased ITT with probiotics in meta-regression. Subgroup analyses demonstrated statistically greater reductions in ITT with probiotics in subjects with *vs* without constipation and in older *vs* younger subjects [both SMD: 0.59 (95%CI: 0.39-0.79) *vs* 0.17 (95%CI: -0.08-0.42), $P = 0.01$]. Medium to large treatment effects were identified with *Bifidobacterium Lactis* (*B. lactis*) HN019 (SMD: 0.72, 95%CI: 0.27-1.18, $P < 0.01$) and *B. lactis* DN-173 010 (SMD: 0.54, 95%CI: 0.15-0.94, $P < 0.01$) while other single strains and combination products yielded small treatment effects.

CONCLUSION: Overall, short-term probiotic supplementation decreases ITT with consistently greater treatment effects identified in constipated or older adults and with certain probiotic strains.

Key words: Constipation; Gastrointestinal; Intestinal transit time; Meta-analysis; Probiotics

Core tip: Clinical trials of probiotics for gut health often utilize intestinal transit time (ITT) as a measure of clinical success although treatment effects are not consistent across studies. We performed the first systematic review and meta-analysis of randomized controlled trials to investigate the efficacy of probiotic supplementation on ITT in adults and to identify factors that influence these outcomes. Overall, short-term probiotic supplementation decreases ITT with consistently greater treatment effects identified in constipated or older adults and with certain probiotic strains.

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trials. *World J Gastroenterol* 2013; 19(29): 4718-4725 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i29/4718.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i29.4718

INTRODUCTION

Functional gastrointestinal (GI) disorders are symptom-based conditions that are not explained by definable structural or biochemical causes[1]. The prevalence of at least one functional GI disorder in the last 3 mo has been reported to be as high as 69% in the general population[2]. Slow intestinal transit is a common symptom of functional GI disorders, particularly those involving the bowel[3]. Therapies intended to ameliorate GI-related symptoms by decreasing intestinal transit time (ITT), such as laxatives, are a mainstay treatment of slow-transit bowel disorders although no known therapy is highly efficacious, safe, and cost effective[4].

Probiotics are live micro-organisms that confer a health benefit on the host when administered in adequate dosages[5], which have been extensively studied for treatment of functional GI disorders[6,7]. Additionally, there is speculation that probiotics may even improve gut health in healthy adults. For example, the European Food Safety Authority (EFSA) guidance on health claims related to gut function states that reduced ITT may be considered a beneficial physiological effect in the non-diseased general population, provided that diarrhea does not develop[8]. Consequently, ITT often serves as a primary study endpoint in probiotic clinical trials of gut health.

Based on the recent emphasis in this study endpoint in clinical trials and because accurate estimates of ITT effect size are mandatory for performing power calculations and estimating sample size in clinical trials, we performed the first systematic review and meta-analysis on the efficacy of probiotic supplementation on ITT in adults.

MATERIALS AND METHODS

The main objective of this systematic review and meta-analysis of RCTs was to assess the efficacy of probiotic supplementation on ITT in adults. The PRISMA Statement for reporting systematic reviews and meta-analyses served as a template for this report[9].

Eligibility criteria and information sources

Studies that were eligible for consideration in this systematic review were RCTs published in English-language journals and indexed in MEDLINE or EMBASE with no date restrictions on the effects of probiotic supplementation on ITT in adults. The following search terms were used for probiotic supplementation (with "*" characterizing a wildcard and "OR" being used as a Boolean function): probiotic*; lactobacill*; bifidobacteri*; yogurt; yoghurt; fermented milk. The following search terms were used for ITT: gastrointestinal; transit; gut; motility; colonic; constipation; irritable bowel. To identify clinical trials, we applied the filters Clinical Trial or Randomized Controlled Trial. The results of each of the three sections were combined by utilizing the "AND" Boolean. In addition, we attempted to identify additional studies by hand-searching references of included studies and relevant review articles.

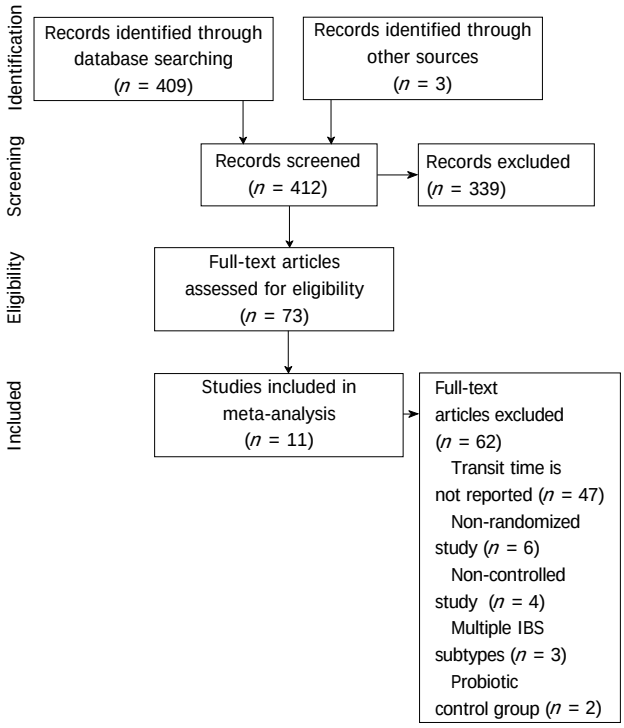

Figure 1 PRISMA flow diagram. IBS: Irritable bowel syndrome.

Study selection

One reviewer (Miller LE) initially assessed study eligibility. Titles and abstracts were screened to exclude all manuscripts published in non-English journals. Next, review articles, commentaries, letters, and case reports were excluded. We also excluded obviously irrelevant articles. Lastly, we excluded studies of subjects where ITT reduction was undesirable or uninterpretable (*i.e.*, subjects with diarrhea or cohorts with multiple IBS subtypes). Full-text of the remaining manuscripts was retrieved and reviewed. Publications that failed to report ITT or that described non-randomized, non-controlled, or otherwise irrelevant studies were excluded. The last search was performed in December 2012.

Data collection process

Data were extracted and entered into a pre-designed database by one reviewer (Miller LE) and the entries were checked by the other reviewer (Ouwehand AC). Disagreements were settled by consensus.

Data items

The following variables were recorded in a pre-designed database: general manuscript information (author, in-

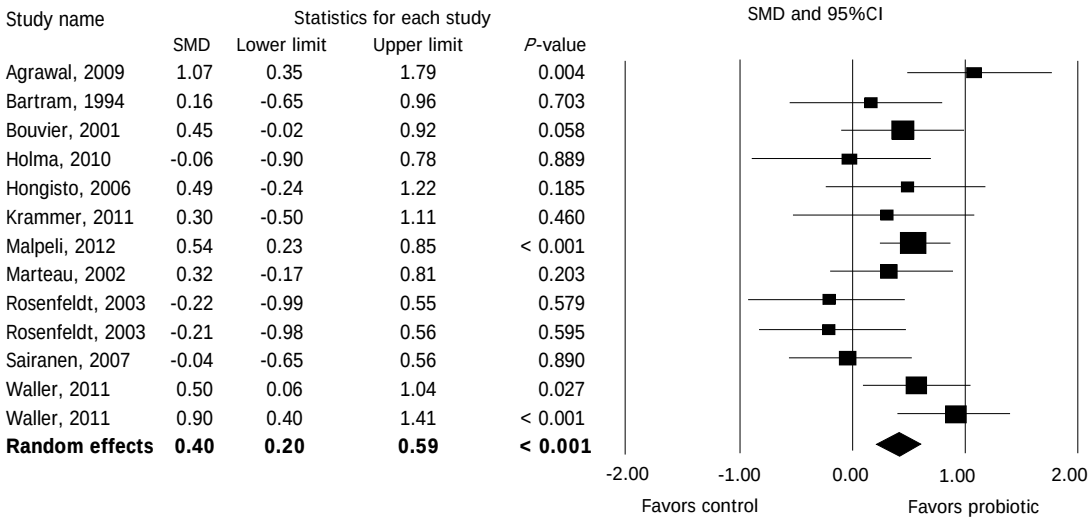

Figure 2 Forest plot of standardized mean difference in intestinal transit time across studies. Random effects model. I^2 = 29%, P = 0.15. SMD: Standardized mean difference.

stitution name and location, journal, year, volume, page numbers), study design characteristics (study quality, study design, sample size, method of ITT assessment, probiotic strain, daily dosage, product delivery method, and treatment duration), subject characteristics (age, gender, body mass index, and condition), and ITT before and after probiotic supplementation.

Quality assessment

We used the Jadad scale to assess study quality of RCTs[10]. Studies were scored according to the presence of three key methodological features: randomization, blinding and subject accountability. Randomization was scored from 0 to 2 with 2 implying appropriate methods of randomization were described, 1 if the study was merely described as "randomized", and 0 when no details were provided to evaluate randomization. A score of 0 was given if the study was described as randomized, but the method of randomization was clearly inappropriate. Similarly, blinding was scored from 0 to 2 with 2 points awarded if subjects and investigators were blinded using appropriate methods, 1 point if the study was described merely as blinded, and 0 points if the study was described as blinded, but the method of blinding was clearly inappropriate. Subject accountability was scored 0 or 1 with 1 point awarded if all subjects were accounted for in the analysis and reasons for withdrawals were provided. A score of 0 was given when information regarding withdrawals was incomplete. *A priori*, studies with a Jadad score of 3 to 5 were deemed higher quality and those with a score of 0 to 2 were classified as lower quality.

Statistical analysis

A random effects meta-analysis model was selected *a priori* based on the assumption that the true effect may vary among studies based on known differences in probiotic strain, study design characteristics, and subject characteristics. The standardized mean difference (SMD) and 95%CI was selected to report treatment effects because different measures of ITT (*e.g.*, whole gut, colonic, oro-cecal, *etc.*) were utilized in the included studies. The SMD is a measure of effect size for continuous outcomes defined as the mean difference between groups divided by the pooled standard deviation. SMD values of 0.2, 0.5 and 0.8 are defined as small, medium, and large, respectively[11]. A forest plot was used to illustrate the individual study findings and the random effects meta-analysis results. We used the I^2 statistic to estimate heterogeneity of effects across studies with values of $\leqslant$ 25%, 50%, and $\geqslant$ 75% representing low, moderate, and high inconsistency, respectively[12]. An alpha error $P <$ 0.05 and/or $I^2 \geqslant$ 50% were taken as indicators of substantial heterogeneity of treatment effects. Publication bias was visually assessed with a funnel plot (not shown) and quantitatively assessed using Egger's test[13]. Meta-regressions and pre-defined subgroup analyses were undertaken to quantify the relationship of individual moderators on ITT SMD. All analyses were performed using Comprehensive Meta-analysis (version 2.2, Biostat, Englewood, NJ, United States).

RESULTS

Study selection

Our initial database search retrieved 409 titles and abstracts and hand searching relevant bibliographies identified 3 additional records. After screening records for inclusion criteria, 73 full text articles were reviewed for eligibility. Ultimately, 11 RCTs with 13 treatment effects representing 464 distinct subjects were included in the final analysis[14-24]. A flow chart of study identification and selection is shown in Figure 1.

Study characteristics

Sample sizes were generally small, ranging from 10 to 36 per treatment group for parallel groups designs and

Table 1 Study characteristics

Ref.	Study design	Active: control (*n*:*n*)	Transit time outcome, method	Probiotic strain	Daily dosage (10^9 cfu)	Delivery method	Treatment duration (d)
Agrawal *et al*[14]	Parallel groups	17:17	CTT, radiopaque markers	*B. lactis* DN-173 010	25.0	Active: Yogurt + probiotic Control: Nonfermented milk-based product	28
Bartram *et al*[15]	Cross-over	12	OATT, radiopaque markers	*B. longum*	> 0.5	Active: Yogurt with 2.5 g lactulose + probiotic Control: Yogurt	21
Bouvier *et al*[16]	Parallel groups	36:36	CTT, radiopaque markers	*B. lactis* DN-173 010	97.5	Active: Probiotic fermented milk Control: Heat-treated probiotic fermented milk	11
Holma *et al*[17]	Parallel groups	12:10	TITT, radiopaque markers	*L. rhamnosus* GG	20	Active: Buttermilk + probiotic and white wheat bread Control: White wheat bread	21
Hongisto *et al*[18]	Parallel groups	16:14	TITT, radiopaque markers	*L. rhamnosus* GG	15	Active: Yogurt + probiotic and low fiber toast Control: Low fiber toast	21
Malpeli *et al*[19]	Cross-over	83	OCTT, carmine red dye	*B. lactis* BB12 *L. casei* CRL 431	2-20 2-12	Active: Yogurt with 0.625 g inulin and oligofructose + probiotic Control: Yogurt	15
Marteau *et al*[20]	Cross-over	32	CTT, radiopaque markers	*B. lactis* DN-173 010	18.75	Active: Yogurt + probiotic Control: Yogurt	10
Rosenfeldt *et al*[21]	Cross-over	13	GTT, radiopaque markers	*L. rhamnosus* 19070-2, *L. reuteri* DSM 12246	20 20	Active: Freeze-dried powder + probiotic Control: Skimmed milk powder w/dextrose	18
Rosenfeldt *et al*[21]	Cross-over	13	GTT, radiopaque markers	*L. casei subsp. alactus* CHCC 3137, *L. delbrueckii subsp. lactis* CHCC 2329, *L. rhamnosus* GG	20 20 20	Active: Freeze-dried powder + probiotic Control: Skimmed milk powder w/dextrose	18
Sairanen *et al*[22]	Parallel groups	22:20	CTT, radiopaque markers	*B. longum* BB536, *B. lactis* 420, *L. acidophilus* 145	2.4-18[1] 0.48	Active: Probiotic fermented milk Control: Fermented milk	21
Waller *et al*[23]	Parallel groups	33:34	WGTT; radiopaque markers	*B. lactis* HN019	1.8	Active: Capsule, maltodextrin, probiotic Control: Capsule, maltodextrin	14
Waller *et al*[23]	Parallel groups	33:34	WGTT; radiopaque markers	*B. lactis* HN019	17.2	Active: Capsule, maltodextrin, probiotic Control: Capsule, maltodextrin	14
Krammer *et al*[24]	Parallel groups	12:12	CTT, radiopaque markers	*L. casei* Shirota	6.5	Active: Probiotic fermented milk drink Control: Nonfermented milk drink	28

[1]Represents the reported range of total *Bifidobacterium* spp. cfu: Colony-forming units; CTT: Colonic transit time; GTT: Gastrointestinal transit time; OATT: Oro-anal transit time; OCTT: Oro-cecal transit time; TITT: Total intestinal transit time; WGTT: Whole gut transit time. *L. rhamnosus*: *Lactobacillus rhamnosus*; *B. lactis*: *Bifidobacterium lactis*; *L. casei*: *Lactobacillus casei*.

from 12 to 83 for cross-over designs. The average detectable effect size, based on sample size and study design by assuming $P = 0.05$ and statistical power = 80%, was 0.8 (range: 0.3 to 1.3). Eleven RCTs contributed one treatment effect each. The study of Rosenfeldt *et al*[21] contributed two treatment effects (two different probiotic formulations) and the study of Waller *et al*[23] contributed two treatment effects (same probiotic strain, two different dosages). Eight of the 11 studies were parallel groups designs while 3 were cross-over studies. The most commonly studied probiotic strains were *Bifidobacterium lactis* (*B. lactis*) DN-173 010 (3 treatment effects), *B. lactis* HN019 (2 treatment effects), and *Lactobacillus rhamnosus* (*L. rhamnosus*) GG (2 treatment effects). Daily probiotic dosages varied substantially across studies, ranging from 5×10^8 to 9.75×10^{10} cfu per day (median 1.72×10^{10} cfu per day). Supplementation periods ranged from 10 to 28 d (median 18 d). Intestinal transit time was quantified using radiopaque markers in 10 studies and with carmine red dye in 1 study[19]. The most commonly

Table 2 Subject characteristics

Ref.	Age (yr)	Female gender	BMI (kg/m^2)	Condition
Agrawal *et al*[14]	40	100%	25	IBS-C
Bartram *et al*[15]	23	58%	-	None
Bouvier *et al*[16]	33	50%	22	None
Holma *et al*[17]	44	92%[1]	24	Constipation
Hongisto *et al*[18]	43	100%	24	Constipation
Malpeli *et al*[19]	41	100%	-	Constipation
Marteau *et al*[20]	27	100%	21	None
Rosenfeldt *et al*[21]	25	0%	-	None
Rosenfeldt *et al*[21]	25	0%	-	None
Sairanen *et al*[22]	39	64%	25	None
Waller *et al*[23]	44	65%	31	Constipation
Waller *et al*[23]	44	65%	32	Constipation
Krammer *et al*[24]	50	100%	-	Constipation

[1]Percentage estimated from larger study cohort. BMI: Body mass index; IBS-C: Irritable bowel syndrome, constipation predominant; "-": Represents missing data.

Table 3 Assessment of study quality

Ref.	Jadad scale			
	Randomization (range: 0-2)	Double blinding (range: 0-2)	Subject account (range: 0-1)	Total score[1] (range: 0-5)
Agrawal *et al*[14]	1	2	1	4
Bartram *et al*[15]	1	2	0	3
Bouvier *et al*[16]	1	2	0	3
Holma *et al*[17]	1	0	1	2
Hongisto *et al*[18]	1	0	0	1
Malpeli *et al*[19]	0	2	1	3
Marteau *et al*[20]	1	2	1	4
Rosenfeldt *et al*[21]	1	1	0	2
Rosenfeldt *et al*[21]	1	1	0	2
Sairanen *et al*[22]	1	1	0	2
Waller *et al*[23]	2	2	1	5
Waller *et al*[23]	2	2	1	5
Krammer *et al*[24]	1	1	1	3

[1]Higher scores represent better study quality.

Table 4 Meta-regression of study- and subject-related factors on intestinal transit time

Variable	Unit of measure	Intercept	Point estimate	Explained variance	*P*-value
Constipation	0 = no, 1 = yes	0.171	0.415	39%	0.01
Age	Per 10 years	-0.445	0.230	27%	0.03
Female gender proportion	Per 10%	0.024	0.053	23%	< 0.05
Body mass index[1]	Per 5 kg/m^2	-0.544	0.200	25%	0.11
Daily probiotic dosage	Per 10 × 10^9 cfu	0.454	-0.013	1%	0.62
Treatment duration	Per 1 wk	0.535	-0.048	1%	0.67

[1]Body mass index not reported for 5 treatment effects.

tested product format was yogurt or other forms of fermented milk. Two studies were confounded by inclusion of other components in the active product that may influence ITT such as lactulose[15] and the combination of inulin and oligofructose[19] (Table 1). Seven treatment effects were calculated based on subjects with constipation or irritable bowel syndrome-C while 6 were based on healthy subjects. Subjects were predominantly female with a mean age ranging from 23 to 50 years and mean body mass index ranging from 21 to 32 kg/m^2 (Table 2).

Study quality assessment

Overall, the quality of RCT reporting was medium with a median Jadad score of 3 (range: 1-5). Eight of 13 treatment effects were based on higher quality (Jadad score 3-5) trials. The method of randomization was unclear in most studies. Descriptions of blinding were adequate overall. Subject accountability in RCTs was mentioned in only 7 of 13 cases (Table 3).

Synthesis of results

Overall, probiotic supplementation was associated with reduced ITT, with an SMD of 0.40 (95%CI: 0.20-0.59, $P < 0.001$) (Figure 2). There was low heterogeneity among studies ($I^2 = 29\%$, $P = 0.15$) with no evidence of publication bias (Egger's regression test: $P = 0.13$). Only 4 of 13 individual treatment effects statistically favored probiotic supplementation.

Additional analyses

We performed meta-regression analysis including pre-defined covariates to explore the potential predictors of SMD. Constipation ($r^2 = 39\%$, $P = 0.01$), higher mean age ($r^2 = 27\%$, $P = 0.03$), and higher percentage of female subjects ($r^2 = 23\%$, $P < 0.05$) were predictive of decreased ITT with probiotics in meta-regression (Table 4). Additionally, we performed a pre-defined subgroup analysis to observe the influence of study- and subject-related characteristics on SMD (Table 5). Subgroup analyses demonstrated statistically greater reductions in ITT with probiotics in subjects with *vs* without constipation and in older *vs* younger subjects (both SMD: 0.59 *vs* 0.17, $P = 0.01$). Study design, body mass index, treatment duration, and daily probiotic dosage had no influence on probiotic treatment effects in any analysis. Analysis of outcomes by probiotic strain identified medium to large treatment effects with *B. lactis* HN019 (SMD: 0.72, $P < 0.01$) and *B. lactis* DN-173 010 (SMD: 0.54, $P < 0.01$) while treatment effects with other single strains and combination products were small (SMD: 0.17-0.25) and not statistically significant (Table 6).

DISCUSSION

Summary of evidence

Clinical trials of probiotic supplementation often utilize ITT as a primary efficacy outcome. However, inconsistent treatment effects among trials have been observed, likely due to differences among study designs, probiotic strains, dosing regimens, and subject characteristics. We performed the first systematic review and meta-analysis on this

Table 5 Subgroup analysis of study- and subject-related factors on intestinal transit time

Study	SMD	95%CI	*P*-value (within groups)	*P*-value (between groups)
Subject condition				
Constipation/IBS-C (n = 7)	0.59	0.39-0.79	< 0.001	0.01
Healthy (n = 6)	0.17	-0.08-0.42	0.18	
Age				
⩾ 40 years (n = 7)	0.59	0.39-0.79	< 0.001	0.01
< 40 years (n = 6)	0.17	-0.08-0.42	0.18	
Study design				
Parallel groups (n = 8)	0.49	0.24-0.75	< 0.001	0.23
Cross-over (n = 5)	0.25	-0.06-0.56	0.11	
Body mass index[1]				
⩾ 25 kg/m^2 (n = 4)	0.61	0.27-0.95	< 0.001	0.29
< 25 kg/m^2 (n = 4)	0.34	-0.02-0.70	0.06	
Female gender proportion				
⩾ 75% (n = 6)	0.44	0.17-0.76	< 0.01	0.47
< 75% (n = 7)	0.32	0.05-0.60	0.02	
Treatment duration				
< 20 d (n = 7)	0.43	0.18-0.67	< 0.001	0.62
⩾ 20 d (n = 6)	0.32	-0.02-0.66	0.07	
Daily probiotic dosage				
⩾ 10^{10} cfu (n = 8)	0.41	0.14-0.68	< 0.01	0.84
< 10^{10} cfu (n = 5)	0.36	0.05-0.68	0.02	

[1]Body mass index not reported for 5 treatment effects. IBS-C: Irritable bowel syndrome, constipation predominant; SMD: Standardized mean difference.

Table 6 Subgroup analysis of probiotic strains on intestinal transit time

Probiotic strain	Treatment effects (n)	SMD	95%CI	*P*-value
B. lactis HN019	2	0.72	0.27-1.18	< 0.01
B. lactis DN-173 010	3	0.54	0.15-0.94	< 0.01
L. rhamnosus GG	2	0.25	-0.38-0.87	0.44
Other single strains	2	0.23	-0.41-0.87	0.48
Strain combinations	4	0.17	-0.18-0.52	0.34

SMD: Standardized mean difference. *B. lactis*: *Bifidobacterium lactis*; *L. rhamnosus*: *Lactobacillus rhamnosus*.

Table 7 Sample size requirements for randomized controlled trials based on standardized mean difference

SMD	Study design	
	Parallel groups[1]	Cross-over
0.2	786	156
0.3	350	71
0.4	198	41
0.5	128	27
0.6	90	19
0.7	66	15
0.8	52	12

[1]Total sample size, assuming 1:1 active-to-control group ratio. Assumes two-sided alpha of 0.05 and statistical power of 80%. Attrition estimate not included. SMD: Standardized mean difference.

topic and demonstrated that, overall, short-term (10-28 d) probiotic supplementation is able to reduce ITT in adults. We also demonstrated that the treatment effect of probiotics is strongly dependent on: (1) the presence or absence of constipation; (2) subject age; and (3) probiotic strain.

Clinical relevance of findings

Presence of constipation and older age were predictive of greater ITT treatment effects with probiotic supplementation. Constipation was the primary influencer of probiotic treatment effects on ITT, explaining 39% of the variance in SMD. The independent influence of subject age, after accounting for constipation, is unknown and may be confounded since the seven studies that enrolled the oldest subjects were the same studies that enrolled constipated subjects. The number of treatment effects per strain is limited; *B. lactis* DN-173 010 (3), *B. lactis* HN019 (2) and *L. rhamnosus* GG (2). Drawing definite conclusions is therefore perilous, but the finding that the former two strains have notably greater treatment effects on ITT suggests that these strains could be considered when aiming to relieve slow intestinal transit.

The clinical importance of ITT is highly dependent on the underlying pathology. In healthy adults with no evidence of GI disturbances or delayed transit, there is arguably little benefit in lowering ITT[25]. In contrast to this position, EFSA considers that a reduction in ITT within the normal range to be a possibly beneficial physiological effect in healthy adults[8]. Overall, probiotic supplementation for this sole purpose cannot be strongly recommended given the questionable clinical benefit and the small effect size (SMD: 0.17) identified in this meta-analysis. In adults with constipation or IBS, a reduction in ITT is moderately associated with improvements in stool form and frequency[25,26]. Therefore, probiotic supplementation appears to be a reasonably effective option to achieve this therapeutic goal provided diarrhea does not develop. Current evidence suggests that probiotics contribute to lowering intestinal pH, decreasing colonization and invasion by pathogenic organisms, and modifying the host immune response with few known side effects[27]. However, there is no strong evidence from RCTs that probiotics improve symptoms such as abdominal pain or bloating in these patients[28]. The clinical importance of decreased ITT in the absence of symptom amelioration is controversial and requires further exploration.

Relevance of findings to clinical trial designs

Interestingly, this meta-analysis identified a positive benefit of probiotic supplementation on ITT although only 4 of 13 treatment effects demonstrated such a benefit. This is likely because the majority of clinical trials were underpowered due to small sample size. In fact, only 1 treatment effect was identified from a study with a minimum detectable effect size ⩽ 0.5 (moderate effect) and only 5 had a minimum detectable effect size ⩽ 0.8 (large effect). Considering the overall ITT SMD with probiotic supplementation is only 0.4, it is clear that small sample size and, consequently, inadequate statistical power was

the main driver of the high failure rate of individual studies.

The use of estimated SMD is an integral component of study design development and sample size estimation for RCTs. Sample sizes for RCTs based on estimated SMD are shown in Table 7. Based on the SMDs calculated in this meta-analysis, enrollment of approximately 90 subjects would be required in a study of probiotics for constipation or irritable bowel syndrome-C with a parallel groups design or 19 subjects if utilizing a cross-over design. In comparison, for a trial of healthy volunteers, required sample sizes would be 786 and 156 for parallel groups and cross-over designs, respectively, in order to achieve adequate statistical power. Although cross-over trials always require a smaller sample size for a given SMD since subjects serve as their own controls, the main disadvantages of this design include a longer time on study, higher attrition rates due to the extended trial duration, and difficulties in estimating an appropriate washout duration. As such, cross-over designs are inappropriate for clinical trials with extended treatment durations or long or unknown washout periods.

Strengths and limitations

The strengths of this systematic review and meta-analysis include selection of only RCTs to minimize bias and the comprehensive assessment of the impact of moderator variables on the primary outcome. Nevertheless, our analysis was associated with several limitations. First, treatment duration in the reviewed studies ranged from 10 to 28 d and, therefore, the treatment effect of longer term probiotic supplementation on ITT is unknown. Second, the therapeutic benefit of probiotics is considered to be strain-specific; however, the small number of studies performed with each strain prevented robust strain-specific comparisons. Third, there was a significant over-representation of subjects who were young to middle-aged, female, and with a normal body mass index. Abundant caution must be exercised when extrapolating the treatment effects observed in this review to a broader population. Finally, we noted significant heterogeneity among ITT measurement methods as well as product delivery methods and additional included ingredients (*e.g.*, prebiotics) among studies. There is potential for these differences to confound the results of our analysis.

In conclusion, short-term probiotic supplementation decreases ITT with consistently greater treatment effects identified in constipated or older adults and with certain probiotic strains.

COMMENTS

Background

Functional gastrointestinal (GI) disorders are common in the general population, with slow intestinal transit a common symptom. No known therapy is highly efficacious, safe, and cost effective for treatment of slow-transit bowel disorders. Probiotics are live micro-organisms that confer a health benefit on the host when administered in adequate dosages and have been extensively studied for treatment of functional GI disorders.

Research frontiers

Clinical trials of probiotic supplementation on intestinal transit time (ITT) yield widely variable outcomes. The reasons for these discrepant outcomes have not been explored to date. Authors performed the first systematic review and meta-analysis on the efficacy of probiotic supplementation on ITT in adults with a secondary focus on identifying the factors that influence these outcomes.

Innovations and breakthroughs

Authors demonstrated that, overall, short-term (10-28 d) probiotic supplementation reduces ITT in adults. However, the treatment effect of probiotics is strongly dependent on: (1) the presence or absence of constipation; (2) subject age; and (3) probiotic strain.

Applications

The effect of probiotics on ITT is highly dependent on probiotic strain and patient characteristics. The reason for these differences requires exploration in future clinical trials. Thus far, no evidence supports the use of probiotics to decrease ITT in younger subjects or in those without constipation.

Terminology

Probiotics are live micro-organisms that confer a health benefit on the host when administered in adequate dosages. Intestinal transit time is a general term that refers to the time taken for a food bolus to travel through the gastrointestinal system. The standardized mean difference is a statistical measure of effect size for continuous outcomes, defined as the mean difference between groups divided by the pooled standard deviation.

Peer review

Considering the high prevalence of functional GI disorders nowadays and the numerous studies on the role of probiotics in treating such conditions, it is important to know where we stand. This meta-analysis demonstrates the efficacy of probiotic supplementation in improving intestinal transit time. It is very well written, with methods clearly presented. Conclusions are drawn regarding the clinical importance of these findings and their relevance to clinical trials design, representing valuable information.

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BRIEF ARTICLE

Comparison of double balloon enteroscopy in adults and children

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Abstract

AIM: To compare results of double balloon enteroscopy (DBE) procedures in pediatric and adult patients.

METHODS: The medical files of patients who underwent DBE at Gazi University School of Medicine, Ankara, Turkey between 2009 and 2011 were examined retrospectively. Adult and pediatric patients were compared according to DBE indications, procedure duration, final diagnosis, and complications. DBE procedures were performed in an operating room under general anesthesia by two endoscopists. An oral or anal approach was preferred according to estimated lesion sites. Overnight fasting of at least 6 h prior to the start of the procedure was adequate for preprocedural preparation of oral DBE procedures. Bowel cleansing was performed by oral administration of sennosides A and B solution, 2 mL/kg, and anal saline laxative enema. The patients were followed up for 2 h after the procedure in terms of possible complications.

RESULTS: DBE was performed in 35 patients (5 pediatric and 30 adult). DBE procedures were performed for abdominal pain, chronic diarrhea, bleeding, chronic vomiting, anemia, and postoperative evaluation of anastomosis. Final diagnosis was diffuse gastric angiodysplasia (n = 1); diffuse jejunal angiodysplasia (n = 1); ulceration in the bulbus (n = 1); celiac disease (n = 1); low differentiated metastatic carcinoma (n = 1); Peutz-Jeghers syndrome (n = 1); adenomatous polyp (n = 1) and stricture formation in anastomosis line (n = 1). During postprocedural follow-up, abdominal pain and elevated amylase levels were noted in three patients and one patient developed abdominal perforation.

CONCLUSION: With the help of technological improvements, we may use enteroscopy as a safe modality more frequently in younger and smaller children.

Key words: Double-balloon enteroscopy; Small bowel disease; Polyp; Angiodysplasia; Peutz-Jeghers syndrome

Core tip: Small bowel diseases are encountered frequently in adults and children. For diagnosing small bowel disease, endoscopy, barium series, ultrasound, and computed tomography methods were used before advanced techniques such as capsule endoscopy and double balloon enteroscopy (DBE). Many centers began to use DBE widely in adults and children. Interventional procedures of DBE in children are also as safe as in adult patients. Although a small number of children were included in the present study, this is believed to be the first comparison of DBE in adult and pediatric patients.

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of double balloon enteroscopy in adults and children. *World J Gastroenterol* 2013; 19(29): 4726-4731 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i29/4726.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i29.4726

INTRODUCTION

Considerable progress has been made in the area of diagnostic or therapeutic endoscopic interventions since Hirschowitz *et al* introduced the fiberoptic endoscope in 1950[1]. Endoscopic imaging of the whole small intestine is more difficult than the colon due to its length, free mobility and tortuosity within the abdominal cavity. Capsule endoscopy (CE) is one of the latest techniques, and provides single pass visualization of the intestines but without biopsy or therapeutic capabilities. Small bowel diseases are common in children and adults. However, surgical approaches were the first choice for diagnostic and therapeutic applications before using techniques like CE and double balloon enteroscopy (DBE). DBE provides significant progress in diagnosis and management of small intestinal diseases. The diagnostic and therapeutic benefits of DBE in adults are well documented and DBE is widely used in many centers. Although DBE is a safe method in adults, its use in children was limited to case reports, which were followed by center reports about the DBE experience[2-6].

In the present study, we report our preliminary comparison of DBE in pediatric and adult patients, focusing on the evolving indications, limitations, associated risks, and complications of this endoscopic therapeutic technique, in the setting of a tertiary care referral center.

MATERIALS AND METHODS

Patients

The medical files of the patients who underwent DBE at Gazi University School of Medicine, Ankara, Turkey between 2009 and 2011 were examined retrospectively. A total of 36 oral or anal procedures were performed in 35 patients. Endoscopy and colonoscopy were performed in all patients prior to DBE. The indications for DBE in each patient were based on clinical, laboratory, radiology, endoscopy and colonoscopy findings, and biopsy results. Unexplained chronic abdominal pain, chronic diarrhea, gastrointestinal bleeding, anemia, and postoperative evaluation of anastomosis line were the indications for DBE.

DBE procedure

DBE included a complete small bowel enteroscope, an overtube, balloons and a special air pump. The Fujinon EN-450T5 DBE system (Tokyo, Japan) used a 200-cm working length enteroscope with an outer diameter of 8.5 and 2.8-mm working channel. The flexible overtube had a length of 140 cm and outer diameter of 12 mm. The enteroscope and overtube had balloons fitted at the distal tip. An air pressure controlled pump system had a maximum inflatable pressure of 45 mmHg. For oral DBE procedures, there was no difference in preprocedural preparation of adult and pediatric patients and overnight fasting of at least 6 h prior to the start of the procedure was adequate. Bowel cleansing was performed by oral administration of sennosides A and B solution, 2 mL/kg, and anal saline laxative enema. DBE procedures were performed in an operating room under general anesthesia by two endoscopists. The patients were followed up for 2 h after the procedure to assess possible complications.

Ethics

Information about the DBE procedure, including its requirements and potential complications, were explained to all adult patients and parents of the children before the procedure. Informed consent was obtained from all the participants.

Statistical analysis

The SPSS 16.0 software package was used for statistical analysis. Numerical data were expressed as a percentage, and measurement data were expressed as the mean ± SD. Differences were evaluated with the χ^2 test.

RESULTS

DBE procedures were performed in 35 patients (5 children and 30 adults). Fourteen of the adult patients were female (46.7%), and 16 were male (53.3%). Among pediatric patients, four were male (80%) and one was female (20%). The mean ages in the adult and pediatric patients were 48.63 ± 12.70 and 12 ± 3 years, respectively. Demographic data of the patients are listed in Table 1.

Indications for DBE procedures were abdominal pain (n = 11), chronic diarrhea (n = 8), anemia (n = 4), bleeding (n = 9), postoperative evaluation of anastomosis line (n = 2), and chronic vomiting in a patient with percutaneous endoscopic jejunostomy tube (n = 1). During postprocedural follow-up, three patients developed abdominal pain and elevated amylase levels, thus follow-up periods were extended. One patient developed intestinal perforation.

Diagnosis was made by enteroscopy, evaluation of biopsy or surgical material, or clinical and laboratory data. Results were as follows: diffuse gastric angiodysplasia (n = 1); diffuse jejunal angiodysplasia (n = 1); ulceration in the bulbus (n = 1); scalloping of the folds in the bulbus and bowel mucosa compatible with celiac disease (n = 3); ulceration crater in the jejunum with 8-9 mm diameter covered with white exudate (low differentiated metastatic carcinoma, n = 1); polyps (3 patients with Peutz-Jeghers syndrome and 1 with adenomatous polyp); and stricture formation in anastomosis line (n = 1) (Table 2). In 23 patients, monitoring of the small bowel mucosa throughout the process was normal. Demographic characteristics of pediatric patients are given in Table 3.

Table 1 Demographic features of double balloon enteroscopy patients

Indications for DBE	Procedure time (mean, min)	*n*	Sex	Oral/anal
Chronic abdominal pain	64.8	11	3 F/8 M	Oral approach DBE [1]Patient oral + anal approach DBE
Anemia	62.5	4	1 F/3 M	Oral approach DBE
Diarrhea	73.5	8	4 F/4 M	Oral approach DBE
Bleeding	66	9	6 F/3 M	Oral approach DBE
Evaluation of anastomosis line	38	2	1 F/1 M	Oral approach DBE
Chronic vomiting	64	1	1 M	Oral approach DBE

[1]Diffuse jejunal angiodysplasia was noted in a female patient being investigated for anemia etiology. F: Female; M: Male; DBE: Double balloon enteroscopy.

Table 2 Comparison of double balloon enteroscopy findings and final diagnosis of study population

	Pediatric (*n* = 5)	Adult (*n* = 30)	*P*
Male/female	4/1	16/14	< 0.05
Procedure time (min, 95%CI)	74 (65-89)	114 (76-125)	< 0.05
Route	5 oral	29 oral, 1 anal + oral	
Findings/intervention (*n*)	Multiple polyps (2) /polypectomy	Diffuse gastric angiodysplasia (1)/APC [1]Diffuse jejunal angiodysplasia in (1) /APC Ulceration in bulbus (1) Scalloping of the folds in bulbus and bowel mucosa (3) Ulceration crater in jejunum with 8-9 mm diameter covered with white exudate (1) Multiple polyps (3)/polypectomy Polyp (1)/polypectomy anastomotic stricture (1)/balloon dilation	
Final diagnosis (*n*)	Irritable bowel syndrome (3) PJS (2)	Angiodysplasia (2) Peptic ulcer (1) Celiac disease (3) Metastatic carcinoma (1) Anastomotic stricture (1) PJS (3) Adenomatous polyp (1) No definitive diagnosis (18)	
Minor complications (*n*)	Sore throat (4) Abdominal discomfort (3)	Abdominal discomfort (21) Hyperamylasemia (5) Sore throat (4)	
Major complications (*n*)	None	Perforation (1)	

Argon plasma coagulation (APC) was performed, unfortunately intestinal perforation identified after the procedure. PJS: Peutz–Jeghers syndrome.

Table 3 Demographic features of pediatric patients

Patient	Complaint	Age (yr) /gender	Enteroscopy findings	Intervention	Final diagnosis
1	Abdominal pain / Diarrhea	12/male	Normal mucosa	-	Irritable bowel syndrome
2	Abdominal pain	11.6/male	Multiple polyps	Polypectomy	Peutz-Jeghers syndrome
3	Anemia	12/female	Multiple polyps	Polypectomy	Peutz-Jeghers syndrome
4	Abdominal pain	10/male	Normal mucosa	-	Irritable bowel syndrome
5	Abdominal pain	10 yr and 5 mo/male	Normal mucosa	-	Irritable bowel syndrome

DISCUSSION

Small bowel diseases are encountered frequently in adults and children. For diagnosing small bowel disease, endoscopy, barium series, ultrasound, computed tomography (CT) methods were used before advanced techniques such as CE and DBE. Segments of the small intestine that cannot be reached are now viewed *via* DBE and progress has been made in diagnosis and treatment of lesions in this region. Although a small number of children were included in the present study, this is believed to be the first comparison of DBE in pediatric and adult patients. Demographic characteristics of pediatric patients.

Analyzing pediatric patients, patient 1 had abdominal pain and diarrhea whereas patients 4 and 5 had abdominal pain only. In all three patients acute phase reactants and leukocyte count were normal and stools were negative for blood. There were no pathological imaging signs and endoscopic and colonoscopic examinations were considered as normal. Abdominal pain was the most common indication for enteroscopy in our limited number of pediatric patients. Evaluating the adult group of patients, abdominal pain constituted the most common indication for enteroscopy, with 11 patients (33%). Reviewing the literature for adult enteroscopy, Pata *et al*[7] reported that obscure bleeding constituted the major indication, with 42.1%, whereas abdominal pain ranked last with 9.47%. He *et al*[8] reported that abdominal pain with 15 patients (25.4%) ranked second below melena with 36 patients (61.01%) as the major indications. In our adult series, three of the 11 patients with abdominal pain had special features. Stool examinations of all three patients were normal. Endoscopic and colonoscopic examinations did not reveal any clear pathological sign. However, due to visualization of local irregularities and scalloping in jejunal bowel mucosa in some areas, celiac serology was performed and biopsy results were compatible with celiac disease.

According to the classical knowledge on celiac disease, it is known that four or more biopsy specimens from the bulbus and duodenum are sufficient for diagnosis. Enteroscopy was not needed for diagnosis of celiac disease. Today, we are aware of using enteroscopy

for viewing the whole small bowel in refractory or complicated celiac disease. Höroldt *et al*[9] reported that endoscopic duodenal biopsies of 31 patients with suspected celiac disease were evaluated as normal. However, 60% of these patients were diagnosed with celiac disease, with the biopsies taken from the jejunum by enteroscopy. Similarly, Rondonotti *et al*[10] reported that among 23 patients with suspected celiac disease, four were diagnosed with celiac disease with the help of enteroscopic jejunal biopsies. Reviewing the literature, and as shown in our study group, duodenal biopsies may not be sufficient in 10%-17% of patients with suspected celiac disease, and biopsies have to be taken from distal parts of the small intestine with the help of an enteroscope[11-13].

In our study, four patients had polyps and resections were done. Patient 2 was being followed for Peutz-Jeghers syndrome in a different center and was referred to our department due to development of ileus symptoms. In abdominal CT scans, a mass of 3 cm × 2.5 cm was noted. Enteroscopy was performed and a giant three-leaf-clover-shaped polyp completely filling the intestinal lumen was seen (Figure 1). Complete resection was done without postprocedural complications. Patient 3 was also being followed for Peutz-Jeghers syndrome and prominent anemia. The patient was positive for occult blood in stool examination. During endoscopy, a jejunal polyp was seen in an area that was out of reach. Polypectomy was performed with the help of an enteroscope without any complications.

Enteroscopy was performed in one adult patient who had Peutz-Jeghers syndrome in childhood due to anemia and recurrent abdominal pain. Polypectomy was performed for multiple polyps with the help of an enteroscope, without any complications. One adult patient being investigated for anemia with normal endoscopy and colonoscopy was referred to our center for enteroscopy. Jejunal polyps were seen and complete resection was performed without any complications. Biopsies were found to be compatible with adenomatous polyps.

Yoon *et al*[14] reported the resection of a giant ileal polyp of 2.5 cm × 2.5 cm *via* enteroscopy. Miyata *et al*[15] reported a case of small bowel intussusception due to inflammatory polyps that was preoperatively diagnosed using DBE. Thus, minimal laparoscopic resection could be performed by shortening intussusception. Until recently, primary surgical resection and intraoperative endoscopy were the only available possibilities to treat polyps in the mid-small bowel in patients with polyps. DBE has changed this approach and now it is possible not only perform endoscopic surveillance and diagnose these lesions, but also resect them.

One of our adult patients investigated for abdominal pain and anemia presented with occult blood. Endoscopy and colonoscopy did not reveal any pathological findings. During enteroscopy, an ulceration crater in the jejunum of 8-9 mm diameter covered with white exudate was noted. Biopsy evaluation was reported as low differentiated metastatic carcinoma.

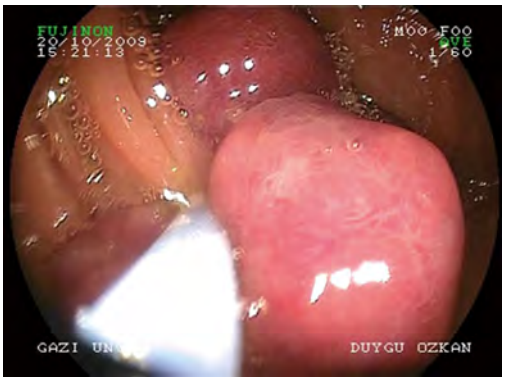

Figure 1 A giant three-leaf cloverleaf shaped polyp completely filling the intestinal lumen.

Fry *et al*[16] reported a relative incidence of 9.6% for small bowel tumors encountered during DBE in a series of 40 patients. Nakatani *et al*[17] reported 10 years DBE experience with 705 patients. All patients were investigated for obscure gastrointestinal bleeding and 12 intestinal tumors were identified. According to the above mentioned study, detection rates for intestinal tumors with DBE, CE and CT were 92%, 60% and 67%, respectively. Considering adult small bowel tumors, probably one of the major benefits of DBE may be defined as diagnostic and/or therapeutic capability without the need for surgery.

Although not met in our pediatric patients, diffuse gastric and jejunal angiodysplasia was noted in two adult patients, and argon plasma coagulation (APC) therapy was performed. May *et al*[18] reported 44 cases of angiodysplasia in a series of 66 patients and APC was the treatment option. As in patients with small bowel tumors, DBE may be compared better alternative to surgery in patients with angiodysplasia[19,20].

Initially, studies about DBE in childhood were limited to case reports, which were followed by center reports. To date, there have been seven studies about DBE procedures in childhood. In 2007, Leung[21] performed DBE in 15 pediatric patients; five of whom were < 10 years old. Liu *et al*[22] performed DBE with a diagnostic rate of 77.8% in 31 pediatric patients aged 3-14 years. In 2010, Lin *et al*[23] reported 13 successful DBE procedures in 11 pediatric patients ranging from 8 to 20 years. Thomson *et al*[24] and Nishimura *et al*[25] reported DBE in 14 and 48 pediatric patients, respectively. In 2012, Shen *et al*[26] reported 35 DBE procedures performed in 30 pediatric patients, whereas Uchida *et al*[27] performed 17 DBE procedures to 12 children. Evaluation of obscure gastrointestinal bleeding, surveillance and treatment of polyposis syndromes, investigation of abdominal pain, chronic diarrhea, and suspected mucosal inflammation comprised the indications in all the above-mentioned pediatric DBE patients. Reported complications encountered in these patients were post-polypectomy bleeding, sore throat, abdominal discomfort, and oral secretion aspiration. One intestinal perforation was reported af-

ter resection of a Peutz-Jeghers polyp in a 3-year-old boy during enteroscopy. Reviewing the studies and case reports, it seems that the younger children with lower weight have a higher possibility of complications such as perforation. In our study, perforation occurred in an adult patient with diffuse angiodysplasia after APC. Post-procedural sore throat and abdominal discomfort were noted in all our pediatric patients, whereas abdominal discomfort was the major complaint in adult patients.

The mean procedure time was significantly shorter for pediatric patients (74 min *vs* 114 min). The possible explanation for this is the relatively shorter small bowel length of pediatric patients. Although the majority of the pediatric patients had polypectomy, none of them had major complications. Although the lumen of the small bowel is narrower in children, interventional endoscopic procedures are relatively safe. Only one patient in the adult group had perforation (possibly due to APC) and the clinical course was uneventful.

As a result, many centers have begun to use DBE widely in adults and children. DBE is a safe method for small bowel lesions in children, as in adults. Interventional procedures are also as safe in pediatric as adult patients; however, larger trials are needed to reach a firm conclusion.

COMMENTS

Background

Small bowel diseases are encountered frequently in adults and children. For diagnosing small bowel disease, endoscopy, barium series, ultrasound, and computed tomography were used before advanced techniques such as capsule endoscopy and double balloon enteroscopy (DBE). Segments of the small intestine that cannot be reached are now viewed *via* DBE and progress has been made in diagnosis and treatment of lesions in this region.

Research frontiers

Studies about DBE in childhood were limited to case reports, which were followed by center reports. To date, there have been seven studies about DBE procedures in childhood. This is believed to be the first comparison of DBE in pediatric and adult patients.

Innovations and breakthroughs

Postprocedural sore throat and abdominal discomfort were noted in all our pediatric patients, whereas abdominal discomfort was the main complaint in the adult patients. The mean procedure time was significantly shorter for pediatric patients. The possible explanation for this is the relatively shorter small bowel length in pediatric patients. Although the lumen of the small bowel is narrower in pediatric patients, interventional endoscopic procedures are relatively safe. DBE is a safe method for small bowel lesions, as in adults.

Applications

DBE is a reliable method in children as well as adults. Diagnostic and therapeutic use of DBE will gradually increase in the future.

Peer review

The study is interesting in that it evaluated the use of DBE in children, which has not yet been universally adopted. It appears to be safe and clinically effective.

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BRIEF ARTICLE

Utility of single and double balloon endoscopy in patients with difficult colonoscopy: A randomized controlled trial

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Abstract

AIM: To compare the utility of single-balloon colonoscopy (SBC) or double-balloon colonoscopy (DBC) for difficult colonoscopies.

METHODS: Between August 2008 and June 2010, patients in whom total colonoscopy failed within 30 min of insertion were assigned randomly to undergo either SBC or DBC. No sedatives were used. After the endoscopy, all patients were asked to evaluate pain during the procedure on a 10-point analog scale (1 = no pain; 10 = worst imaginable pain) with a questionnaire. The study outcomes were the cecal intubation rate and time, endoscopic findings, complications, and pain score.

RESULTS: The SBC and DBC groups included 11 and 10 patients, respectively. All but one SBC patient achieved total colonoscopy successfully. The cecal intubation times were 18 min (range: 10-85 min) and 12.8 min (range: 9.5-42 min) in the SBC and DBC groups, respectively (P = 0.17). No difference was observed in the prevalence of colon polyps between the SBC and DBC groups (45% *vs* 30%, P = 0.66). SBC showed advanced colon cancer in the ascending colon, which was inaccessible using conventional colonoscopy. The respective pain scores were 5 (1-10) [median (range)] and 5 (1-6) in the SBC and DBC groups (P = 0.64). No complications were noted in any patient.

CONCLUSION: The utility of single- and double-balloon endoscopy for colonoscopy seems comparable in patients with incomplete colonoscopy using a conventional colonoscope.

Key words: Difficult colonoscopy; Double-balloon endoscopy; Single-balloon endoscopy; Double-balloon colonoscopy; Single-balloon colonoscopy

Core tip: We compared the utility of single-balloon colonoscopy and double-balloon colonoscopy for difficult colonoscopy. Both single-balloon endoscopy (SBE) and double-balloon endoscopy (DBE) make possible performance of total colonoscopy in patients with incomplete colonoscopy using a conventional colonoscope. The utility of SBE and DBE for colonoscopy seems to be comparable. We recommend that patients with incomplete total colonoscopy undergo SBE or DBE.

Yamada A, Watabe H, Takano N, Togo G, Yamaji Y, Yoshida H, Kawabe T, Omata M, Koike K. Utility of single and double balloon endoscopy in patients with difficult colonoscopy: A randomized controlled trial. *World J Gastroenterol* 2013; 19(29): 4732-4736 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i29/4732.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i29.4732

INTRODUCTION

Optical colonoscopy is the gold standard for colorectal examination. Despite advances in colonoscopes and endoscopy techniques, total colonoscopy is still demanding technically. Total colonoscopy is unsuccessful in 5%-10% of patients for a number of reasons[1]. Difficult cecal intubation is associated with female gender, old age, a low body mass index, diverticular disease, and previous abdominal surgery[2-5]. Solutions to this problem are the use of pediatric colonoscopes[6] or a transparent hood[7].

Balloon endoscopy is an effective method for investigating the small intestine[8,9]. Two different types of balloon endoscopy are available: single-balloon endoscopy (SBE) and double-balloon endoscopy (DBE). Both can be performed using either the antegrade or retrograde approach. A retrograde approach might facilitate successful total colonoscopy and allow endoscopic therapy in patients who had incomplete colonoscopy with a conventional colonoscope. Although several studies have assessed the utility of single-balloon colonoscopy (SBC)[10-12] or double-balloon colonoscopy (DBC)[13-15] for colorectal examination, a difference between SBC and DBC has not yet been identified.

Therefore, we compared the utility of SBC and DBC for difficult colonoscopy in an exploratory randomized controlled trial.

MATERIALS AND METHODS

Study protocol

Consecutive patients after a prior incomplete colonoscopy with a conventional colonoscope were candidates for this study. Incomplete colonoscopy was defined as failure to identify two landmarks, the ileocecal valve and appendiceal orifice, within 30 min or cancellation of colonoscopy due to intolerable pain during the procedure. The exclusion criteria were the following: incomplete colonoscopy due to poor bowel preparation or colonic stenosis, prior colectomy, inflammatory bowel disease, malignant tumor, poor general condition, pregnancy, having undergone successful total colonoscopy within 1 year, age younger than 20 years, and refusal to provide written informed consent.

To eliminate patient selection bias, the enrolled patients were assigned randomly to either the SBC or DBC group in a 1:1 ratio. Randomization was performed using a computer-generated list of random numbers. The endoscopists and patients were not blinded to the group assignment.

The study was conducted according to the Declaration of Helsinki and approved by the ethics committee of our institution. The Japanese clinical trial registration scheme (UMIN-CTR) registration number for the study was UMIN000001684. Written informed consent was obtained from each study participant.

Endoscopic procedure

Each balloon endoscopic examination was conducted by an endoscopist who had performed at least 30 balloon endoscopies. SBC was performed using an SIF-Q260 (Olympus Medical Systems, Tokyo, Japan), and DBC was performed using an EN-450T5 (FUJIFILM Medical, Tokyo, Japan). The study patients were administered 2 L of polyethylene glycol (PEG) solution before the procedure. Scopolamine butylbromide (20 mg) or glucagon (1 IU) was administered. No sedatives were used. Air insufflation was used during the both procedures. Fluoroscopy was used when stretching the scope or when the scope was stacked. We withdrew the endoscope when either the insertion time exceeded 90 min or the patient requested that the procedure be stopped. Cecal intubation was defined as successful when the ileocecal valve and appendiceal orifice were identified. Ancillary procedures such as polypectomy and biopsy were performed while withdrawing the scope after cecal intubation. While the patients were in the recovery room after the examination, they were asked to evaluate the pain during the examination on a 10-point analog scale (1 = no pain, 10 = worst imaginable pain) in a questionnaire.

Table 1 Baseline of characteristics of the study patients *n* (%)

	SBC group (n = 11)	DBC group (n = 10)	P value
Male/female	7/4	6/4	0.99[1]
Age (yr)	71.7 ± 8.0	71.5 ± 7.8	0.94[2]
BMI (kg/m^2)	22.3 ± 4.4	22.6 ± 3.3	0.87[2]
Past history of abdominal surgery	4 (36)	2 (20)	0.64[1]

[1]Fisher's exact test; [2]Student's *t*-test. All variables are means ± SD. SBC: Single-balloon colonoscopy; DBC: Double-balloon colonoscopy; BMI: Body mass index.

Study outcomes

The primary outcome was the successful cecal intubation rate. Secondary outcome measures were the cecal intubation time, endoscopic findings, complications, and pain score during the examination.

Statistical analysis

Categorical data including the total enteroscopy rate and diagnosis rate were compared using Fisher's exact test. Continuous variables were compared using Student's *t*-test. The cecal insertion time and X-ray fluoroscopy time were compared with the Mann-Whitney *U*-test. Differences with $P < 0.05$ were considered to indicate statistical significance. All statistical analyses were performed using JMP, ver. 9.0 (SAS Institute, Cary, NC, United States).

RESULTS

Baseline characteristics

During the study period from August 2008 to June 2010, 21 patients were enrolled and assigned randomly to undergo either SBC (n = 11) or DBC (n = 10). Table 1 shows the baseline characteristics of the study patients. There was no significant difference between the SBC and

Table 2 Cecal intubation rate and endoscopy-related outcomes of balloon colonoscopy

	SBC group (n = 11)	DBC group (n = 10)	P value
Successful cecal intubation, n (%)	10 (91)	10 (100)	0.99[1]
Cecal intubation time[3] (min) [median (range)]	18.0 (10-85)	12.8 (9.5-42)	0.17[2]
Total X-ray fluoroscopic time (min) [median (range)]	3 (1-7)	1 (1-5)	0.12[2]
Pain score[4] [median (range)]	5 (1-10)	5 (1-6)	0.64[2]

[1]Fisher's exact test; [2]Mann-Whitney *U*-test; [3]The cecal intubation time was compared for cases with successful cecal intubation; [4]After balloon endoscopy, the patients were asked to evaluate the pain during the procedure on a 10-point analog scale (1 = no pain, 10 = worst imaginable pain). SBC: Single-balloon colonoscopy; DBC: Double-balloon colonoscopy.

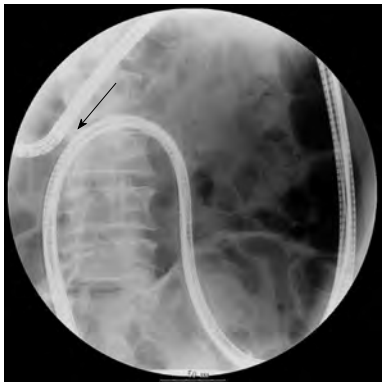

Figure 1 Failed cecal intubation with single-balloon colonoscopy. The patient had previously undergone abdominal surgery for cholecystitis. Fluoroscopy shows adhesion between the sigmoid and transverse colons (arrow).

DBC groups.

Cecal intubation rate and endoscopic procedural results

Table 2 shows the cecal intubation rate and endoscopy results. Using balloon endoscopy, cecal intubation was achieved in all cases but one patient who previously had abdominal surgery for cholecystitis and fluoroscopy showed adhesion between the sigmoid and transverse colons (Figure 1). The cecal intubation rate was 91% in the SBC group and 100% in the DBC group (P = 0.99). The median cecal intubation time of the successful cases did not differ between the SBC and DBC groups [18.0 min (range: 10-85 min) *vs* 12.8 min (range: 9.5-42 min), respectively, P = 0.17], neither did the X-ray fluoroscopy time [3 min (range: 1-7 min) *vs* 1 min (range: 1-5 min), P = 0.12). There was no difference in the pain score during the endoscopic procedure between the SBC and DBC groups [median (range), 5 (range: 1-10) *vs* 5 (range: 1-6), P = 0.64].

Diagnostic yield

The diagnostic yield in each group is shown in Table 3. Colorectal polyps were detected in 8 of the 21 (38%) patients. All polyps were histologically confirmed as adenoma. The detection rate was 45% and 30% with SBC and DBC, respectively; the difference was not significant (P = 0.66). Moreover, SBC showed advanced colon cancer in the ascending colon, which was inaccessible by conventional colonoscopy (Figure 2).

Table 3 Comparison of the diagnostic yields of single-balloon colonoscopy and double-balloon colonoscopy n (%)

	SBC group (n = 11)	DBC group (n = 10)	P value[1]
Advanced cancer	1 (9)	0 (0)	0.99
Colon polyp	5 (45)	3 (30)	0.66
Diverticulosis	3 (27)	4 (40)	0.66

[1]Fisher's exact test. SBC: Single-balloon colonoscopy; DBC: Double-balloon colonoscopy.

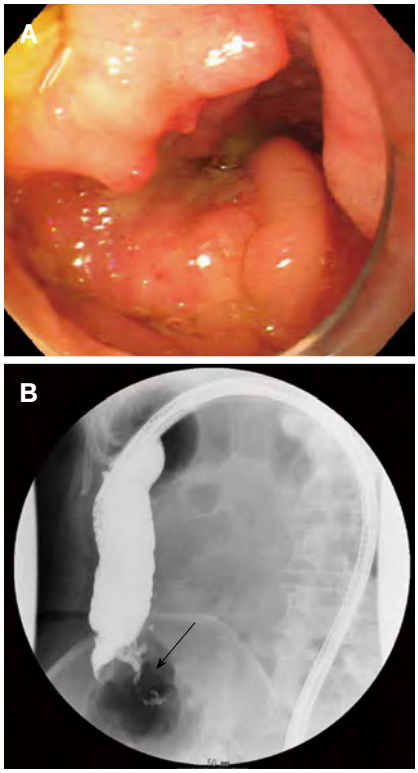

Figure 2 Advanced colon cancer detected at single-balloon colonoscopy. A: Endoscopic image; B: Selective contrast image. The lesion was located in the ascending colon, which was not accessible by conventional colonoscopy. With single-balloon colonoscopy, the lesion was detected in 11 min.

Complications

No complications were noted in this study.

DISCUSSION

In our series, both SBE and DBE had high total colonoscopy rates in patients with incomplete colonoscopy using a conventional colonoscope. The utility of SBE and DBE for colonoscopy seems comparable.

Both SBE and DBE were initially designed for small

bowel endoscopy and have proved their value for small bowel examination[9]. The endoscope and overtube are advanced sequentially with serial fixing and shortening of the small intestine using the balloons, in order to minimize looping and advance the scope. This balloon technique has already been used successfully for patients in whom colonoscopy is difficult. The reported total colonoscopy rate is 95%-100% for SBE[10-12], and 88%-100% for DBE[13-16]. Our total colonoscopy rates were comparable for SBE and DBE (91% *vs* 100%, $P = 0.99$). Previously, we reported that the total enteroscopy rate for the small intestine was higher with DBE than with SBE (57% *vs* 0%, $P = 0.002$)[17]. DBE grips the small intestine at the tip of the endoscope more easily than SBE, which makes possible deep insertion of the endoscope without redundant loops. When used for colonoscopy, the potential disadvantage of SBE seen in small intestinal endoscopy is negligible.

In our series, the cecal intubation time was comparable between SBC and DBC. Teshima *et al*[12] reported that SBE was faster than DBE because only one balloon cycle is used, as opposed to two. Compared with small intestinal endoscopy, colonoscopy is faster and requires fewer balloon cycles. Consequently, the simpler manipulation with SBE does not shorten the cecal intubation time.

The importance of total colonoscopy is well recognized, especially for older patients because of the increase in right-sided colon cancer with age[18,19]. Indeed, SBE detected advanced colon cancer in the ascending colon, which was inaccessible to conventional colonoscopy. In addition, we performed all procedures without any sedatives or complications. The study indicated that SBE and DBE can be performed safely in patients with incomplete total colonoscopy using a conventional colonoscope. Therefore, we recommend that patients with incomplete total colonoscopy undergo SBE or DBE.

The present study showed that SBC and DBC can be performed safely without sedation even in patients with incomplete total colonoscopy using a conventional colonoscope. In terms of colonoscopy, several papers reported that conventional colonoscopy without sedation is feasible, effective and well tolerated[20,21]. On the other side, most of previous papers regarding SBC and DBC used sedative drugs[11,12,14-16]. Although the pain score during for procedure was slightly high, all of the present study patients did not request to stop the procedure.

There were some limitations to this study. The number of participants was relatively small and a larger prospective non-inferiority trial is needed to elucidate any difference in the utility of SBE and DBE. However, the procedures had very high diagnostic yields in both groups, suggesting that both SBC and DBC are effective modalities for colonic examination.

In conclusion, both single- and double-balloon endoscopy make possible performance of total colonoscopy in patients with incomplete colonoscopy using a conventional colonoscope. The utility of SBE and DBE for colonoscopy seems to be comparable.

COMMENTS

Background

Despite advances in colonoscopes and endoscopy techniques, total colonoscopy still fails in some patients. Balloon endoscopy is an effective tool for investigating the small intestine. Two different types of balloon endoscopy are available commercially: single-balloon endoscopy (SBE) and double-balloon endoscopy (DBE). A retrograde approach might facilitate successful total colonoscopy and allow endoscopic therapy in patients who had incomplete colonoscopy with a conventional colonoscope. A difference between single-balloon colonoscopy (SBC) and double-balloon colonoscopy (DBC) has not yet been identified.

Research frontiers

SBE and DBE can be used to complete examination of the colon in patients with incomplete colonoscopy using a conventional colonoscope. It also allows therapeutic interventions.

Innovations and breakthroughs

SBE and DBE have been an important endoscopic breakthrough for successful total colonoscopy and endoscopic therapy in patients who had incomplete colonoscopy with a conventional colonoscope. In this study, the authors compared the utility of SBC and DBC for difficult colonoscopy in an exploratory randomized controlled trial. The study indicated that both SBE and DBE make possible performance of total colonoscopy in patients with incomplete colonoscopy using a conventional colonoscope without any sedation. The utility of SBE and DBE for colonoscopy seems to be comparable.

Applications

This study suggests that patients with incomplete total colonoscopy undergo SBE or DBE.

Terminology

DBE consists of an endoscope and a soft overtube. A latex balloon is attached to the tip of the endoscope and another to the tip of the overtube. Each balloon can be inflated and deflated by a pressure controlled air pump system. SBE is simpler to perform than DBE because it has only 1 balloon at the tip of the overtube. The equipment and techniques are different between DBE and SBE. However, the principle of insertion is the same; gripping the intestine by using balloon inflation prevents redundant loop formation and thus facilitates deep insertion of the endoscope.

Peer review

The authors compared SBE with DBE in patients with previous incomplete colonoscopy because of several reasons. They achieved excellent total colonoscopy rates (91% *vs* 100%) even in these difficult cases. These results indicate the utility of SBE and DBE in patients with incomplete conventional colonoscopy.

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BRIEF ARTICLE

Clinical outcome in patients with hepatocellular carcinoma after living-donor liver transplantation

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Abstract

AIM: To investigate risk factors for hepatocellular carcinoma (HCC) recurrence after living donor liver transplantation (LDLT) and efficacy of various criteria.

METHODS: From October 2000 to November 2011, 233 adult patients underwent LDLT for HCC at our institution. After excluding nine postoperative mortality cases, we analyzed retrospectively 224 patients. To identify risk factors for recurrence, we evaluated recurrence, disease-free survival (DFS) rate, survival rate, and various other factors which are based on the characteristics of both the patient and tumor. Additionally, we developed our own criteria based on our data. Next, we compared our selection criteria with various tumor-grading scales, such as the Milan criteria, University of California, San Francisco (UCSF) criteria, TNM stage, Barcelona Clinic Liver Cancer (BCLC) stage and Cancer of the Liver Italian Program (CLIP) scoring system. The median follow up was 68 (6-139) mo.

RESULTS: In 224 patients who received LDLT for HCC, 37 (16.5%) experienced tumor recurrence during the follow-up period. The 5-year DFS and overall survival rates after LDLT in all patients with HCC were 80.9% and 76.4%, respectively. On multivariate analysis, the tumor diameter {5 cm; $P < 0.001$; exponentiation of the B coefficient [Exp(B)], 11.89; 95%CI: 3.784-37.368} and alpha fetoprotein level [AFP, 100 ng/mL; $P = 0.021$; Exp(B), 2.892; 95%CI: 1.172-7.132] had significant influences on HCC recurrence after LDLT. Therefore, these two factors were included in our criteria. Based on these data, we set our selection criteria as a tumor diameter $\leqslant$ 5 cm and AFP $\leqslant$ 100 ng/mL. Within our new criteria (140/214, 65.4%), the 5-year DFS and overall survival rates were 88.6% and 81.8%, respectively. Our criteria ($P = 0.001$), Milan criteria ($P = 0.009$), and UCSF criteria ($P = 0.001$) showed a significant difference in DFS rate. And our criteria ($P = 0.006$) and UCSF criteria ($P = 0.009$) showed a significant difference in overall survival rate. But Milan criteria did not show significant difference in overall survival rate ($P = 0.137$). Among stages 0, A, B and C of BCLC, stage C had a significantly higher recurrence rate ($P = 0.001$), lower DFS ($P = 0.001$), and overall survival rate ($P = 0.005$) compared with the other stages. Using the CLIP scoring system, the group with a score of 4 to 5 showed a high recurrence rate ($P = 0.023$) and lower DFS ($P = 0.011$); however, the overall survival rate did not differ from that of the lower scoring group. The TNM system showed a trend of increased recurrence rate, decreased DFS, or survival rate according to T stage, albeit without statistical significance.

CONCLUSION: LDLT is considered the preferred therapeutic option in patients with an AFP level less than 100 ng/mL and a tumor diameter of less than 5 cm.

Key words: Hepatocellular carcinoma; Living donor

liver transplantation; Selection criteria; Milan criteria; University of California, San Francisco criteria; Barcelona Clinic Liver Cancer; Cancer of the Liver Italian Program

Core tip: Liver transplantation (LT) for hepatocellular carcinoma (HCC) is known to be the best therapeutic option. To obtain a good result, it is important to select appropriate LT candidates from among HCC patients. For living-donor liver transplantation (LDLT), investigation of the efficacy of such criteria will facilitate adoption of extended criteria in LDLT. We defined the selection criteria according to risk factors for recurrence based on our results and compared them with other criteria or scoring systems, such as the Milan and University of California, San Francisco criteria, tumor node metastasis and Barcelona Clinic Liver Cancer staging, and the Cancer of the Liver Italian Program scoring system.

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INTRODUCTION

On the hepatocellular carcinoma (HCC), two alternative treatment options exist with curative intention, such as liver resection and liver transplantation (LT). LT, unlike hepatic resection, has the advantage that it can treat not only the tumor but also the underlying liver cirrhosis. Most LT candidates in the past have been patients with advanced HCC. The high tumor recurrence rates and low survival rates of these patients were discouraging[1,2]. However, LT was established as a suitable treatment for HCC since Mazzaferro *et al*[3] reported the Milan criteria in 1996. The Milan criteria improved the overall survival and disease-free survival (DFS) rates. Thereafter, LT has achieved the best results in well-selected candidates, and most international transplantation communities have adopted the Milan criteria for the treatment of HCC.

However, unfortunately, about 70% of HCC patients are diagnosed with advanced stage disease[4]. Even among patients who meet the Milan criteria, 20% or more will be removed from the waiting list because progression of HCC excludes them from the Milan criteria[5-7]. Additionally, the treatment outcome of a patient excluded from the waiting list because of HCC progression who selects other treatments is known to be very poor. Furthermore, many centers have reported good results despite expansion of the selection criteria. Therefore, the current Milan criteria are too strict, and most centers agree on the need for their expansion. Additionally, in Asian countries, potential donors for deceased donor liver transplantation (DDLT) are lacking. Therefore, living donor liver transplantation (LDLT) is emerging as an additional therapeutic option. In LDLT, it is possible to match the donor and recipient, so the decision to operate is based on the risk to the donor and the expected benefit to the recipient. Patient selection in LDLT is generally wider compared with that in DDLT. However, ethical issues exist concerning LDLT, such as the risk of the donor and the high recurrence rate of the recipients due to advanced-stage disease. Appropriate criteria are necessary for these issues. Therefore, criteria for LDLT and DDLT that are suitable for each center are necessary.

To date, various groups have attempted to expand the Milan criteria while maintaining long-term survival rates[8-12]. One was the proposal of the University of California, San Francisco (UCSF) criteria by Yao *et al*[8] in 2001. Most centers agree on expanding the criteria because more patients can benefit from transplantation, and the DFS and overall survival are comparable to the Milan criteria. Other systems include the TNM system that reflects cancer stage, and the Barcelona clinic liver cancer (BCLC) staging and the cancer of the liver Italian program (CLIP) staging systems that are well-known guidelines for therapy and prognosis prediction that are used in the United States and Europe. Regarding LT, investigation of the efficacy of these criteria for therapy of HCC is important.

The purpose of the present study was to evaluate the characteristics of patients and tumors according to recurrence. Subsequently, we defined the selection criteria according to our results and compared our criteria with other criteria or scoring systems, such as the Milan and UCSF criteria, TNM and BCLC staging, and the CLIP scoring system.

MATERIALS AND METHODS

From October 2000 to November 2011, 233 patients underwent LDLT for HCC at our center. After excluding nine postoperative mortality cases, 224 patients were evaluated. We defined the postoperative mortality as expire of patients within a month after transplantation. All LDLT patients were adults, and the right lobe was used for transplantation. The Institutional Review Board of our center approved the study design.

All patients with HCC planned for transplantation were evaluated preoperatively by computed tomography (CT) of the abdomen and chest, enhanced magnetic resonance image (MRI), positron emission tomography-CT (PET-CT), bone scintigraphy, gastrofiberscopy, and colonoscopy. Alpha fetoprotein (AFP) and protein induced by vitamin K absence or angiotensin-Ⅱ (PIVKA-Ⅱ) were also evaluated as tumor markers. Contraindications of LT in patients with HCC included tumor thrombus in the main portal vein, regional lymph node metastasis, and distant metastasis. We reviewed the tumor diameter and number of tumors based on the pathologic report.

LDLT was performed according to a standard technique using a modified right lobe with middle hepatic vein reconstruction. For ascites, aspiration and cytology were performed before beginning the operation. When lymph node enlargement was present, or in cases with suspicious metastatic disease, an intraoperative biopsy was performed. The operation was performed only in cases with negative biopsy results. Immunosuppression treatment included a regimen of a calcineurin inhibitor (Cyclosporine or Tacrolimus) as part of a dual- or triple-drug regimen with prednisone and mycophenolate mofetil (MMF). An interleukin-2 receptor blocker was administered on both the day of the operation and the fourth postoperative day. Steroids were withdrawn 1 mo after surgery, and MMF was withdrawn 6 mo after surgery. Only a low dose of a single calcineurin inhibitor was administered after this period. The immunosuppression protocol did not differ from other benign diseases. When recurrence was detected during the follow-up period, the immunosuppressive agent was changed or decreased.

For early detection of cancer recurrence, AFP and PIVKA-Ⅱ were checked monthly during the first year, and then bimonthly thereafter. Abdomen CT, chest CT, and bone scintigraphy were routinely performed every 6 mo during the first 2 years, and then were performed annually. When tumor recurrence was suspected, MRI and/or PET-CT were performed.

We evaluated recurrence, the DFS rate, the survival rate, and various other factors to identify the risk of recurrence. Additionally, based on our data, we developed patient selection criteria suitable for our center. Furthermore, using our LDLT data, we evaluated and assessed criteria, such as the most frequently used Milan criteria, the UCSF criteria, and important therapeutic guidelines such as TNM staging, BCLC staging, and the CLIP scoring system.

Statistical analysis

Numeric data were presented as means and standard deviations or as medians and ranges. Continuous variables (means, standard deviations, medians, and ranges) were analyzed using an independent *t*-test or χ^2 test. Multiple regression analyses were performed using Cox proportional hazards models for identification of factors independently associated with recurrence in 95%CI. DFS and 5-year survival rates were estimated using the Kaplan-Meier method, and survival curves were compared using the log-rank test. Statistical analysis was performed using SPSS version 19.0 (IBM SPSS statistics 19). Statistical significance was accepted for *P* values less than 0.05.

RESULTS

Patient characteristics

The mean age of all patients was 51.9 ± 6.92 years (range, 34-66 years), and 184 patients (82.1%) were male. Underlying liver disease was caused by HBV infection (87.9%) most commonly, followed by HCV infection (5.8%) and other causes (6.3%). The average Child-Pugh score was 8.15 ± 2.40 (range, 5-15), and the average Model for end-stage liver disease (MELD) score was 12.8 ± 7.63 (range, 1-37). The graft *vs* recipient weight ratio (GRWR) was 1.21% ± 0.27% (range, 0.6%-2.3%). Preoperative treatments were performed in 167 patients (74.6%). Of the 224 patients, 133 (59.4%) met Milan criteria, and 154 patients (68.8%) met UCSF criteria. During the follow-up period, 50 patients expired. The cause of death was HCC recurrence in 31 patients (62%), technical complications in nine patients, (18%), sepsis in five patients (10%), graft failure in three patients (6%), and other causes in two patients (4%). The median follow-up duration was 68 (range, 6-139) mo.

Recurrence and related factors

Of the 224 patients, recurrence occurred in 37 (16.5%) during the follow-up period. The number of patients who met the Milan criteria was 16 of 133 (12.0%), and of those who did not meet the Milan criteria 20 of 83 (24.1%) ($P = 0.021$). Most recurrences occurred within 2 years, with 26 patients (76%) experiencing recurrence within 1 year, and 30 (81%) within 2 years. Two patients experienced recurrence 5 years after transplantation. The primary recurrence site was intrahepatic in 10 patients (27%) and extrahepatic in 27 (73%). Of the extrahepatic metastasis sites, the lung was the most common primary recurrence site [10 patients (27%)]. Other recurrence sites were the brain, bone, adrenal gland, diaphragm, omentum, para-aortic lymph nodes, and neck lymph nodes.

Patient demographics and tumor characteristics were compared between the group without recurrence and the group with recurrence. The levels of AFP and PIVKA-Ⅱ were compared to the averages of 50, 100 and 200 ng/mL. The AFP level showed a significant difference when compared to that of 100 ng/mL ($P = 0.025$). Additionally, although the compared number of PIVKA-Ⅱ cases was small, the comparison itself was significant at 50, 100 and 200 ng/mL. Furthermore, the mean AFP and PIVKA-Ⅱ levels seemed higher in the recurrent patient group; however, no significant differences were noted (AFP, $P = 0.568$; PIVKA-Ⅱ, $P = 0.576$). The group that had received preoperative treatment showed a higher recurrence rate ($P = 0.025$). However, age, gender, cause of disease, Child-Pugh scores, MELD scores, and GRWRs showed no statistically significant difference. The mean maximal and total tumor diameters were higher in the recurrent group, being 5.54 ± 5.65 and 7.82 ± 6.56 cm, respectively, in the recurrent group and 2.76 ± 1.98 and 4.21 ± 2.88 cm, respectively, in the non-recurrent group ($P = 0.006$ and 0.005, respectively). Additionally, when the recurrent and non-recurrent groups were divided according to maximal diameter and total diameter over 5 cm, both groups had significantly higher recurrence rates ($P < 0.001$ and $P = 0.011$, respectively). The tumor number was significantly different between the non-recurrent and recurrent groups when the cutoff numbers were 5 and 7 ($P = 0.046$ and $P = 0.049$, respectively). Microvascular invasion was

Table 1 Patient and tumor characteristics according to recurrence rate, univariate analysis

Variables	Non-recurrent (n = 187)	Recurrent HCC (n = 37)	P-value
Patient characteristics			
Age (yr)	52.05 ± 6.93	50.89 ± 6.88	0.354
Gender (male: female)	151 (80.7):36 (19.3)	33 (89.2):4 (10.8)	0.221
HBV: HCV: Other cause	164:11:12	33:2:2	0.965
Child-Pugh score	8.15 ± 2.41	8.14 ± 2.37	0.972
MELD score	12.76 ± 7.25	13.05 ± 9.45	0.858
GRWR (%)	1.20 ± 0.28	1.24 ± 0.24	0.454
≤ 1 (n = 29)	26 (89.7)	3 (10.3)	0.327
> 1 (n = 193)	159 (82.4)	34 (17.6)	
AFP (ng/mL)	170.5 ± 806.2	249.94 ± 481.37	0.568
≤ 100 (n = 163)	142 (87.1)	21 (12.9)	0.025
> 100 (n = 59)	44 (74.6)	15 (25.4)	
PIVKA-Ⅱ (mAU/mL)	189.6 ± 1150.3	397.2 ± 674.5	0.576
≤ 100	99 (94.3)	6 (5.7)	0.018
> 100	14 (77.8)	4 (22.2)	
Pre-transplant treatment			0.025
No (n = 57)	53 (93.0)	4 (7.0)	
Yes (n = 167)	134 (80.2)	33 (19.8)	
Pathologic characteristics			
Maximal diameter (cm)	2.76 ± 1.98	5.54 ± 5.65	0.006
≤ 5 (n = 196)	170 (86.7)	26 (13.3)	< 0.001
> 5 (n = 20)	10 (50.0)	10 (50.0)	
Total diameter (cm)	4.21 ± 2.88	7.82 ± 6.56	0.005
≤ 9 (n = 185)	162 (87.6)	23 (12.4)	0.001
> 9 (n = 19)	11 (57.9)	8 (42.1)	
Tumor number	2.46 ± 2.18	3.06 ± 3.11	0.282
≤ 5 (n = 193)	164 (85.0)	29 (15.0)	0.046
> 5 (n = 22)	15 (68.2)	7 (31.8)	
Microvascular invasion			0.039
No (n = 163)	140 (85.9)	23 (14.1)	
Yes (n = 44)	32 (72.7)	12 (27.3)	
E-S grade			0.300
Low (Ⅰ, Ⅱ) (n = 108)	93 (86.1)	15 (13.9)	
High (Ⅲ, Ⅳ) (n = 81)	65 (80.2)	16 (19.8)	

Data are expressed as absolute n (%) or mean ± SD. HBV: Hepatitis B virus; HCV: Hepatitis C virus; MELD: Model for End-Stage Liver Disease; GRWR: Graft *vs* recipient weight ratio; AFP: Alpha fetoprotein; PIVKA-Ⅱ: Protein induced by vitamin K absence or angiotensin-Ⅱ; E-S grade: Edmondson-Steiner grade.

significant different between the non-recurrent and recurrent groups (P = 0.039). There was no difference in the Edmondson-Steiner (E-S) grade (P = 0.3; Table 1).

To identify factors related to recurrence, a multivariate analysis of factors that had shown statistical significance in univariate analysis was performed. The prognostic factors affecting recurrence included a serum AFP level > 100 ng/mL, a PIVKA-Ⅱ level > 100 mAU/mL, a tumor diameter > 5 cm, a total tumor diameter > 9 cm, a tumor number > 5, microvascular invasion, and pretransplantation treatment in univariate analyses. In multivariate analysis, a maximal tumor diameter > 5 cm {exponentiation of the B coefficient [Exp(B)], 11.89; 95%CI: 3.784-37.368; P < 0.001} and an AFP level > 100 ng/mL [Exp(B), 2.892; 95%CI: 1.172-7.132; P = 0.021] had a significant influence on recurrence (Table 2). Based on these data, we set our selection criteria [Catholic Medical Center (CMC) criteria] as a tumor diameter ≤ 5 cm and AFP ≤ 100 ng/mL. When both

Table 2 Risk factors for recurrence; multivariate analysis

Variables	P-value	Exp(B)	95%CI
AFP (100 ng/mL)	0.021	2.892	1.172-7.132
Pre-transplant treatment	0.114	2.421	0.742-7.897
Maximal diameter (5 cm)	< 0.001	11.891	3.784-37.368
Total diameter (9 cm)	0.142	2.633	0.754-9.194
Tumor number (5)	0.712	1.373	0.262-7.203
Microvascular invasion	0.27	1.768	0.653-4.784

AFP: Alpha fetoprotein; Exp(B): Exponentiation of the B coefficient.

criteria were met, the case was classified as within criteria (n = 138, 66.0%), and when at least one criterion was not met, the case was classified as beyond criteria (n = 71, 34.0%).

Impacts of the various criteria

We applied our data to various conventional criteria and therapeutic guidelines and compared the results with those of our CMC criteria. The data were applied to the Milan and UCSF criteria, TNM and BCLC stagings, the CLIP scoring system, and our CMC criteria were evaluated in terms of recurrence. In the Milan criteria and UCSF criteria, the patient group not meeting the criteria demonstrated a significantly higher recurrence rate than the group meeting the criteria (Milan, P = 0.021; UCSF, P = 0.002). In the CMC criteria specified above, the within-criteria group showed a recurrence rate of 10.0%, and the beyond-criteria group showed a recurrence rate of 28.4%. The difference was statistically significant (P = 0.001). In BCLC staging, the C stage showed a significantly higher recurrence rate compared with the 0, A, and B stages (P < 0.001); however, the differences among the 0, A, and B stages were not significant. In evaluating the CLIP scoring system, scores of 0 and 1, 2 and 3, and 4 and 5 were classified as three groups. The group with scores of 4 and 5 had a significantly higher recurrence rate than the other two groups (P = 0.023). The group with scores of 0 and 1, as well as that with scores of 2 and 3, showed no difference in recurrence. However, in the TNM staging, although recurrence showed an increasing trend with higher T status, there were no significant differences in recurrence (P = 0.325; Table 3).

The DFS and overall survival rates according to various criteria were compared. The 5-year DFS and survival rates of total patients were 80.9% and 76.4%, respectively. The Milan criteria showed a statistically significant difference in only the DFS rate (P = 0.009). However, the UCSF criteria showed a statistically significant difference in the DFS rate (P = 0.001) and overall survival rate (P = 0.009). Regarding CMC criteria, the 5-year DFS rate (P < 0.0001) and survival rate (P = 0.0006) showed statistically significant differences. Classification according to T status showed a decreasing trend in the 5-year DFS rate (P = 0.190) and survival rate (P = 0.394) with increasing T stage, albeit without statistical significance (P = 0.190). In the BCLC staging, the 5-year DFS and survival rates among stages 0, A, and B showed no statistically sig-

Table 3 Recurrence rates according to various criteria *n* (%)

Variables	Non-recurrent	Recurrent	*P*-value
Milan criteria			0.021
Within (n = 133)	117 (88.0)	16 (12.0)	
Beyond (n = 83)	63 (75.9)	20 (24.1)	
UCSF criteria			0.002
Within (n = 154)	136 (88.3)	18 (11.7)	
Beyond (n = 62)	44 (71.0)	18 (29.0)	
CMC criteria			0.001
Within (n = 140)	126 (90.0)	14 (10.0)	
Beyond (n = 74)	53 (71.6)	21 (28.4)	
TNM			0.325
T1 (n = 81)	71 (87.7)	10 (12.3)	
T2 (n = 127)	103 (81.1)	24 (18.9)	
T3 (n = 7)	5 (71.4)	2 (28.6)	
BCLC			< 0.001
0 (n = 36)	33 (91.7)	3 (8.3)	
A (n = 82)	73 (89.0)	9 (11.0)	
B (n = 53)	47 (88.7)	6 (11.3)	
C (n = 45)	27 (60.0)	18 (40.0)	
CLIP			0.023
0, 1 (n = 89)	73 (82.0)	16 (18.0)	
2, 3 (n = 110)	97 (88.2)	13 (11.8)	
4, 5 (n = 12)	7 (58.3)	5 (41.7)	

UCSF: University of California, San Francisco; CMC: Catholic Medical Center; BCLC: Barcelona Clinic Liver Cancer; CLIP: Cancer of the Liver Italian Program; HCC: Hepatocellular carcinoma.

Table 4 Disease-free survival and overall survival rates according to various criteria

Criteria		Disease-free survival			Overall survival		
		3 yr	5 yr	*P*-value	3 yr	5 yr	*P*-value
Milan	Within (n = 130)	0.880	0.867	0.009	0.808	0.798	0.137
	Beyond (n = 81)	0.730	0.704		0.733	0.706	
UCSF	Within (n = 150)	0.881	0.869	0.001	0.822	0.813	0.009
	Beyond (n = 61)	0.681	0.645		0.677	0.643	
CMC	Within (n = 138)	0.899	0.886	< 0.001	0.840	0.818	0.006
	Beyond (n = 71)	0.682	0.656		0.663	0.663	
TNM	T1 (n = 80)	0.890	0.870	0.190	0.830	0.830	0.394
	T2 (n = 123)	0.788	0.773		0.753	0.725	
	T3 (n = 7)	0.571	0.571		0.556	0.556	
BCLC	0 (n = 35)	0.943	0.902	< 0.001	0.882	0.882	0.005
	A (n = 80)	0.878	0.878		0.805	0.787	
	B (n = 52)	0.854	0.854		0.878	0.826	
	C (n = 44)	0.578	0.544		0.549	0.549	
CLIP	0,1 (n = 88)	0.795	0.771	0.011	0.776	0.776	0.272
	2,3 (n = 106)	0.885	0.870		0.819	0.792	
	4,5 (n = 12)	0.556	0.556		0.509	0.509	

UCSF: University of California, San Francisco; CMC: Catholic Medical Center; BCLC: Barcelona Clinic Liver Cancer; CLIP: Cancer of the Liver Italian Program.

nificant differences. However, the 5-year DFS rate (P < 0.001) and survival rate (P = 0.005) of stage C were significantly lower than those of the other stages. The CLIP scoring system showed a statistically significant difference in only DFS (P = 0.011; Table 4).

The Milan criteria showed significant differences regarding DFS, but not survival rate. We subdivided each factor of the Milan criteria and compared them with respect to the recurrence rate, and DFS and survival rates. Additionally, we subdivided factors of the CMC criteria and compared them with those of the Milan criteria (Table 5). Of the total, 133 (59.4%) patients were within the Milan criteria, and 140 (62.5%) were within the CMC criteria. When applying Milan criteria, the difference was significant when the cutoff value was a single tumor under 5 cm but was not significant when the cutoff value was less than three tumors, or two or three tumors less than 3 cm. When factors of the CMC criteria were subdivided, the recurrence rate, DFS, and overall survival rates were all significantly different. Although the number was small, most cases with a tumor size over 5 cm and an AFP level over 100 ng/mL recurred with a very poor prognosis; no patients survived beyond 3 years (Figure 1).

DISCUSSION

LT is a curative treatment modality for HCC. LT is particularly important in cases where resection is impossible due to factors such as liver cirrhosis. In countries with a shortage of deceased donor organs, LDLT can be the mainstay of therapy rather than DDLT. However, there is concern that LDLT has disadvantages regarding HCC recurrence compared with DDLT. The LDLT selection criteria are likely applied more widely than DDLT. Due to the short waiting time, LDLT candidates have no opportunity to be screened for an aggressive tumor biology. Because of their relatively small size LDLT grafts are subject to additional mechanical injury at the start of reperfusion, and angiogenesis and cell division signaling pathways may be initiated more frequently. The rapid graft regeneration in LDLT may also be associated with acceleration of tumor growth[13]. In fact, some studies have reported higher recurrence rates of LDLT compared with DDLT[14,15]. However, a recent meta-analysis concluded that the DFS rates of LDLT and DDLT do not differ significantly[16]. In our study, the recurrence rate was 16.5%, and for DDLT performed on patients within the Milan criteria, the recurrence rate was 12.0%. The total 5-year DFS rate and overall survival rate were 80.9% and 76.4%, respectively, and these results were generally acceptable.

The Milan criteria (MC) are often used to determine which patients will benefit from LT. However, when LT is strictly confined to those within MC, many patients who may benefit from LT will be lost. In advanced-stage Ⅲ HCC patients, survival rates are about 59% after LT, which is comparable to patients with benign disease (65%)[17]. LT is undoubtedly superior to transarterial chemoembolization or chemotherapy in patients beyond MC, who may still benefit from transplantation. Unlike DDLT, LDLT is private, not public, and is performed in beyond-MC patients more easily. As reported by four major LT centers, 29.6% of LDLT procedures were performed in beyond MC patients[18]. At our institution, 37.5% of LDLT patients exceeded MC. Many centers have center-based criteria for selection of patient

Table 5 Comparison of the Milan and Catholic Medical Center criteria

Criteria		Recurrence rate	*P*-value	5-yr DFS	*P*-value	5-yr survival	*P*-value
Milan criteria							
Single	≤ 5 cm (n = 91)	13%	< 0.001	0.862	< 0.001	0.829	< 0.001
	> 5 cm (n = 12)	61.5%		0.333		0.333	
No. 2 or 3	≤ 3 cm (n = 39)	9.8%	0.889	0.878	0.891	0.711	0.251
	> 3 cm (n = 22)	8.7%		0.848		0.747	
Number	≤ 3 (n = 164)	15.4%	0.209	0.824	0.228	0.781	0.316
	> 3 (n = 47)	21.7%		0.737		0.698	
CMC criteria							
Size ≤ 5 cm	AFP ≤ 100 (n = 138)	9.6%	0.001	0.886	< 0.001	0.818	< 0.001
Size ≤ 5 cm	AFP > 100 (n = 52)	20.0%		0.765		0.749	
Size > 5 cm	AFP ≤ 100 (n = 15)	38.9%		0.514		0.508	
Size > 5 cm	AFP > 100 (n = 4)	75.0%		0		0	

CMC: Catholic Medical Center; DFS: Disease free survival; AFP: Alpha fetoprotein (mg/dL).

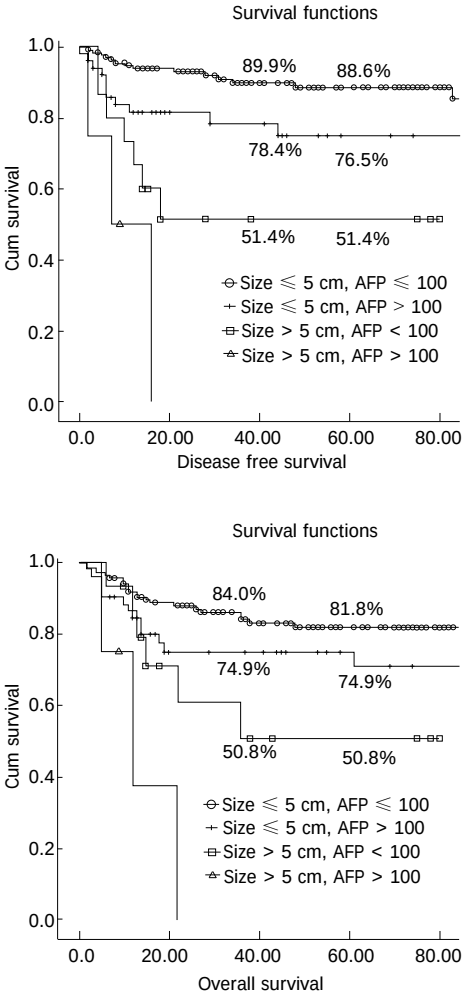

Figure 1 Comparison of the disease free survival and overall survival rates in patients with hepatocellular carcinoma according to Catholic Medical Center criteria. AFP: Alpha fetoprotein (mg/dL).

who exceed MC. The UCSF, Tokyo, and up-to-seven criteria are based on HCC size and number, which are surrogate markers of tumor volume[8-10]. Recently, the expression level of pre-operative serum tumor markers that affect HCC recurrence, such as pre-operative AFP and PIVKA-Ⅱ levels, have become a hot topic of research. Some authors have reported that an AFP level prior to LT of > 200 or > 1000 ng/mL affected HCC recurrence[8,19,20]. Conversely, the Kyoto and Kyushu University criteria included the PIVKA-Ⅱ level as a factor affecting tumor recurrence[11,12].

According to our results, a largest tumor diameter > 5 cm and AFP level C 100 ng/mL significantly influenced recurrence. Tumor number, which is important in the Milan criteria, did not show statistical significance. Indeed, many tumors discovered postoperatively are around 1 cm in size and are difficult to identify preoperatively. A discrepancy between the preoperatively and postoperatively discovered tumor numbers exists. Therefore, using tumor number as an important criterion may be problematic. Our results showed significant differences with the Milan criteria regarding recurrence and DFS; however, there was no significant difference regarding the survival rate, and so we subdivided each factor. There was a significant difference with a "single tumor smaller than 5 cm", a finding that ascribes importance to size. However, no significant difference was found regarding the two other factors, a finding that confers importance on the tumor number. These findings correlate with the finding that tumor number was not a significant factor in multivariate analysis. Additionally, biologic factors are more important than morphologic factors in terms of predicting tumor behavior. Thus, inclusion of AFP, which is a biologic factor rather than representative of the tumor number, seems to be more appropriate. Therefore, we decided that, for our criteria (CMC criteria), the largest diameter of the tumor should be less than 5 cm, and the AFP level should be less than 100 ng/mL.

Next, we applied our data to current HCC guidelines, staging systems, and the Milan and UCSF criteria, which are relatively widely used LT criteria, and compared the recurrence, DFS, and overall survival rates with those of our criteria. The Milan and UCSF criteria both adequately reflected recurrence, DFS, and survival. However, when compared with only our data, the UCSF criteria included more patients and showed a better correlation between criteria and prognosis. CMC criteria showed statistically significant differences in recurrence, DFS, and overall

survival rates. Notably, although the case number was small, in cases beyond both criteria, the recurrence rate was as high as 75%, and the 5-year DFS and overall survival rates were 0%, suggesting a contraindication to LT.

Many HCC staging systems are extant; however, the most commonly used is TNM staging, the 7th edition of which was recently published by the American Joint of Committee on Cancer (AJCC). The TNM system effectively stratifies post-hepatectomy HCC patients into stages Ⅰ, Ⅱ, and Ⅲ[21]. According to this system, we analyzed our results to evaluate the efficacy of the TNM system for LDLT. The recurrence, DFS, and overall survival rates worsened with increasing T stage; however, no statistically significant difference was found. T3 cases seemed to show a stronger trend, although the number was small. A statistically significant difference may be found if additional cases are examined.

Because HCC patients also have liver cirrhosis, not only the stage of HCC but also liver function and the general condition of the patient must be considered when deciding the treatment modality. Many HCC guidelines exist, but the most commonly used are the BCLC and CLIP scoring systems. The BCLC scoring system is used frequently in the United States and Europe for HCC therapy and considers factors such as the patient's general condition, Child-Pugh score, portal pressure, and tumor size and number. LT is recommended only for early stage A (single, 3 nodules, < 3 cm). Because LT can be performed regardless of Child-Pugh score or portal pressure, only the tumor factor is considered. Regarding transplantation, it is important to investigate the results according to BCLC stage. Vitale *et al*[22] reported that LT showed a survival benefit for patients with HCC and advanced liver cirrhosis (BCLC stage D) and in those with intermediate tumors (BCLC stage B-C). In our study, the recurrence, DFS, and overall survival rates showed no significant differences among BCLC stages 0, A, and B. However, these rates showed significant differences between stage C, which includes gross portal vein thrombosis, and the other stages. Therefore, although LT is recommended only for stage A according to the BCLC guidelines, LT may be performed on stage 0, A, and B patients with comparable results. Additionally, our study showed a stage C recurrence rate of 40.0% and 5-year DFS and survival rates of 54.4% and 54.9%, respectively. Although those data indicate a poor prognosis, as the recommended minimum prerequisite 5-year survival is 50%, LDLT may be performed in select stage C patients when the patient and family agree.

The CLIP scoring system was established in 1993. It considers Child-Pugh stage, tumor morphology, AFP level, and degree of portal vein thrombosis, and is considered an important index of prognosis. The CLIP scoring system for HCC is accurate and easy to implement[23]. However, to our knowledge, few studies have investigated the association between the CLIP scoring system and LDLT. In our study, we divided patients into three groups according to CLIP scores. In the lower-scoring groups (scores 0-3), recurrence rates, DFS rates, and overall survival rates showed no statistically significant differences. However, the group with scores of 4 and 5 showed a significant difference compared with the lower-scoring groups. Although it is difficult to reach a definite conclusion due to the small number of cases, patients with a CLIP score of 3 or lower may be suitable for LT, whereas those with a score of 4 or 5 may demonstrate worse results.

In conclusion, the recurrence rate was 16.5%, and the 5-year DFS and overall survival rates were 80.9% and 76.4%, respectively, after performance of LDLT in HCC patients. Factors influencing recurrence were a maximal tumor diameter greater than 5 cm and an AFP level greater than 100 ng/mL. When both criteria are not met, LT is contraindicated. Milan criteria, UCSF criteria, TNM staging, BCLC staging, and the CLIP scoring system showed different outcomes depending on the degree of criteria. Therefore, all of these staging and scoring systems are useful for determining LDLT for HCC patients. These findings should be confirmed by future prospective studies that include larger numbers of cases.

COMMENTS

Background

Liver transplantation (LT) for hepatocellular carcinoma (HCC) is known to be the best therapeutic option. To obtain a good result, it is important to select appropriate LT candidates from among HCC patients. Currently, many centers have their own criteria or treatment guidelines, which generally consist of factors that influence survival.

Research frontiers

The Milan criteria is a well-established tool for assessing the prognosis of HCC. We expanded the criteria while maintaining long-term survival rates. Authors decided that, for our criteria (CMC criteria), the largest diameter of the tumor should be less than 5 cm, and the alpha fetoprotein (AFP) level should be less than 100 ng/mL. When both criteria are not met, authors think LT is contraindicated.

Innovations and breakthroughs

Most centers agree on expanding the criteria because more patients can benefit from transplantation. Other systems include the TNM system that reflects cancer stage, and the Barcelona Clinic Liver Cancer (BCLC) and the Cancer of the Liver Italian Program (CLIP) staging systems that are well-known guidelines for therapy and prognosis prediction that are used in the United States and Europe. Regarding LT, investigation of the efficacy of these criteria for therapy of HCC is important. Authors defined the selection criteria according to risk factors for recurrence based on authors' results and compared them with other criteria or scoring systems, such as the Milan and UCSF criteria, TNM and BCLC staging, and the CLIP scoring system.

Applications

For living donor liver transplantation (LDLT), investigation of the efficacy of such criteria will facilitate adoption of extended criteria in LDLT.

Peer review

It is an important study which establishes a new and useful scoring system for HCC patients undergoing living donor liver transplantation. The paper is interesting, as it introduces a new (biochemical) parameter for the evaluation of HCC candidates to LDLT.

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BRIEF ARTICLE

Risk of sedation for diagnostic esophagogastroduodenoscopy in obstructive sleep apnea patients

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Abstract

AIM: To investigate whether patients with obstructive sleep apnea (OSA) are at risk of sedation-related complications during diagnostic esophagogastroduodenoscopy (EGD).

METHODS: A prospective study was performed in consecutive patients with OSA, who were confirmed with full-night polysomnography between July 2010 and April 2011. The occurrence of cardiopulmonary complications related to sedation during diagnostic EGD was compared between OSA and control groups.

RESULTS: During the study period, 31 patients with OSA and 65 controls were enrolled. Compared with the control group, a higher dosage of midazolam was administered (P = 0.000) and a higher proportion of deep sedation was performed (P = 0.024) in the OSA group. However, all adverse events, including sedation failure, paradoxical responses, snoring or apnea, hypoxia, hypotension, oxygen or flumazenil administration, and other adverse events were not different between the two groups (all P > 0.1). Patients with OSA were not predisposed to hypoxia with multivariate logistic regression analysis (P = 0.068).

CONCLUSION: In patients with OSA, this limited sized study did not disclose an increased risk of cardiopulmonary complications during diagnostic EGD under sedation.

Key words: Conscious sedation; Obstructive sleep apnea; Endoscopy; Complications; Safety

Core tip: Patients with obstructive sleep apnea (OSA) are known to be vulnerable to cardiopulmonary complications during deep sedation and anesthesia; however, little is known about the risk of conscious sedation during esophagogastroduodenoscopy (EGD). This prospective study evaluated the cardiopulmonary complications related to conscious sedation during diagnostic EGD between OSA group (n = 31) and control group (n = 65). All adverse events, including sedation failure, paradoxical responses, snoring or apnea, hypoxia, hypotension, oxygen or flumazenil administration, and other adverse events were not different between groups. Therefore, the risk of cardiopulmonary complications during diagnostic EGD under sedation may not be increased in patients with OSA.

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INTRODUCTION

Esophagogastroduodenoscopy (EGD) is generally a safe procedure, and has been routinely performed with sedation in the general population. Sedation reduces patient discomfort by inducing analgesia and amnesia and improves patient tolerance during EGD[1-3]. However, sedation itself is also the cause of many potentially serious adverse events during EGD. The frequency of serious adverse events associated with EGD is about 0.5%[4,5], and over 50% of these adverse events are cardiopulmonary complications related to the sedation[4-6]. The most common cardiopulmonary adverse event is hypoxia, although its incidence is variable depending on the definition of hypoxia, the patient population, and the level of sedation[7-9].

Obstructive sleep apnea (OSA) is characterized by intermittent and recurrent episodes of partial or complete obstruction of the upper airway during sleep. The administration of sedatives in patients with OSA may worsen obstruction of the pharynx and depression of the upper airway muscles[10]. Therefore, patients with OSA are known to be vulnerable to cardiopulmonary complications during deep sedation and anesthesia[10-13]. However, little is known about the adverse events of moderate sedation for diagnostic EGD in patients with OSA. Khiani *et al*[14] reported that there was no significant difference in the rates of hypoxia during EGD with sedation between high- and low-risk OSA cases, but patients with confirmed OSA were not included in this study.

The purpose of this study was to determine whether patients with confirmed OSA undergoing diagnostic EGD under sedation are more likely to become hypoxic. A prospective, case-control study was performed to compare the rate of hypoxia between an OSA group and a control group during diagnostic EGD under sedation.

MATERIALS AND METHODS

Patient population

A prospective study was performed in consecutive patients with OSA, who were recruited from a sleep center laboratory at the Kyung Hee University Hospital in Gang Dong, Seoul, Republic of Korea between July 2010 and April 2011. Patients with confirmed OSA who underwent EGD under moderate sedation for their routine health checkup at initial diagnosis or at a follow-up visit in an outpatient clinic were considered eligible for this study. Consecutive healthy subjects, who underwent EGD under moderate sedation for a routine health checkup between February 2011 and April 2011, were also enrolled as a control group. As the prevalence of gastric cancer is high in South Korea, healthy subjects often routinely undergo screening EGD for a regular medical checkup without any gastrointestinal symptoms in Korea. The occurrence of hypoxia and other cardiopulmonary complications related to sedation for diagnostic EGD were compared between an OSA and a control groups.

Patients with confirmed OSA between 18 and 70 years old who provided informed consent were eligible for this study. The diagnosis of OSA was confirmed based on the results of full-night, in-laboratory polysomnography. Asymptomatic patients who scored higher than 15 on the apnea hypopnea index or respiratory disturbance index were diagnosed with OSA, as were patients with symptoms or signs of disturbed sleep who scored higher than 5 on the hypopnea index or respiratory disturbance index[11]. Patients who declined to participate in the study; were younger than 18 years or older than 70 years; had an EGD examination within the last 12 wk; were pregnant; had a history of substance abuse; were in poor health as determined by a score greater than grade Ⅲ in the American Society of Anesthesiologists classification; had lung disease requiring home oxygen; had a baseline oxygen saturation less than 90% as measured by pulse oximetry without sedation; or had previous gastric surgery were excluded from the study.

A study coordinator administered a questionnaire to patients, which included questions about alcohol consumption, cigarette smoking, or co-morbidities such as hypertension or diabetes mellitus (DM). Current smokers were defined as those who smoked at least one cigarette per day for the previous 12 mo and alcohol consumption was defined as drinking over 40 g of alcohol per day. Hypertension was defined as a blood pressure of $\geqslant$ 140 mmHg or taking anti-hypertensive medication. DM was defined as fasting glucose of $\geqslant$ 126 mg/dL or previously diagnosed DM. All data were collected and stored securely.

EGD and sedation

EGDs were performed by an expert staff endoscopist (Cha JM) in a standard manner using a standard video scope (EG-590WR; Fujinon Inc., Saitama, Japan). Sedation was performed in accordance with guidelines published by the American Society of Gastroenterological Endoscopy[2]. An individualized dose of midazolam was administered by registered nurses according to study protocol based on patient age and weight. In this study, sedation was initiated with a standard dose of midazolam at 0.07 mg/kg[15,16]. Those not adequately sedated 180 s later were provided an additional 1-2 mg of midazolam until the patient reached a state of moderate sedation or until the maximum dose of 0.1 mg/kg had been administered. The goal was to achieve moderate sedation (*i.e.*, conscious sedation), which was defined as depression of consciousness during which the patient responds purposefully to verbal commands, either alone or accompanied by light tactile stimulation[3]. The level of sedation was evaluated by an independent nurse using the Modified Observer's Assessment of Alertness/Sedation (MOAA/S) scale[17], 2 min after initiation of sedation and when the EGD was inserted. MOAA/S scores range from 0 to 6, and moderate sedation was defined as a MOAA/S score from 3 to 4. The total duration of the EGD was recorded with a stopwatch, from the beginning of sedation until the end of the procedure. During EGD, the following data

were collected: midazolam dose, MOAA/S score, total duration of EGD, oxygen administration, patient physiologic parameters (*e.g.*, SaO_2 and systolic blood pressure), whether the patient snored during the procedure, and episodes of hypoxia or other cardiopulmonary complications. Potential complications were described to patients before the procedure, and all patients provided verbal and written consent prior to the EGD under sedation. After EGD, patients were observed for at least 30 min in a recovery room for possible complications.

Monitoring and data collection

All outcome parameters during the procedure were assessed by the endoscopist and registered nurses. Baseline blood pressure and SaO_2 were recorded before administration of midazolam. All patients were continuously monitored for blood pressure, SaO_2, respiratory activity, and electrocardiography during the procedure. The assistant nurse's responsibilities were limited to sedating and monitoring ventilatory effort, which was visually monitored by chest excursions, respiratory effort, and respiratory rate at regular intervals. A change in tone denoted a rise or fall in saturation, and alarms were set to sound if the value fell below 90%. Oxygen was supplemented when the oxygen saturation level dropped to between 81%-89% for more than 15 s, or below 80% more than 5 s.

Sedation failure was defined as any procedure that required the use of flumazenil for successful completion of the procedure, or that was terminated by the endoscopist due to patient agitation. Hypoxia was defined as a pulse oximetry measurement of SaO_2 less than 90% for at least 5 s. Paradoxical response was defined as hostility, rage and even physical violence, necessitating the restraint of such patients after the administration of midazolam. Hypotension was defined as systolic blood pressure less than 90 mmHg or a drop in systolic blood pressure of more than 20 mmHg from baseline systolic blood pressure.

Ethics

This work was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. This study was approved ethically by the Institutional Review Board of the Kyung Hee University Hospital at Gangdong (KHNMC IRB 2010-013). All patients provided informed written consent.

Statistical analysis

The prevalence of hypoxia related to moderate sedation for diagnostic EGD was assumed to be approximately 0.5% in control group, as the risk of hypoxia in diagnostic EGD with moderate sedation was approximately 0.5% in previous studies[4,5,8,18]. We assumed that a difference in hypoxia rate of 20% between the OSA and control groups was clinically significant and would be sufficient for clinicians to avoid sedation in patients with OSA. It was calculated that 56 participants (28 in each group) would be required to have an 80% chance of ruling out a 20% difference with 95% confidence (one-sided analysis).

Continuous data are described by mean and SD. Categorical data are presented as numbers and percentages. Continuous variables were compared using the *t* test. Categorical variables were compared using the χ^2 test or Fisher's exact test, when appropriate. We computed OR and 95%CI using logistic regression analysis. A P value < 0.05 was considered significant. All statistical analyses were performed using SPSS statistical software version 13.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

During the study period, 61 patients with OSA were eligible. Data of 31 patients with OSA were analyzed after exclusion of 30 patients: unwillingness to participate in the study ($n = 22$), EGD examination within the last 12 wk ($n = 6$) and previous gastric surgery ($n = 2$). For the control group, 69 subjects were eligible and data of 65 subjects were analyzed after exclusion of 4 subjects due to unwillingness to participate ($n = 2$) and an EGD examination within the last 12 wk ($n = 2$). In total, 31 patients with confirmed OSA and 65 control subjects were enrolled in this study. The study group of 96 subjects included 50 men (52.1%) and 46 women (47.9%), with a mean age of 48.3 years. Mean body mass index (BMI) was 24.2 kg/m^2; 24.0% ($n = 23$) of subjects were classified as overweight and 37.5% ($n = 36$) of subjects were classified as obese according to Asia-Pacific guidelines. In total, a mean dose of 5.0 mg midazolam was used for sedation of diagnostic EGD.

Table 1 shows the patient characteristics and baseline clinical data of the two study groups, including age, sex, height, weight, BMI, smoking and drinking status, Charlson's comorbidity score, history of hypertension or DM, baseline blood pressure and SaO_2. As expected, a higher proportion of males, subjects with a higher BMI and subjects with hypertension were more frequently included in the OSA group than in the control group. However, other clinical variables and baseline SaO_2 were not different between the two groups.

Table 2 shows the procedural characteristics and adverse events in the two study groups. A higher dosage of midazolam was administered in the OSA group than in the control group ($P < 0.001$), which makes sense considering midazolam doses were based on weight, and patients with OSA weighed more on average. MOAA/S scores were not significantly different between two groups. The target level of sedation for this study was moderate sedation, and 58.3% of subjects reached moderate sedation while 41.7% reached deep sedation. For the level of sedation, the proportion of deep sedation was significantly higher in the OSA group than in the control group ($P = 0.024$). Most patients (99.0%) reached the target level of sedation within 2 min, and only one in the OSA group needed an additional dose of sedatives. The frequency of all adverse events, including sedation failure, paradoxical responses, snoring or apnea, hypoxia, hypotension, oxygen or flumazenil administration, and other

Table 1 Characteristics and baseline clinical data of subjects in the obstructive sleep apnea and control groups

	OSA group (n = 31)	Control group (n = 65)	P value
Patient characteristics			
Age (yr)	51.3 ± 9.6	47.1 ± 11.8	0.082
Sex (male)	23 (74.2)	27 (41.5)	0.003
Height (meters)	167.1 ± 6.8	164.4 ± 8.0	0.104
Weight (kg)	74.3 ± 11.2	62.6 ± 12.0	0.000
BMI (kg/m^2)	26.5 ± 3.0	23.1 ± 3.3	0.000
Smoking	9 (29.0)	9 (13.8)	0.075
Drinking	11 (35.5)	24 (36.9)	0.891
Charlson score (points)	1.0 ± 0.2	1.0 ± 0.0	0.325
Hypertension	10 (32.3)	9 (13.8)	0.034
Diabetes mellitus	4 (12.9)	7 (10.8)	0.759
Baseline clinical data			
Systolic BP (mmHg)	127.6 ± 16.9	114.3 ± 22.2	0.004
Diastolic BP (mmHg)	79.3 ± 10.6	66.1 ± 14.4	0.000
SaO_2 (%)	97.6 ± 1.7	98.0 ± 2.2	0.418

Data are expressed as absolute n (%) or mean ± SD. OSA: Obstructive sleep apnea; BMI: Body mass index; BP: Blood pressure; SaO_2: Arterial oxygen saturation.

Table 2 Procedural characteristics and adverse events in the obstructive sleep apnea and control groups

	OSA group (n = 31)	Control group (n = 65)	P value
EGD data			
Duration of EGD (s)	261.4 ± 79.6	304.4 ± 154.2	0.074
Midazolam dosage (mg)	5.3 ± 0.9	4.5 ± 0.8	0.000
MOAA/S score (points, at 2 min)	2.5 ± 1.2	2.9 ± 1.0	0.146
Level of sedation for EGD			0.024
Moderate sedation	13 (41.9)	43 (66.2)	
Deep sedation	18 (58.1)	22 (33.8)	
Adverse events			
Sedation failure	0 (0.0)	0 (0.0)	-
Paradoxical responses	4 (12.9)	4 (6.2)	0.268
Snoring or apnea	5 (16.1)	7 (10.8)	0.458
Hypoxia (SaO_2 < 90%)	1 (3.2)	7 (10.8)	0.211
Hypotension	0 (0.0)	0 (0.0)	-
Other adverse events	0 (0.0)	0 (0.0)	-
Oxygen administration	1 (3.2)	1 (0.2)	0.588
Flumazenil administration	0 (0.0)	0 (0.0)	-

Data are expressed as absolute n (%) or mean ± SD. OSA: Obstructive sleep apnea; EGD: Esophagogastroduodenoscopy; MOAA/S: Modified Observer's Assessment of Alertness/Sedation; SaO_2: Arterial oxygen saturation.

Table 3 Multivariate logistic regression analysis of possible risk factors for a hypoxia

Parameter	OR (95%CI)	P value
Age (continuous)	1.056 (0.958-1.164)	0.276
Sex (female *vs* male)	1.003 (0.153-6.563)	0.997
Smoking (no *vs* yes)	0.753 (0.056-10.127)	0.830
Alcohol (no *vs* yes)	0.592 (0.095-3.706)	0.576
Diabetes mellitus (no *vs* yes)	1.842 (0.187-18.128)	0.601
Hypertension (no *vs* yes)	1.102 (0.120-10.117)	0.932
Body mass index (< 25 kg/m^2 *vs* ≥ 25 kg/m^2)	1.304 (0.192-8.845)	0.786
Midazolam dose (< 5 mg *vs* ≥ 5 mg)	10.726 (0.815-141.100)	0.071
OSA (no *vs* yes)	0.117 (0.012-1.168)	0.068

OSA: Obstructive sleep apnea.

adverse events was not different between two groups (all $P > 0.1$). In cases of hypoxia, only one (3.2%) patient became transiently hypoxic in the OSA group, and seven cases (10.8%) became transiently hypoxic in the control group ($P = 0.211$). In total, 96.8% of patients remained stable without oxygenation supplementation in the OSA group, and 99.8% remained stable without oxygenation supplementation in the control group ($P = 0.588$).

To determine independent predictors of hypoxia, we performed logistic regression analysis adjusted for age, sex, smoking, alcohol, DM, hypertension, BMI (< 25 kg/m^2 *vs* ≥ 25 kg/m^2), midazolam dose (< 5 mg *vs* ≥ 5 mg), and OSA (Table 3). In this analysis, all variables including the confirmed diagnosis of OSA were not predisposed to risk of hypoxia (OR = 0.117, 95%CI: 0.012-1.168, $P = 0.068$).

DISCUSSION

This is the first prospective study to evaluate the risk of sedation for diagnostic EGD in patients with OSA. Although higher doses of midazolam were administered and a higher proportion of deep sedations were performed in the OSA group than in the control group, all adverse events associated with sedation for diagnostic EGD were not significantly different between the two study groups. In addition, the confirmed diagnosis of OSA was not predisposed to hypoxia with multivariate logistic regression analysis.

Anesthesia and deep sedation have been shown to increase pharyngeal collapse, decrease ventilator response, and impair the arousal response, leading to cardiopulmonary complications in patients with OSA[10-13,19-22]. However, little is known about the risk of sedation for diagnostic EGD, which is usually targeted for moderate sedation, in patients with OSA. The risk of hypoxia associated with sedation for diagnostic EGD in patients with OSA might be lower than expected due to the following three reasons. First, the sedation level for diagnostic EGD is generally targeted for moderate sedation. The risk of hypoxia in patients with OSA might be lower in cases of moderate sedation than deep sedation or anesthesia because increasing depth of sedation is associated with a progressive increase in upper airway collapsibility[23,24]. Second, diagnostic EGD is a relatively short procedure, which may be associated with a lower risk of hypoxia, as longer endoscopic procedures were associated with increased risk of hypoxia compared to shorter endoscopic procedures[25]. Finally, the position of the patient during diagnostic EGD is in the left lateral decubitus position, which may be associated with a lower risk of hypoxia. Patients who undergo anesthesia or surgery in the supine position may be at greater risk of upper airway collapsibility than those who are in the left lateral decubitus position. Therefore, the risk of hypoxia associated with sedation for diagnostic EGD in patients with OSA might

not be high.

As patients with OSA are classified at increased risk for sedation-related complications[26], physicians are often reluctant to recommend sedation for diagnostic EGD. However, the risk of sedation for diagnostic EGD in patients with OSA is not supported by the direct evidence. Our findings suggest that the risk of sedation for diagnostic EGD in patients with OSA might be rare. In the literature, hypoxia has been used as a surrogate for cardiopulmonary outcomes, and its reported incidence during gastrointestinal endoscopy under sedation ranges widely from 10% to 70%[9,18,27-38]. Although the study methodology, definition of hypoxia, patient populations, type of endoscopy, use of supplemental oxygen, and type of sedatives should be considered for the interpretation of risk factors of hypoxia during endoscopy under sedation, a confirmed diagnosis of OSA is rarely pointed out as a risk factor of hypoxia during endoscopy under moderate sedation.

This study is unique as this is the first prospective study that evaluated the risk of sedation for diagnostic EGD in patients with OSA. Khiani *et al*[14] suggested that patients at risk for OSA can safely undergo sedation for routine endoscopic procedures; however, only the Berlin questionnaire was used for OSA risk stratification and patients with OSA were not included in this study. Sharara *et al*[39] suggested that snoring during colonoscopy is a strong predictor of OSA, however, only a sleep questionnaire was also used in this study. Mador *et al*[40] showed that presence of OSA does not clearly increase the risk of cardiopulmonary complications in endoscopy procedures under moderate sedation, which is a consistent finding with ours. Although all patients with OSA were confirmed with overnight polysomnography in this study, it was also limited due to its retrospective nature and nonstandard definition of hypopnea. Furthermore, the method of sedation is not controlled, and EGD and colonoscopy were analyzed together in this retrospective analysis. In contrast, our prospective study included all patients with confirmed OSA with a polysomnography and evaluated adverse effects with pre-defined criteria. As an OSA diagnosis is confirmed in only 50%-60% of subjects suspected of having OSA with questionnaire[41,42], subjects without OSA might be included in previous questionnaire based studies[14,39].

The present study has several limitations. First, 30 OSA cases were excluded from 61 eligible patients with confirmed diagnosis of OSA. However, there was minimal selection bias, if any, as data was prospectively collected and patients were excluded based on criteria determined before starting enrollment. One reason for the high rate of exclusions in our study is that most patients with OSA were concerned about sedation risks. They have been recommended for a diagnostic EGD without sedation due to hypoxia risk for a long time. Therefore, our study may have a helpful clinical implication for them. Second, the sample size was small because we only included confirmed OSA cases. At the inception of this study, we estimated that the OSA group would comprise a much larger number of cases and improve the power of this study. The sample size of our study was small (n = 96), however; the statistical significance may be sufficiently verified with our study design as only 56 participants were required in our sample size calculation. Third, there is a limitation for the generalizability of our results to all patients with OSA because our patients were recruited from a single university outpatient clinic, and procedures were purely diagnostic EGD with midazolam alone. Our results cannot be generalized to EGDs that include therapy or more extensive diagnosis such as Barrett's surveillance or sedation with propofol or opioids.

In conclusion, this limited sized study did not disclose an increased risk of cardiopulmonary complications during diagnostic EGD with moderate sedation in patient with OSA. This suggests that the majority of patients with OSA would safely undergo diagnostic EGD with sedation. However, our findings should be more clearly established in the future in large studies.

COMMENTS

Background

Obstructive sleep apnea (OSA) is characterized by intermittent and recurrent episodes of partial or complete obstruction of the upper airway during sleep. The administration of sedatives in patients with OSA may worsen obstruction of the pharynx and cause depression of the upper airway muscles. Therefore, patients with OSA are known to be vulnerable to cardiopulmonary complications during deep sedation and anesthesia. However, little is known about the adverse events of moderate sedation for diagnostic esophagogastroduodenoscopy (EGD) in patients with OSA.

Research frontiers

The research hotspot is to investigate whether patients with OSA are at risk of sedation-related complications during diagnostic EGD.

Innovations and breakthroughs

This is the first prospective study to evaluate the risk of sedation for diagnostic EGD in patients with OSA. Although higher doses of midazolam were administered and a higher proportion of deep sedation was performed in the OSA group than in the control group, all adverse events associated with sedation for diagnostic EGD were not significantly different between the two study groups. In addition, the confirmed diagnosis of OSA was not predisposed to hypoxia with multivariate logistic regression analysis. This study is outstanding in that the risk of sedation for diagnostic EGD in patients with OSA was investigated in a prospective design with pre-defined criteria and the diagnosis of all OSA patients was confirmed with full-night, in-laboratory polysomnography. Although the diagnosis of OSA was made with a questionnaire in previous studies, subjects without OSA might be included as an OSA diagnosis is confirmed in only 50%-60% of subjects suspected of having OSA with a questionnaire.

Applications

As patients with OSA are classified at increased risk for sedation-related complications, physicians are often reluctant to recommend sedation for diagnostic EGD. In fact, most patients with OSA were concerned about sedation risks in our study, as they have been recommended for a diagnostic EGD without sedation for a long time. However, the risk of sedation for diagnostic EGD in patients with OSA is not supported by the direct evidence. Therefore, this study may have a helpful clinical implication for patients with OSA when they perform diagnostic EGD under sedation.

Terminology

OSA: The diagnosis of OSA was confirmed based on the results of full-night, in-laboratory polysomnography. Asymptomatic patients who scored higher than 15 on the apnea hypopnea index or respiratory disturbance index were diagnosed with OSA, as were patients with symptoms or signs of disturbed sleep who scored higher than 5 on the hypopnea index or respiratory disturbance index.

Moderate sedation: The level of sedation was evaluated by an independent nurse using the Modified Observer's Assessment of Alertness/Sedation (MOAA/S) scale, and moderate sedation was defined as a MOAA/S score from 3 to 4. Hypoxia: Hypoxia was defined as a pulse oximeter measurement of SaO_2 less than 90% for at least 5 s.

Peer review

It is generally a well-written paper: it has an excellent core tip section to "advertise" the paper, well-summarized results section adding meaningfully to the existing literature on an important subject and the rest of the sections are also well-written. The bibliography is focused and well-researched. The results are interesting and suggest that the majority of patients with OSA would safely undergo diagnostic EGD with sedation.

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BRIEF ARTICLE

A feasible modified biopsy method for tissue diagnosis of gastric subepithelial tumors

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Abstract

AIM: To evaluate the diagnostic yield and safety of a modified technique for the histological diagnosis of subepithelial tumors (SETs).

METHODS: A retrospective review of patients who underwent a modified technique for the histological diagnosis of gastric SETs, consisting of a mucosal incision with a fixed flexible snare (MIF) and deep-tissue biopsy under conventional endoscopic view, from January 2012 to January 2013 was performed. Eleven patients with gastric SETs 10-30 mm in diameter and originating from the third or fourth layer on endoscopic ultrasonography were included.

RESULTS: The mean age was 59.8 (range, 45-76) years, and 5 patients were male. The mean size of the SETs was 21.8 (range, 11-30) mm. The number of biopsy specimens was 6.3 (range 5-8). The mean procedure time was 9.0 min (range, 4-17 min). The diagnostic yield of MIF biopsies was 90.9% (10/11). The histological diagnoses were leiomyoma (4/11, 36.4%), aberrant pancreas (3/11, 27.3%), gastrointestinal stromal tumors (2/11, 18.2%), an inflammatory fibrinoid tumor (1/11, 9.1%); one result was non-diagnostic (1/11, 9.1%). There were six mesenchymal tumors; the specimens obtained in each case were sufficient for an immunohistochemical diagnosis. There was no major bleeding, but one perforation occurred that was successfully controlled by endoscopic clipping.

CONCLUSION: The MIF biopsy was simple to perform, safe, and required a shorter procedure time, with a high diagnostic yield for small SETs.

Key words: Subepithelial tumors; Stomach; Biopsy; Endoscopy; Diagnostic techniques

Core tip: Tissue acquisition from subepithelial tumors (SETs) is essential for a differential diagnosis. Several techniques have been introduced to obtain SET tissue samples. However, the diagnostic efficacy was limited or the procedure was complex and difficult. We investigated a modified technique for the histological diagnosis of SETs, consisting of a mucosal incision with a fixed flexible snare (MIF) and deep-tissue biopsy at the incision site under a conventional endoscopic view. The results of this study suggest that the MIF biopsy is simple to perform, safe, fast, and provides a high diagnostic yield for small SETs.

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INTRODUCTION

Gastric subepithelial tumors (SETs) are typically found incidentally during screening endoscopies. The exact incidence of SETs on routine endoscopy is unknown, although one retrospective study reported a prevalence of 0.36%[1]. A wide range of diseases may present as SETs in the upper gastrointestinal tract, including lipoma, leiomyoma, aberrant pancreas, varices, carcinoid, gastrointestinal stromal tumors (GISTs), and lymphomas. Thus, tissue diagnosis for SET differentiation is particularly important because these lesions may have different prognoses and have different therapeutic protocols, such as resection or observation.

Gastric SETs are difficult to definitively diagnose by conventional imaging studies, such as ultrasonography, computed tomography, and magnetic resonance imaging. Endoscopic ultrasonography (EUS) is currently the most effective diagnostic tool for the differential diagnosis of SETs because it can help determine the depth and originating layer of the gastrointestinal wall of the lesion[2]. However, EUS morphological characteristics alone do not provide an accurate diagnosis. EUS has limited utility in distinguishing between benign and malignant lesions[3]. In particular, if the SET is found to be a hypoechoic lesion located in the third or fourth layer on EUS findings, tissue acquisition should be strongly considered for a histological diagnosis[3].

Generally, histological diagnosis may not be necessary in large SETs (more than 3 cm in diameter) or symptomatic lesions because such SETs require resection regardless of the pathological confirmation[4,5]. In contrast, small SETs, such as GISTs less than 3 cm in diameter, do not usually require resection because most are benign. However, the current concept is that every GIST has at least malignant potential, even small GISTs of 1 cm in diameter[6,7].

Presently, there is no consensus regarding the management strategy and surveillance of asymptomatic and small SETs[5,8]. For a definitive diagnosis of SETs, tissue acquisition from a subepithelial lesion is essential for a differential diagnosis and an assessment of the malignant potential.

However, conventional endoscopic biopsies do not typically provide sufficient submucosal tissue specimens for diagnosis because SETs are located deep and are covered with normal mucosa. Thus, several techniques have been introduced to obtain SET tissue samples. However, the diagnostic efficacy seems to be limited for immunohistological diagnosis with these methods, such as EUS-guided fine-needle aspiration (EUS-FNA), EUS-guided trucut biopsy (EUS-TCB), and stacked biopsy[9-15].

We thus investigated a modified technique for the histological diagnosis of SETs, consisting of mucosal incision with a fixed flexible snare (MIF) and deep-tissue biopsy at the incision site under a conventional endoscopic view.

MATERIALS AND METHODS

Patients

A retrospective review of patients who underwent MIF biopsies from January 2012 to January 2013 was conducted. Among the patients with incidental SETs 10-30 mm in diameter, the inclusion criteria were SETs found in the third and fourth layers, with hypoechoic or mixed echogenic patterns on EUS. We excluded patients with typical findings of a vessel, cyst, or lipoma on EUS. We also excluded patients with EUS characteristics suggestive of malignancy, including those with hyperechogenic foci, anechoic necrotic zones, irregular extraluminal borders, or adjacent malignant-appearing lymphadenopathy[16].

Informed consent, with adequate explanation of the biopsy and possible complications, was obtained from each patient. This study was approved by the Institutional Review Board of Gachon University Gil Medical Center (IRB No. GDIRB2013-05).

Procedure details

All procedures were performed by one endoscopist (Chung JW) using a conventional single-channel endoscope (GIF Q260 or H260; Olympus Optical Co., Ltd., Tokyo, Japan) with patients under conscious sedation without a transparent hood. An endoscopic-knife (fixed flexible snare; Kachu Technology, Seoul, Korea) connected to an electrosurgical unit (VIO 300D; ERBE, Tübingen, Germany) in "ENDO CUT 1" mode was used for the incision of the mucosa covering the SETs (Figure 1). The length of the tip in this endoscopic knife was 1.5 mm. Under a direct conventional endoscopic view, a mucosal incision was made over the convex zone of the lesion (Figure 2). After the mucosal incision using the fixed flexible snare, we performed a conventional forceps (FB-25K-1; Olympus) biopsy, deep into the incision site of the covering mucosa. Finally, we obtained 5-8 biopsy samples. According to the judgment of the endoscopist, incision site bleeding was controlled using argon plasma coagulation (APC 2; ERBE); the site was closed prophylactically with 2-4 endoclips (HX-610-90L or HX 610-135L; Olympus) in some patients.

Before the MIF biopsy, EUS was performed to characterize the SETs using conventional radial EUS (UM2000; Olympus). All patients were closely monitored for any procedure-related complication in the recovery room and were discharged 2-3 h after the procedure was finished. Oral intake was started 8 h after the procedure. Patients who underwent MIF biopsy empirically received proton pump inhibitors for 1 wk after the procedure. If there was no symptom and/or sign associated with complications, routine follow-up endoscopy was not performed. All patients were instructed to visit our hos-

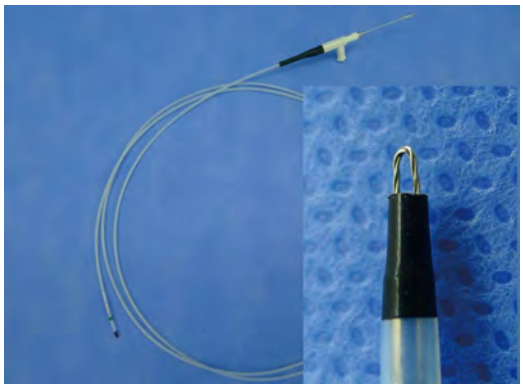

Figure 1 **The fixed flexible snare is an endoscopic instrument for mucosal incision and dissection.**

pital immediately if they had symptoms and/or signs of complications (abdominal pain, hematemesis, melena, dizziness). Patients without symptoms and/or signs of complications visited the outpatient clinic 1-2 wk after the procedure.

Perforation was defined as a split in the muscle layer that occurred during the procedure or the presence of free air detected in post-procedure imaging studies. Major bleeding was defined as bleeding that resulted in a drop in hemoglobin of 2 g/dL or more, that required blood transfusion and/or endoscopic re-intervention, or if surgical intervention caused the hemorrhage. Minor bleeding was defined as bleeding that was controlled by endoscopic hemostasis (argon plasma coagulation or clip) during the procedure.

Pathologic examination

The forceps biopsy specimens were fixed in a 10% formalin solution and embedded in paraffin wax. The pathologic examinations included identification of cell type, overall cellularity, cytoplasmic features, nuclear atypia, mitotic index, and immunohistochemical findings. The mitotic index was determined on 50 consecutive high-power fields (HPFs). Immunohistochemical analyses of CD117 (c-kit), CD34, desmin, smooth muscle actin, S-100, and Ki-67 markers were performed with commercially available antibodies to classify the tumor subtype. Positive reactions for CD117 and CD34 were considered diagnostic of a GIST. Mesenchymal lesions that were positive for desmin and smooth muscle actin and negative for CD117 and CD34 were diagnosed as smooth muscle tumors such as leiomyoma. Positivity for S-100 protein and negativity for desmin, smooth muscle actin, and CD117 were diagnostic of neural tumors.

Statistical analysis

Statistical analyses were performed using SPSS software (Ver. 12.0 for Windows; SPSS, Chicago, IL, United States). Continuous data are presented as the means (range), and categorical data are presented as absolute numbers and percentages.

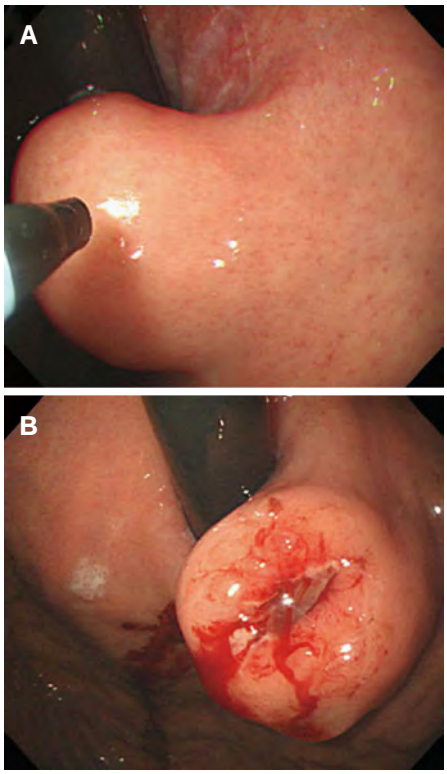

Figure 2 **Mucosal incision with a fixed flexible snare and conventional forceps biopsy.** A: A fixed flexible snare performing a mucosal incision over the center of a subepithelial lesion in the cardia of the stomach; B: The lesion after the forceps biopsy following the mucosal incision.

RESULTS

The patient characteristics, location and size of the SETs, histological results, and procedure details are summarized in Table 1. In total, 11 patients were enrolled during the study period. The mean age was 59.8 years (range, 45-76 years); there were five males and six females. The mean size (longest diameter) of the tumors was 21.8 mm (range, 11-30 mm). The number of biopsy specimens was 6.3 (range, 5-8). The mean procedure time was 9.0 min (range 4-17 min).

The MIF biopsy provided specimens that were sufficient for a definitive histological diagnosis in 90.9% (10/11) of cases. The histological diagnoses were leiomyoma (36.4%, 4/11), aberrant pancreas (27.3%, 3/11), GIST (18.2%, 2/11), and inflammatory fibrinoid tumor (9.1%, 1/11), and one result was non-diagnostic (9.1%, 1/11; Table 1). There were six mesenchymal tumors (4 leiomyomas, 2 GISTs), and the specimens obtained were large enough for immunohistochemical diagnoses. Both cases (case No. 3, 5) with GISTs had a spindle cell-type tumor with intermediate mitotic activity (mitotic index 5-10/50 HPFs). These patients had undergone surgical resection (wedge resection), and the results of the biopsy and the surgical resection were consistent.

The patient with a non-diagnostic result (case No. 6) refused a re-biopsy and did not want further evaluation or surgical resection. Thus, this patient was followed an-

Table 1 Endoscopic and clinicopathological characteristics of the patients and subepithelial tumors lesions

Case	Gender	Age (yr)	Location	EUS			MIF biopsy[1]			
				Layer	Echogenicity	Size (mm)	Procedure time (min)	Biopsy number (pieces)	Additional procedure	Pathology
1	Male	71	Angle	Fourth	Hypoechoic	21	12	7	Clip	IFT
2	Female	46	LB	Third	Mixed	15	10	6	APC	Aberrant pancreas
3	Female	69	Fundus	Fourth	Hypoechoic	20	5	5	Clip	GISTs
4	Male	76	Cardia	Fourth	Hypoechoic	21	10	6	Clip	Leiomyoma
5	Female	65	HB	Fourth	Mixed	27	7	6	No	GISTs
6	Female	47	LB	Third	Hypoechoic	30	11	8	No	CAG
7	Female	45	Angle	Third	Mixed	28	4	7	No	Aberrant pancreas
8	Male	71	Angle	Fourth	Mixed	26	9	6	Clip	Aberrant pancreas
9	Female	46	Cardia	Fourth	Hypoechoic	11	7	7	No	Leiomyoma
10	Male	62	HB	Fourth	Mixed	22	17	6	Clip	Leiomyoma
11	Male	60	Cardia	Fourth	Hypoechoic	19	7	5	Clip	Leiomyoma

[1]MIF biopsy is defined as a modified technique for the histological diagnosis of SETs: consisting of a mucosal incision with a fixed flexible snare (MIF) and deep-tissue biopsy at the incision site under a conventional endoscopic view. EUS: Endoscopic ultrasonography; SETs: Subepithelial tumors; IFT: Inflammatory fibrinoid tumor; LB: Low body: APC: Argon plasma coagulation: GISTs: Gastrointestinal stromal tumors; HB: High body: CAG: Chronic atrophic gastritis.

nually, and a final histological diagnosis was not reached. One perforation (case No. 10) was observed, and it was successfully controlled by endoscopic clipping. No major bleeding was recorded, but 63.6% (7/11) of patients showed minor bleeding.

DISCUSSION

We present a modified biopsy technique for the histological diagnosis of SETs. The diagnostic accuracy of MIF biopsies was 90.9% in our study. Adequate samples for diagnosis were obtained from 10 of 11 patients. The success rate was higher than other previously reported conventional methods.

Despite an endoscopist's intention to obtain tissue from submucosal lesions, conventional methods such as large-capacity "jumbo" forceps biopsies acquire submucosa for diagnosis with an approximately 17% yield[17]. Recent studies have investigated EUS-based methods, which have several limitations, despite a higher success rate than previously reported methods. EUS-FNA can obtain only a limited number of cells and cannot determine the structure of the organization, although the method typically has a 60%-80% success rate[10,14,18,19]. EUS-TCB generally has a similar yield to EUS-FNA, with no additional benefit[10]. Additionally, with EUS-TCB, it is not easy to obtain sufficient tissue with intact tissue architectural details for determining the mitotic index. However, it can provide a higher success rate than EUS-FNA in some patients requiring immunostaining. Combined EUS-FNA and EUS-TCB has been reported to have a diagnostic yield as high as 77%, although with a longer procedure time and higher cost[14].

The MIF biopsy is a simple technique, first making an incision in the mucosa covering the SETs, followed by acquiring SET tissues at the incision site with conventional biopsy forceps. Another advantage of this method is that it is not difficult regardless of the location of the lesion. In contrast, EUS-FNA and EUS-TCB are limited by technical problems in approaching the antrum and at angles because of the stiffness of the device and the rubbery consistency of the subepithelial mass[9,20].

Recently, there have been efforts to resect gastric SETs using ESD techniques, which provided successful resection of SETs in 74.3%-81.1% of cases, with a mean procedure time of 60.9 (range, 20-170) min[21-23]. There has been no report of life-threatening complications, although the incidence of complications was relatively high, at 12%-17%. In our study, the mean procedure time of the MIF biopsy was short (9 min), and the success rate was high (90.9%). Large GISTs with high potential for malignancy should be removed using surgical or endoscopic approaches. However, resection of all small SETs may be an unnecessarily invasive and money-wasting treatment, considering the risk of complications and cost effectiveness. Thus, a pre-resection histological evaluation is essential for SETs, and the MIF biopsy may provide a useful alternative technique in this regard.

One reported method for the adequate tissue acquisition of SETs is to remove the mucosa covering the SETs using an endoscopic knife for an endoscopic submucosal dissection (ESD) and to then to perform a partial resection of the SET[24]. This method provides a 93.7% diagnostic yield, but the procedure is more complex and difficult than the MIF biopsy we describe. Another ESD technique is mucosal incision-assisted biopsy (MIAB), which allows a mucosal incision at the circumferential margin of the lesion using an ESD-associated technique, followed by submucosal dissection to expose the SETs and then biopsy. This method differs from MIF biopsy, which involves an incision in the mucosa covering the top of the convex zone. MIAB appears to be much more complex and difficult than the MIF biopsy[25].

Another method, similar to the MIF biopsy, was re-

ported recently and involves performing a mucosal incision using a needle-knife sphincterotome (Microknife XL; Boston Scientific Inc., Natick, MA, United States), followed by sampling of the tissues inside and then prophylactic clipping (a SINK biopsy)[26]. The difference between the two methods is that we used a fixed flexible snare and did not routinely perform prophylactic endoscopic clipping. We performed APC or clipping for minor bleeding in 63.6% (6/11) of cases, which did not require additional endoscopy or re-admission for post-procedural bleeding. The mucosal incision with multiple deep biopsies appeared to be relatively safe in terms of bleeding, even without prophylactic APC or clipping.

The MIF biopsy needs to be performed carefully, depending on the shape of the SETs. Mucosal incision with deep biopsies should not be technically difficult if the SET is an exophytic "ball shape" growing toward the gastric lumen. However, a slightly elevated lesion, not a ball-shaped protruding lesion, necessitates a careful procedure. One (case No. 6) of our patients could not be diagnosed after the MIF biopsy, and another patient (case No. 10) experienced perforation; the SETs were slightly elevated, *i.e.*, mounded, in both cases. We suggest that these lesions were difficult to target because they were movable, and it was therefore difficult to identify the correct location when making the mucosal incision. Thus, SETs with such shapes require special attention.

Our data suggest that the MIF biopsy is a safe and effective method for the tissue diagnosis of small SETs. However, we recognize the limitations of this study. This was a retrospective study at a single tertiary academic center, and the sample size was small.

In conclusion, MIF biopsy was simple to perform, safe, required a shorter procedure time, and provided a high diagnostic yield for small SETs. Further comparative, prospective studies with larger sample sizes are required.

COMMENTS

Background

Gastric subepithelial tumors (SETs) are difficult to definitively diagnose by conventional imaging studies. Therefore, tissue acquisition from SETs is essential for a differential diagnosis. Conventional endoscopic biopsies do not typically provide sufficient submucosal tissue specimens for diagnosis. Several techniques have been introduced to obtain SET tissue samples. However, their diagnostic efficacies were limited, or the procedures were complex and difficult. Authors investigated a modified technique for the histological diagnosis of SETs, consisting of a mucosal incision with a fixed flexible snare (MIF) and deep-tissue biopsy at the incision site under a conventional endoscopic view.

Research frontiers

Presently, there is no consensus regarding the management strategy and surveillance of asymptomatic and small SETs. For a definitive diagnosis of SETs, tissue acquisition from a subepithelial lesion is essential for a differential diagnosis and an assessment of the malignant potential. Authors present a modified biopsy technique for the histological diagnosis of small SETs. The diagnostic accuracy of MIF biopsies was 90.9% in our study. The success rate was higher than other previously reported conventional methods.

Innovations and breakthroughs

The results of this study suggest that the MIF biopsy is simple to perform, safe, requires a shorter procedure time, and provides a high diagnostic yield for small SETs. Another advantage of this method is that it is not difficult regardless of the location of the lesion.

Applications

Their data suggest that the MIF biopsy is a safe and effective method for the tissue diagnosis of small SETs. A pre-resection histological evaluation is essential for SETs, and the MIF biopsy may provide a useful alternative technique.

Terminology

The modified technique that uses the MIF biopsy consists of two steps. Under direct conventional endoscopic view, the incision of the mucosa covering the SETs is made over the convex zone of the lesion using an endoscopic knife. After the mucosal incision is created using the fixed flexible snare, a conventional forceps biopsy is performed, deep in the incision site of the covering mucosa.

Peer review

This paper reports the usefulness of a mucosal incision with a fixed flexible snare and a deep-tissue biopsy for the histological diagnosis of gastric subepithelial tumors. A modified biopsy method is feasible for the tissue diagnosis of gastric subepithelial tumors. The article is well written, and the findings are of practical importance.

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BRIEF ARTICLE

A novel technique for endoscopic ultrasound-guided biliary drainage

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Abstract

AIM: To describe a successful endoscopic ultrasound (EUS)-guided biliary drainage technique with high success and low complication rates.

METHODS: The recorded data of consecutive patients who presented to Siriraj Gastrointestinal Endoscopy Center, Siriraj Hospital in Bangkok, Thailand for treatment of malignant obstructive jaundice but failed endoscopic retrograde cholangiopancreatography and underwent subsequent EUS-guided biliary drainage were retrospectively reviewed. The patients' baseline characteristics, clinical manifestations, procedure details, and post-procedure follow-up data were recorded and analyzed. Clinical outcomes were assessed by physical exam and standard laboratory tests. Technical success of the procedure was defined as completion of the stent insertion. Clinical success was defined as improvement of the patient's overall clinical manifestations, in terms of general well-being evidenced by physical examination, restoration of normal appetite, and adequate biliary drainage. Overall median survival time was calculated as the time from the procedure until the time of death, and survival analysis was performed by the Kaplan-Meier method. The Student's *t*-test and the χ^2 test were used to assess the significance of inter-group differences.

RESULTS: A total of 21 cases were enrolled, a single endoscopist performed all the procedures. The mean age was 62.8 years (range: 46-84 years). The sex distribution was almost equal, including 11 women and 10 men. Patients with failed papillary cannulation (33.3%), duodenal obstruction (42.9%), failed selective cannulation (19.0%), and surgical altered anatomy (4.8%) were considered candidates for EUS-guided biliary drainage. Six patients underwent EUS-guided choledochoduodenostomy and 15 underwent EUS-guided hepaticogastrostomy. The technique using non-cauterization and no balloon dilation was performed for all cases, employing the in-house manufactured tapered tip Teflon catheter to achieve the dilation. The technical success and clinical success rates of this technique were 95.2% and 90.5%, respectively. Complications included bile leakage and pneumoperitoneum, occurred at a rate of 9.5%. None of the patients died from the procedure. One patient presented with a biloma, a major complication that was successfully treated by another endoscopic procedure.

CONCLUSION: We present a highly effective EUS-guided biliary drainage technique that does not require cauterization or balloon dilation.

Key words: Endoscopic ultrasound; Biliary drainage; Hepaticogastrostomy; Choledochoduodenostomy; Endoscopic ultrasound-guided

Core tip: A total of 21 patients who underwent endoscopic ultrasound (EUS)-guided biliary drainage following failure of endoscopic retrograde cholangiopancreatography were analyzed. The EUS-guided biliary drain-

age technique, which does not require cauterization or balloon dilation, was found to be effective and safe. The rates of technical and clinical success were 95.2% and 90.5%, respectively. Complications occurred at a relatively low rate (9.5%) and included bile leakage and pneumoperitoneum. No procedure related deaths occurred during the procedure, hospital recovery, or follow-up period. However, one patient developed the major complication of iatrogenic biloma due to stent mal-position, which was successfully resolved by another endoscopic procedure.

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INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) is a standard treatment for benign and malignant biliary obstruction. ERCP has a failure rate of about 3%-10% due to a combination of the following factors: improper cannulation due to periampullary diverticulum or anatomical variation, biliary obstruction from impacted large common bile duct stones, tight malignant obstruction, gastrointestinal anatomical changes, surgically altered anatomy or gastroduodenal obstruction[1]. Common management techniques following a failed ERCP include an additional ERCP performed by a more experienced endoscopist or the implementation of alternative methods for biliary decompression such as surgical bypass or percutaneous transhepatic biliary drainage (PTBD). PTBD is superior to surgical bypass techniques in terms of morbidity and complications[1-5]. However, PTBD still resulted in complications (9%-30%) and a 2%-15% mortality rate was reported. Furthermore, patients reported symptoms including discomfort, unusual physiology and some limitations of the procedure, especially in patients with large amount of ascites[2,3].

Endoscopic ultrasound (EUS), a medical procedure in which endoscopy is combined with ultrasound to provide imaging of internal organs, has expanded its therapeutic potential since the invention of the linear-array echoendoscope. EUS guided biliary access (EUS guided cholangiography) was first reported by Wiersema *et al*[6], followed by EUS-guided choledochoduodenostomy (EUS-CD) by Giovannini *et al*[7], and lastly EUS guided hepaticogastrostomy (EUS-HG) was introduced by Burmester *et al*[8]. There have been many reports regarding the efficacy, safety and feasibility of EUS guided biliary drainage (EUS-BD)[9-24]. The two most common EUS-BD procedures are EUS-CD and EUS-HG[2-10]. Both procedures are initiated by identifying and targeting the biliary tract, followed by puncturing the bile duct depended on the location of obstruction and the reasons for performing drainage, manipulating the guidewire, dilating the created tract, and placing the stent. The success rate of EUS-BD was is 72%-98% while the complication rate is 15%-35%, with complications such as peritonitis and bile leakage being fatal[9-16]. The details of different endoscopic techniques, instruments and accessories used in the procedures are described elsewhere[17-19]. Reasonably, the most important issue for high success and low complication rate of EUS-BD is an appropriate technique for biliary access. The techniques for biliary access were classified into two groups, cauterized or non-cauterized, while the dilation maneuver was also classified as graded dilation or balloon dilation. At present, there are no data directly comparing these two methods. Therefore, the aim of this study was to describe the techniques for EUS-BD used in our institute, which provided good clinical outcomes.

MATERIALS AND METHODS

We retrospectively reviewed data from patients with advanced malignant bile duct obstruction who failed ERCP and underwent EUS-guided biliary drainage in our institute from October 2010 to July 2012. The medical records and electronic based medical records were reviewed. Prachayakul V was the endoscopist who performed all the procedures. We analyzed the following clinical outcomes during the follow-up period: patient demographic data, baseline clinical characteristics, indications for EUS-BD, procedure details, and clinical and laboratory results. We defined clinical outcomes using several different criteria. Technical success was defined as the ability to complete the procedure until stent insertion. Clinical success was defined as the improvement of the overall clinical manifestations of the patients in terms of clinical well-being, loss of appetite, and adequate biliary drainage. Adequate biliary drainage was defined as the reduction of total bilirubin more than 50% of pre-treatment bilirubin. Median survival time was defined as the time from the procedure until the time of death. While most patients experiencing complications were followed-up until they passed away, some were contacted telephonically in order to obtain any missing or additional information related to specific complications.

All participating patients gave written informed consent. The procedures were performed in the endoscopic suite at the Siriraj Hospital's Endoscopy Center with propofol administered total intravenous anesthesia. Patients were positioned in left lateral decubitus, with the punctured site chosen depending on the site of biliary obstruction. The site was chosen after EUS survey, EUS-CD for distal biliary obstruction and EUS-HG for hilar obstruction. Curvilinear echoscope (GF UC-140P, Olympus, Tokyo, Japan) were used in all cases. Either the common bile duct or the intrahepatic bile duct was punctured by using 19-gauge needle (EchoTip® Ultra, Cook Ireland, Limerick, Ireland) and bile was aspirated to confirm the correct position. Contrast medium was injected to get more cholangiography details. The 0.035 jag wire was inserted through a channel in the intrahepatic bile duct and then the tapered tip Teflon catheters which were made by

the author (Aswakul P) (Figure 1) were used for graded dilatation starting from 7 or 8.5 up to 10 Fr in diameter. Next, the fully covered self-expandable metal stent (FCSEMS) with varying sizes (60, 80 or 100 mm) were inserted depending on the location of EUS-BD. We generally used FCSEMS size 60 mm in diameter for EUS-CD and 80-100 mm for EUS-HG. All the procedures were done under fluoroscopic monitoring. The positions of the stents were checked and the bleeding was secured. Patients were observed in the recovery room for 1-2 h and transferred to the regular ward care. Most achieved uneventful discharge within 3-5 d after the procedure. Due to awareness of the positions of the stents and stent shortening, abdominal X-rays were done in all patients within 48-72 h. The patients' physical examination, laboratory tests, and clinical status were recorded. The procedure sequence is shown in Figure 2.

Statistical analysis

Data were analyzed using SPSS version 13.0 software (SPSS, Inc., Chicago, IL, United States). Overall median survival time was evaluated from the time of procedure until the time of death. The data was analyzed using survival analysis with the Kaplan-Meier method. The descriptive data were reported as mean ± SD and percentage. The Student's *t*-test and the χ^2 test were used to assess the inter-group differences according to the clinical data. A *P*-value of < 0.05 was considered statistically significant.

RESULTS

A total of 21 patients were enrolled in the present study. The mean age was 62.8 years (range 46-84 years), and ten were men (47.6%). The most common diagnoses were advanced pancreatic cancer (45.5%), cholangiocarcinoma (18.2%), gallbladder cancer (18.2%) and others (18.1%). The most common clinical manifestation was obstructive jaundice, which occurred in 86.4% of patients. The mean pre-treatment total bilirubin, alkaline phosphatase and carbohydrate antigen 19-9 level were 18.1 ± 7.9 mg/dL, 716.5 ± 395.6 IU/mL and 242.0 ± 325.6 IU/mL respectively. The indications for EUS-BD were failed papillary cannulation (33.3%), duodenal obstruction (42.9%), failed selective cannulation (19.0%) and surgical altered anatomy (4.8%). Six patients underwent EUS-CD while another 15 patients underwent EUS-HG. One patient underwent the procedure twice due to a first time technical failure where during guide wire manipulation the wire slipped out and bile leakage occurred. This event led to reduction of intrahepatic duct size and it was too small for puncturing. The patient underwent another procedure two weeks later and achieved successful biliary drainage. Therefore, only 21 patients would be analyzed for clinical outcomes following treatment.

There were immediate complications reported in two cases (9.5%); a case of pneumoperitoneum related with neotract creation and dilation technique that recovered after conservative treatment and another case of bile leakage. Here, a 53-year-old female who had a known case of advanced cholangiocarcinoma and underwent EUS-guided hepaticogastrostomy, showed higher level of bilirubin within 2 wk after stent insertion due to malposition of the stent. The stent was slipped out of the gastric wall causing acute angulation at hepatogastric site that occurred during stent deployment and led to biloma formation. The patient was managed conservatively without surgery because there was no evidence of clinical peritonitis. A computed tomography scan was done 10 d later and revealed biloma at the intraperitoneum site of the stent. The patient underwent EUS-guided biloma drainage and passed away one month later due to disease progression.

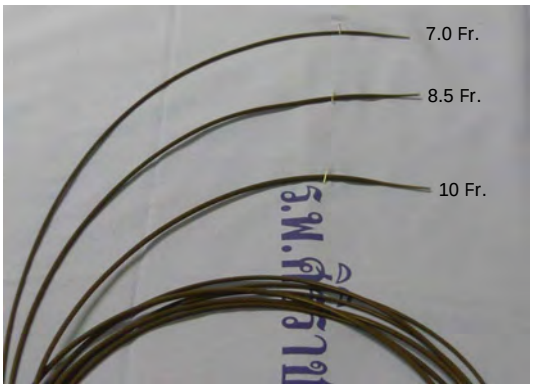

Figure 1 Self-made tapered tip Teflon catheters.

Technical success occurred in 95.5% of patients while clinical success was achieved in 90.5%. Adequate biliary drainage was achieved in 85.7% of the patients within two weeks except only one case where drainage did not occur until the fourth week after the procedure. There were two patients who did not benefit from the drainage procedures; the first case showed no improvement of bilirubin level after the stent insertion due to liver failure from massive liver metastasis and a case of bile leakage where the patient eventually died due to disease progression. The median survival of the patients in this series was 93 d. There were three patients still alive at the time that we finished the study.

DISCUSSION

The result of EUS-BD in the present study was similar to previous case reports in terms of technical and clinical success, at about 75.0%-96.5%[1-13]. However the complications found in this series were lower than those reported in other studies[16-29]. More than 90% of the patients were clinically improved after the procedures even though the median survival time was only 93 d. Most patients passed away because of advancement of the primary malignancy. Regarding of the complications occurring with EUS-BD technique that we used in this study, only one case (4.8%) related with neotract creation and dilation technique. We did not use cauterization because we hypothesized that it might cause more tissue injury and is technically dangerous, especially when the tip of the knife was not in the appropriate position. Difficulty

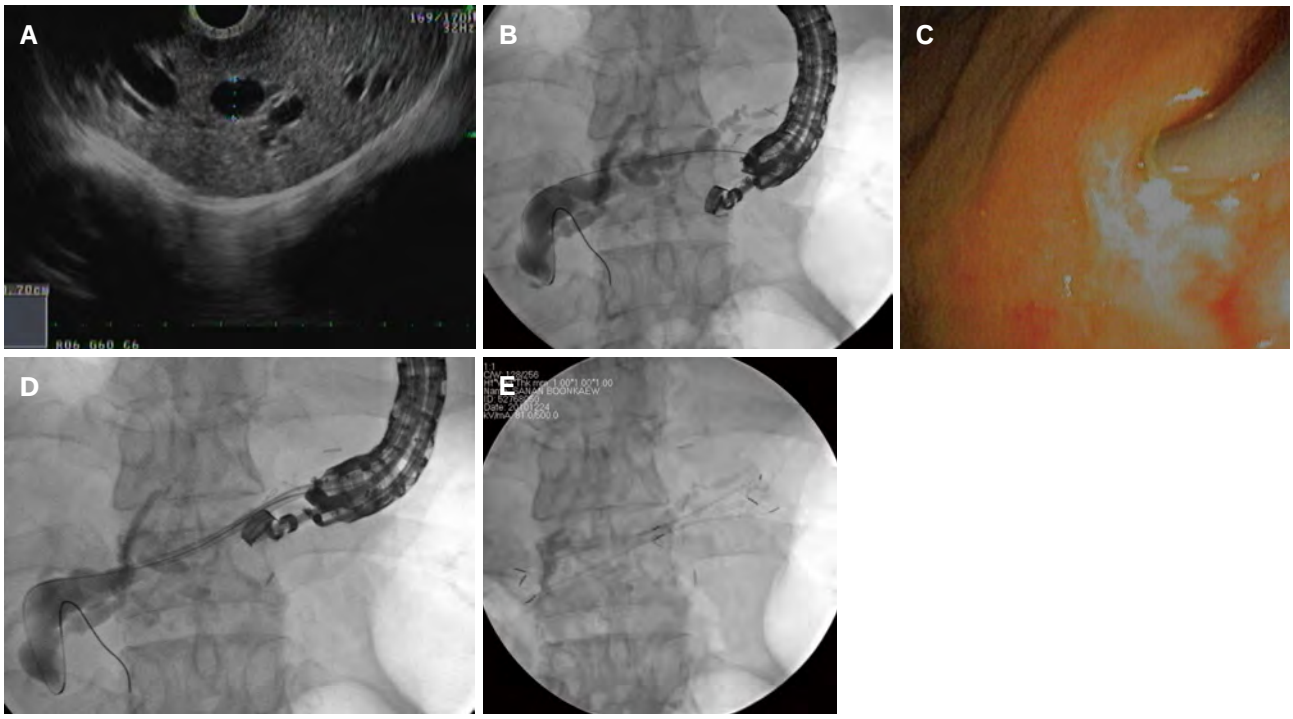

Figure 2 The procedure sequence. A: Endosonographic view of dilated intra hepatic duct; B: Endoscopic ultrasound guided cholangiography; C: Endoscopic view showing dilatation using tapered tip Teflon catheter; D: Fluoroscopic view showing dilatation tapered tip Teflon catheter; E: Post self-expandable metal stent insertion.

inserting the catheter was overcome by using a self-made Teflon catheter with a small the tapered tip diameter (as small as the 0.035 guidewire). We also chose to not use the balloon dilatation technique to create the neo-tract that was slightly larger than the size of the introducer of the SEMS. This worked to minimize the risk of bile leakage and symptomatic gastric perforation. Further, we found this technique was feasible and easy to perform because of the specific characteristic of dilator tip. While a potential challenge to this technique is the need for more frequent guidewire exchange, the presence of a well-trained assistant allowed us to complete the procedure without encountering any problems. While we did encounter some bile leakage, it was due to a mistake during SEMS deployment, not during the neotract creation and dilation. Fortunately, because of smaller dilated tract, the SEMS did not immediately expand, so the bile leakage in this particular case was minimal and gradually occurred leading only to a localized formation of biloma without peritonitis. Another complication of EUS-BD was early stent migration. This led to peritonitis, but was corrected by surgery[30]. We did not experience this complication because the SEMS did not immediately over-expanded due to the smaller diameter of the neo-tract. This made the SEMS fit the tract appropriately under good radial SEMS force, and the tract was slowly formed and fully expanded by 72 h[31]. This technique also minimized the risk of early stent migration. We had four cases of late stent migration (partial migration) when the tract was already well formed four to eight weeks after the procedure and thus no morbidity was found. Consistent with findings of Park *et al*[1], the only one risk factor associated with postprocedure adverse events in performing EUS-BD was the use of a needle knife for fistula tract creation. This study did have some limitations. First, although the tapered tip Teflon catheter used in this case series were very unique, the small sample size may not be reproducible. Second, this was the retrospective review of the data. Therefore, multi-center, larger population, prospective studies should be conducted in the future.

We have reported a technique for EUS-guided biliary drainage that does not use cauterization or balloon dilation. The technique was highly effective with a low complication rate, and has not been previously reported.

COMMENTS

Background

Endoscopic ultrasound-guided biliary drainage (EUS-BD) is a novel alternative treatment for the approximately 3%-10% of patients who fail standard endoscopic retrograde cholangio-pancreatography (ERCP). Techniques for biliary access and drainage were reported in recent years, however there was no data indicating which one is the best technique for minimizing complications and increasing the success.

Research frontiers

This was a retrospective study of 21 patients with advanced malignant bile duct obstruction who failed ERCP and underwent EUS-guided biliary drainage. The techniques used in this case series created the hepaticogastrostomy and choledochoduodenostomy tract in the absence of cauterization and balloon dilatation. Tapered tip Teflon catheters were used for dilation. The procedure had very high technical (95.2%) and clinical (90.5%) success, while maintaining lower complication rates (9.5%) when compared to other studies (15%-35%).

Innovations and breakthroughs

The goal of this study was to demonstrate another option for EUS-guided biliary drainage that used novel instruments and techniques resulting in an enhanced suc-

cess rate and a decreased complication rate.

Applications

This novel EUS-guided biliary drainage technique could be widely used as a safer and more effective alternative to cauterization or balloon dilation techniques that are widely used currently.

Terminology

EUS-BD is a novel alternative treatment for patients who suffered from biliary obstruction and failed endoscopic retrograde cholangiopancreatography. The most common procedures are EUS guided hepaticogastrostomy and EUS-guided choledochoduodenostomy. Regarded as a less invasive procedure when comparing to surgical bypass with a complication rate of 15%-35%, it becomes a preferable treatment option in some particular patients.

Peer review

This was an interesting case series that demonstrated alternative treatment options and techniques for endoscopic ultrasound guided-biliary drainage in patients with advanced malignant bile duct obstruction who failed conventional biliary drainage techniques.

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BRIEF ARTICLE

Quality of life after laparoscopic *vs* open sphincter-preserving resection for rectal cancer

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Abstract

AIM: To compare quality of life (QoL) outcomes in Chinese patients after curative laparoscopic *vs* open surgery for rectal cancer.

METHODS: Eligible Chinese patients with rectal cancer undergoing curative laparoscopic or open sphincter-preserving resection between July 2006 and July 2008 were enrolled in this prospective study. The QoL outcomes were assessed longitudinally using the validated Chinese versions of the European Organization for Research and Treatment of Cancer QLQ-C30 and QLQ-CR38 questionnaires before surgery and at 4, 8, and 12 mo after surgery. The QoL scores at the different time points were compared between the laparoscopic and open groups. A higher score on a functional scale indicated better functioning, whereas a higher score on a symptom scale indicated a higher degree of symptoms.

RESULTS: Seventy-four patients (49 laparoscopic and 25 open) were enrolled. The two groups of patients were comparable in terms of sociodemographic data, types of surgery, tumor staging, and baseline mean QoL scores. There was no significant decrease from baseline in global QoL for the laparoscopic group at different time points, whereas the global QoL was worse compared to baseline beginning at 4 mo but returned to baseline by 12 mo for the open group (P = 0.019, Friedman test). Compared to the open group, the laparoscopic group had significantly better physical (89.9 ± 1.4 *vs* 79.2 ± 3.7, P = 0.016), role (85.0 ± 3.4 *vs* 63.3 ± 6.9, P = 0.005), and cognitive (73.5 ± 3.4 *vs* 50.7 ± 6.2, P = 0.002) functioning at 8 mo, fewer micturition problems at 4-8 mo (4 mo: 32.3 ± 4.7 *vs* 54.7 ± 7.1, P = 0.011; 8 mo: 22.8 ± 4.0 *vs* 40.7 ± 6.9, P = 0.020), and fewer male sexual problems from 8 mo onward (20.0 ± 8.5 *vs* 76.7 ± 14.5, P = 0.013). At 12 mo after surgery, no significant differences were observed in any functional or symptom scale between the two groups, with the exception of male sexual problems, which remained worse in the open group (29.2 ± 11.3 *vs* 80.0 ± 9.7, P = 0.026).

CONCLUSION: Laparoscopic sphincter-preserving resection for rectal cancer is associated with better preservation of QoL and fewer male sexual problems when compared with open surgery in Chinese patients. These findings, however, should be interpreted with caution because of the small sample size of the study.

Key words: Quality of life; Rectal cancer; Laparoscopic surgery; Sphincter-preserving surgery; European Organization for Research and Treatment of Cancer QLQ-C30; European Organization for Research and Treatment of Cancer QLQ-CR38

Core tip: This prospective nonrandomized study dem-

onstrates that laparoscopic sphincter-preserving resection for rectal cancer is associated with better preservation of quality of life (QoL) and fewer male sexual problems when compared with open surgery in Chinese patients in the first postoperative year. Our study has several strengths. First, our study only focused on Chinese patients undergoing curative sphincter-preserving rectal resection, thus minimizing the impact of other potential confounders on the QoL assessment. Second, all our questionnaires were administered by a single research assistant and were completed by the patients during clinic visits. Therefore, we achieved 100% compliance at different time points.

Ng SSM, Leung WW, Wong CYN, Hon SSF, Mak TWC, Ngo DKY, Lee JFY. Quality of life after laparoscopic *vs* open sphincter-preserving resection for rectal cancer. *World J Gastroenterol* 2013; 19(29): 4764-4773 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i29/4764.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i29.4764

INTRODUCTION

Accumulating evidence from recent randomized trials indicates that laparoscopic surgery for rectal cancer is associated with clear short-term benefits and similar tumor clearance when compared with open surgery[1-5]. Researchers currently are particularly eager to know whether the long-term oncologic results are also comparable between the two approaches for rectal cancer[6]. Indeed, long-term survival has always been regarded as the most important study endpoint in these clinical trials[7]. However, functional outcomes and quality of life (QoL) must not be ignored in the quest for surgical and oncologic excellence.

Notably, up to 30% of rectal cancer survivors will develop urinary and sexual dysfunctions after surgery attributable to inadvertent injury of the pelvic autonomic nerves[8]. Bowel dysfunction and fecal incontinence are also not uncommon after sphincter-preserving rectal surgery and radiotherapy[9,10]. These functional hazards will have a significant negative impact on the patients' functioning and QoL for the remainder of his/her life[11].

Therefore, in addition to traditional study endpoints such as postoperative recovery, morbidity, and survival, functional results and QoL have recently become important outcome parameters for defining surgical performance in clinical trials[12]. Within the context of medical and healthcare research, QoL is the patient's subjective perception of the impact of his/her disease and its treatments on his/her physical, psychological, and social functioning and general well-being[13]. Health-related QoL after cancer surgery can be assessed by standardized instruments such as the questionnaires developed by the European Organization for Research and Treatment of Cancer (EORTC), which contain multidimensional generic and disease-specific domains; the EORTC QLQ-C30 and QLQ-CR38 are the most commonly used questionnaires in colorectal cancer trials[14,15].

The magnified vision and less traumatic surgery offered by the laparoscopic approach may allow better preservation of the pelvic autonomic nerves[4,5], and presumably, functional outcomes following laparoscopic surgery for rectal cancer may be better compared to open surgery. However, conflicting results have been reported in the literature[2,16]; some studies have even reported a higher incidence of sexual dysfunction after laparoscopic rectal surgery[17,18]. Furthermore, it is also unclear whether the short-term and long-term clinical benefits associated with the laparoscopic approach will translate into better QoL outcomes for patients with rectal cancer. To date, few studies have specifically compared QoL outcomes between laparoscopic and open surgery for rectal cancer[19]. We therefore conducted this prospective study to compare QoL outcomes in Chinese patients after curative laparoscopic *vs* open sphincter-preserving surgery for rectal cancer. Changes in QoL over time were also longitudinally assessed and compared between the two groups.

MATERIALS AND METHODS

Between July 2006 and July 2008, eligible Chinese patients with rectal cancer undergoing curative laparoscopic or open sphincter-preserving resection at our hospital were enrolled in this prospective study. The study was approved ethically by the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee (CRE-2005.259). All patients provided written informed consent. We excluded the following patients: patients who presented with recurrent disease, patients who required multivisceral en bloc resections, patients who required conversion from laparoscopic to open surgery, patients with intestinal obstruction or perforation, and patients with known dementia or cognitive dysfunction.

The operative approach (laparoscopic or open resection) was decided by the operating surgeon after considering the tumor characteristics and the patient's preference. All operations were performed by surgeons experienced in both laparoscopic and open colorectal surgery. Our laparoscopic techniques for resection of rectal cancer were previously described[20,21]. For mid and low rectal cancer located 5-12 cm from the anal verge, sphincter-preserving total mesorectal excision with protective loop ileostomy was performed. All patients in this study underwent ileostomy closure within 7 mo after the primary surgery. Adjuvant therapy was administered to patients with pathologic stage Ⅱ or Ⅲ disease. Clinical parameters including patient sociodemographic data, types of surgery, tumor staging, and short-term clinical outcomes were prospectively recorded.

After surgery, all patients were followed-up regularly at 4-mo intervals for clinical examination and carcinoembryonic antigen testing. All patients were free of recurrence during the study period.

Quality of life assessment

Patient QoL was assessed using the QLQ-C30 and QLQ-CR38 questionnaires developed by the EORTC[14,15]. The clinical validity and reliability of the Chinese versions of both QLQ-C30 and QLQ-CR38 have been confirmed[22-24]. QLQ-C30 is a generic questionnaire for the assessment of QoL in cancer patients[14]. It includes 30 items, 24 of which are combined to form a global QoL scale, five functional scales (physical, role, emotional, cognitive, and social), and three symptom scales (fatigue, nausea/vomiting, and pain). The other six single items evaluate dyspnea, insomnia, appetite loss, constipation, diarrhea, and financial difficulties. QLQ-CR38 is a specific questionnaire module specifically designed for assessment of QoL in patients with colorectal cancer[15]. It consists of 38 items covering symptoms and side effects related to different colorectal cancer treatment modalities. The module contains four functional scales (body image, sexual functioning, sexual enjoyment, and future perspective) and eight symptom scales/items (micturition problems, chemotherapy side effects, gastrointestinal tract symptoms, male sexual problems, female sexual problems, defecation problems, stoma-related problems, and weight loss).

The questionnaires were scored according to the EORTC Scoring Manual[25]. Each item has four response alternatives (scoring 1-4), "not at all", "a little", "quite a bit", and "very much", except for the global QoL scale, which has seven alternatives (scoring 1-7) from "very poor" to "excellent". All questionnaire responses and scores were linearly transformed to a 0-100 scale. A higher score on the global QoL and functional scales represented a higher level of QoL and functioning, whereas a higher score on the symptom scales/items represented a higher degree of symptoms or dysfunction.

All questionnaires were administered by a single research assistant and completed by the patients before surgery and at 4, 8 and 12 mo after surgery (during clinic visits). Every effort was made to avoid missing data during questionnaire administration.

Statistical analysis

QoL scores were presented as the mean ± SD. For longitudinal assessment of changes of QoL scores over time, the Friedman test was used to identify overall significant differences between QoL scores at the four different time points (before surgery and at 4, 8 and 12 mo after surgery) for each variable. When the overall P value indicated statistical significance (*i.e.*, $P < 0.05$), a *post-hoc* Wilcoxon signed-rank test for paired data using a simplified Bonferroni correction was used to compare pairs of QoL scores (with $P < 0.0083$ considered significant for six pair-wise comparisons). Cross-sectionally, to test for differences in QoL scores between the laparoscopic and open groups at different time points, the Mann-Whitney U test was used. The baseline characteristics of the two groups of patients were compared using the χ^2 test (or Fisher's exact test when appropriate), Student's t test, and the Mann-Whitney U test for categorical, parametric, and non-parametric data, respectively. A P value of less than 0.05 was considered statistically significant, whereas a difference in mean QoL scores of more than 10 points was regarded as clinically significant[26]. Using a 5% significance level, a total sample size of 75 (50 laparoscopic and 25 open) would have a power of 80% to detect a minimum difference of 10 points in mean QoL scores between the two groups.

RESULTS

Between July 2006 and July 2008, 74 patients were enrolled in this study: 49 patients underwent laparoscopic surgery, and 25 patients underwent open surgery. The two groups of patients were comparable in terms of sociodemographic data, types of surgery, tumor staging, and the proportion of patients who received adjuvant therapy (Table 1). The overall short-term morbidity rates of the laparoscopic and open groups were 34.7% and 52%, respectively ($P = 0.152$, χ^2 test). Transient urinary retention and septic complications (including chest infection, wound infection, and urinary tract infection) occurred more frequently in the open group. No patient in this study required reoperation for postoperative complications. With the exception of higher baseline symptom scores for insomnia in the open group, there was no significant difference in baseline mean QoL scores for any of the functional or symptom scales between the two groups (Table 2).

EORTC QLQ-C30

There was no significant decrease from baseline in global QoL scores for the laparoscopic group at the evaluated time points; the statistically significant difference detected with the Friedman test ($P = 0.044$) was due to an increase in global QoL scores from 4 to 8 mo ($P = 0.031$, post-hoc Wilcoxon signed-rank test) (Figure 1A). For the open group, the global QoL was worse than at baseline from 4 mo onward but gradually returned to baseline by 12 mo ($P = 0.019$, Friedman test; a significant decrease occurred between baseline and 4 mo, $P = 0.004$, post-hoc Wilcoxon sign-ranked test) (Figure 1A). Both the laparoscopic and open groups showed a significant decrease in physical functioning from 4 to 12 mo postoperatively ($P < 0.001$, Friedman test) (Figure 1B). Role functioning and social functioning were significantly worse than at baseline from 4 to 12 mo for the open group but remained the same as at baseline for the laparoscopic group (Figure 1C and D). There was no change from baseline in emotional functioning for either group. Cognitive functioning fluctuated over time for the laparoscopic group ($P = 0.035$, Friedman test) but remained the same as at baseline for the open group.

There was no significant change from baseline in fatigue scores for the laparoscopic group; more fatigue was reported in the open group beginning at 4 mo, but it returned to the baseline level by 12 mo ($P = 0.003$, Fried-

Table 1 Sociodemographic and clinicopathologic data of the patients *n* (%)

	Laparoscopic group	Open group	*P* value
Number of patients	49	25	/
Age (yr, mean ± SD)	65.6 ± 11.3	66.7 ± 12.4	0.705[1]
Sex (male/female)	30/19	15/10	0.919[2]
Body mass index (kg/m^2, mean ± SD)	22.4 ± 3.4	21.4 ± 3.5	0.210[1]
Number of patients with comorbidities	27 (55.1)	13 (52)	0.800[2]
Marital status (married/divorced/widow/single)	36/5/6/2	18/3/4/0	0.742[2]
Education level (under primary/primary/secondary/tertiary or above)	10/19/16/4	8/10/6/1	0.623[2]
Tumor location in rectum (upper/middle/lower)	26/15/8	12/10/3	0.697[2]
Types of surgery (AR/LAR with TME)	24/25	12/13	0.936[2]
Number of patients with temporary ileostomy	25 (51)	13 (52)	0.936[2]
Number of patients with complications	17 (34.7)	13 (52)	0.152[2]
Subclinical anastomotic leak	0	1	
Anastomotic bleeding	1	0	
Chest infection	1	3	
Wound infection	2	5	
Urinary tract infection	2	5	
Urinary retention	4	5	
Prolonged ileus	7	3	
Others	1	3	
Reoperation	0	0	
AJCC staging (Ⅰ/Ⅱ/Ⅲ)	3/26/20	2/13/10	0.955[2]
Adjuvant chemotherapy	20 (40.8)	10 (40)	0.946[2]
Adjuvant radiotherapy	11 (22.4)	7 (28)	0.599[2]

[1]Student's *t* test; [2]χ^2 test. AR: Anterior resection; LAR: Low anterior resection; TME: Total mesorectal excision; AJCC: American Joint Committee on Cancer.

man test; a significant increase occurred between baseline and 4 mo, $P = 0.004$, *post-hoc* Wilcoxon sign-ranked test) (Figure 2A). The 4-mo to 12-mo symptom scores remained similar to those at baseline for nausea/vomiting, pain, dyspnea, insomnia, appetite loss, constipation, and diarrhea for both groups. More financial difficulties were reported at 4 mo postoperatively, but this returned to baseline levels by 12 mo for both groups.

Compared to the open group, the laparoscopic group had significantly better global QoL at 4 and 8 mo, better physical, role, and cognitive functioning at 8 mo, less fatigue at 4 and 8 mo, and less nausea/vomiting, appetite loss, and financial difficulties at 8 mo (Table 2). However, at 12 mo after surgery, no significant differences were observed in any of the EORTC QLQ-C30 functional or symptom scales between the two groups.

EORTC QLQ-CR38

There was no significant change from baseline in body image for the open group; for the laparoscopic group, body image was significantly worse compared to baseline beginning at 4 mo but returned to baseline levels by 12 mo ($P = 0.002$, Friedman test). Sexual functioning remained the same as at baseline for both groups (Figure 1E), but the overall scores for sexual functioning were low (Table 2), indicating that the majority of patients were sexually inactive. There was a trend toward worsening of future perspective scores over time for the laparoscopic ($P = 0.074$, Friedman test) and open ($P = 0.094$, Friedman test) groups, but the change was statistically insignificant.

Improvement in micturition problems was noted in the laparoscopic group ($P = 0.031$, Friedman test; a decrease in symptom scores primarily occurred between baseline and 8 mo, $P = 0.019$, post-hoc Wilcoxon sign-ranked test), but there was no significant change from baseline for the open group (Figure 2B). More problems with chemotherapy side effects were reported at 4 mo postoperatively, but returned to baseline levels by 12 mo for patients receiving chemotherapy in both groups (Figure 2C). There was no significant change from baseline in gastrointestinal tract symptoms for either group (Figure 2D). Defecation problems significantly decreased from 4 to 8 mo for patients without stoma in the laparoscopic group ($P = 0.003$, Friedman test; $P = 0.003$, post-hoc Wilcoxon sign-ranked test), but they remained the same as at baseline for the open group. A significant improvement in weight loss over time was observed in both groups (Figure 2E). Compared to the open group, the laparoscopic group had significantly fewer micturition problems at 4 and 8 mo and fewer gastrointestinal symptoms at 8 mo (Table 2).

Sexual enjoyment and sexual problems were not evaluated in the female patients in this study because they were all sexually inactive. Altogether, 19 male patients (14 in the laparoscopic group and 5 in the open group) who had been sexually active before surgery were assessed for changes in QoL related to sexual activities (Table 3). Sexual enjoyment and male sexual problems remained relatively stable at different time points for the laparoscopic group, but less sexual enjoyment and more sexual problems were reported from 4 to 12 mo for the open group (Figures 1F and 2F). Compared to the laparoscopic group, the open group had significantly more sexual

Table 2 Comparison of European Organization for Research and Treatment of Cancer QLQ-C30 and QLQ-CR38 scores between the laparoscopic and open groups at different time points

	Baseline			4 mo			8 mo			12 mo		
	Lap	Open	*P* value	Lap	Open	*P* value	Lap	Open	*P* value	Lap	Open	*P* value
EORTC QLQ-C30												
Functional scales												
Global QoL	72.4 (3.4)	68.3 (5.2)	0.443	65.8 (3.6)	47.3 (5.9)	0.009	73.6 (3.8)	52.0 (5.9)	0.003	71.1 (3.4)	61.0 (7.0)	0.371
Physical	94.7 (1.3)	91.5 (2.6)	0.255	86.4 (2.3)	77.6 (4.1)	0.056	89.9 (1.4)	79.2 (3.7)	0.016	87.1 (2.6)	81.3 (4.0)	0.149
Role	88.8 (3.0)	92.7 (3.1)	0.660	75.9 (4.5)	67.3 (6.6)	0.155	85.0 (3.4)	63.3 (6.9)	0.005	82.7 (3.9)	71.3 (7.1)	0.129
Emotional	71.1 (4.1)	66.0 (5.4)	0.404	76.0 (4.0)	82.0 (4.9)	0.401	79.8 (3.4)	71.3 (6.3)	0.379	79.1 (3.2)	71.0 (6.3)	0.579
Cognitive	71.1 (4.2)	66.7 (5.5)	0.415	67.7 (3.6)	59.3 (5.8)	0.284	73.5 (3.4)	50.7 (6.2)	0.002	61.9 (4.2)	60.0 (6.7)	0.940
Social	82.7 (3.2)	88.0 (4.4)	0.292	73.5 (3.1)	64.7 (5.7)	0.268	76.9 (3.8)	62.7 (7.1)	0.110	76.5 (4.0)	62.7 (7.4)	0.124
Symptom scales/items												
Fatigue	16.6 (2.8)	14.2 (2.9)	0.859	17.7 (2.6)	27.6 (4.3)	0.042	13.8 (2.7)	28.9 (5.9)	0.027	12.0 (2.2)	15.6 (4.0)	0.520
Nausea/vomiting	0 (0)	0.7 (0.7)	0.162	3.1 (1.3)	6.7 (3.7)	0.931	0 (0)	2.7 (2.1)	0.046	0 (0)	1.3 (1.3)	0.162
Pain	14.3 (3.2)	16.7 (4.5)	0.772	20.1 (2.7)	25.3 (5.2)	0.534	16.7 (2.7)	27.3 (5.2)	0.089	15.6 (2.9)	19.3 (5.4)	0.755
Dyspnea	4.1 (1.6)	2.7 (1.8)	0.581	7.5 (2.2)	16 (5.5)	0.208	2.7 (1.3)	12 (6.0)	0.252	4.1 (2.1)	12.0 (5.0)	0.065
Insomnia	33.3 (5.1)	58.7 (8.5)	0.011	32.0 (5.1)	46.7 (8.2)	0.141	27.2 (4.8)	45.3 (7.9)	0.052	30.6 (5.1)	46.7 (8.4)	0.129
Appetite loss	8.8 (2.7)	6.7 (4.3)	0.313	7.5 (2.2)	14.7 (6.4)	0.794	6.8 (2.2)	17.3 (4.8)	0.035	7.5 (3.0)	10.7 (4.2)	0.330
Constipation	18.4 (4.6)	29.3 (8.0)	0.292	11.6 (4.2)	12.0 (4.7)	0.525	15.0 (4.4)	6.7 (2.7)	0.508	18.4 (4.5)	10.7 (5.3)	0.205
Diarrhea	19.7 (4.4)	17.3 (6.1)	0.759	23.1 (4.9)	30.7 (7.2)	0.394	15.0 (4.2)	29.3 (8.2)	0.156	15.0 (4.1)	21.3 (6.6)	0.379
Financial difficulties	13.6 (4.0)	14.7 (4.7)	0.426	24.5 (4.3)	37.3 (6.5)	0.066	23.1 (5.2)	42.7 (7.1)	0.010	13.6 (3.6)	20.0 (6.7)	0.580
EORTC QLQ-CR38												
Functional scales												
Body image	93.9 (1.9)	93.3 (2.6)	0.945	81.0 (4.2)	82.7 (6.0)	0.699	81.2 (3.9)	85.3 (4.8)	0.834	87.8 (3.7)	84.4 (5.6)	0.526
Sexual functioning	18.7 (3.9)	14.0 (5.3)	0.268	18.7 (4.1)	8.7 (5.1)	0.069	19.0 (4.2)	16.0 (5.7)	0.630	19.0 (4.3)	17.3 (5.7)	0.807
Future perspective	54.4 (4.9)	64.0 (7.2)	0.272	54.4 (4.4)	45.3 (6.0)	0.309	44.2 (5.1)	42.7 (7.8)	0.779	44.2 (4.9)	44.0 (6.9)	0.995
Symptom scales/items												
Micturition problems	37.8 (4.4)	38.7 (6.9)	0.907	32.3 (4.7)	54.7 (7.1)	0.011	22.8 (4.0)	40.7 (6.9)	0.020	31.6 (4.5)	41.3 (6.9)	0.246
Chemotherapy side effects[1]	16.7 (4.1)	10.0 (2.6)	0.530	41.1 (7.3)	44.4 (11.1)	0.846	20.6 (4.7)	28.9 (7.8)	0.422	8.9 (3.3)	17.8 (6.0)	0.214
Gastrointestinal tract symptoms	20.7 (2.2)	19.5 (2.8)	0.817	16.6 (1.9)	18.1 (2.8)	0.656	15.9 (1.9)	24.8 (3.3)	0.022	18.0 (2.2)	16.3 (3.3)	0.546
Defecation problems[2]	22.2 (2.3)	15.5 (3.5)	0.133	24.3 (3.6)	21.4 (3.0)	0.821	11.8 (2.5)	23.0 (4.9)	0.062	13.2 (2.9)	16.3 (3.3)	0.235
Weight loss	27.9 (4.7)	38.7 (8.3)	0.376	8.8 (3.2)	24.0 (7.6)	0.079	6.1 (2.1)	17.3 (6.1)	0.094	6.1 (2.1)	10.7 (4.2)	0.383

[1]Only for patients who received chemotherapy, 20 in the laparoscopic group and 10 in the open group; [2]Only for patients without temporary loop ileostomy, 24 in the laparoscopic group and 12 in the open group. Quality of life (QoL) scores are presented as the mean (standard error of mean). Scores ranged from 0 to 100. A higher score on a functional scale indicates better functioning, whereas a higher score on a symptom scale indicates a higher degree of symptoms. Scores in the laparoscopic and open groups were compared by the Mann-Whitney *U* test. EORTC: European Organization for Research and Treatment of Cancer.

problems at 8 mo ($P = 0.013$, Mann-Whitney *U* test) and 12 mo ($P = 0.026$, Mann-Whitney *U* test) postoperatively.

At 12 mo after surgery, no significant differences were observed in any of the EORTC QLQ-CR38 functional or symptom scales between the two groups, with the exception of male sexual enjoyment and sexual problems, which remained worse in the open group.

DISCUSSION

This prospective study was specifically designed to compare QoL outcomes in Chinese patients after curative laparoscopic *vs* open sphincter-preserving resection for rectal cancer. Our study has several strengths. First, although nonrandomized, the baseline characteristics and sociodemographic data of the two groups of patients were similar, and a fair comparison could therefore be made. The social backgrounds of patients, such as marital status and education level, which may impact QoL after surgery[27], have seldom been provided by other studies comparing QoL after laparoscopic and open rectal surgery[1-3,28,29]. Second, other studies have included metastatic cases and abdominoperineal resection in their QoL analysis[1,3,28], whereas our study only focused on Chinese patients undergoing curative sphincter-preserving rectal resection, thus minimizing the impact of other potential confounders on the QoL assessment. Third, all our questionnaires were administered by a single research assistant and were completed by the patients during clinic visits. Therefore, we achieved 100% compliance at different time points, a figure that was not achieved by other studies in which the questionnaires were collected by mail[28,29]. Full compliance with the questionnaires is essential to ensure a precise longitudinal assessment of QoL changes.

Our results showed that laparoscopic sphincter-preserving resection for rectal cancer was associated with better preservation of QoL and fewer male sexual problems when compared with the open approach in the first year after surgery. Other benefits of the laparoscopic approach, such as better physical functioning and fewer micturition and gastrointestinal problems, were evident only in the short term. These findings are in accordance with those reported by Braga *et al*[3], who found that QoL after laparoscopic surgery for rectal cancer was better

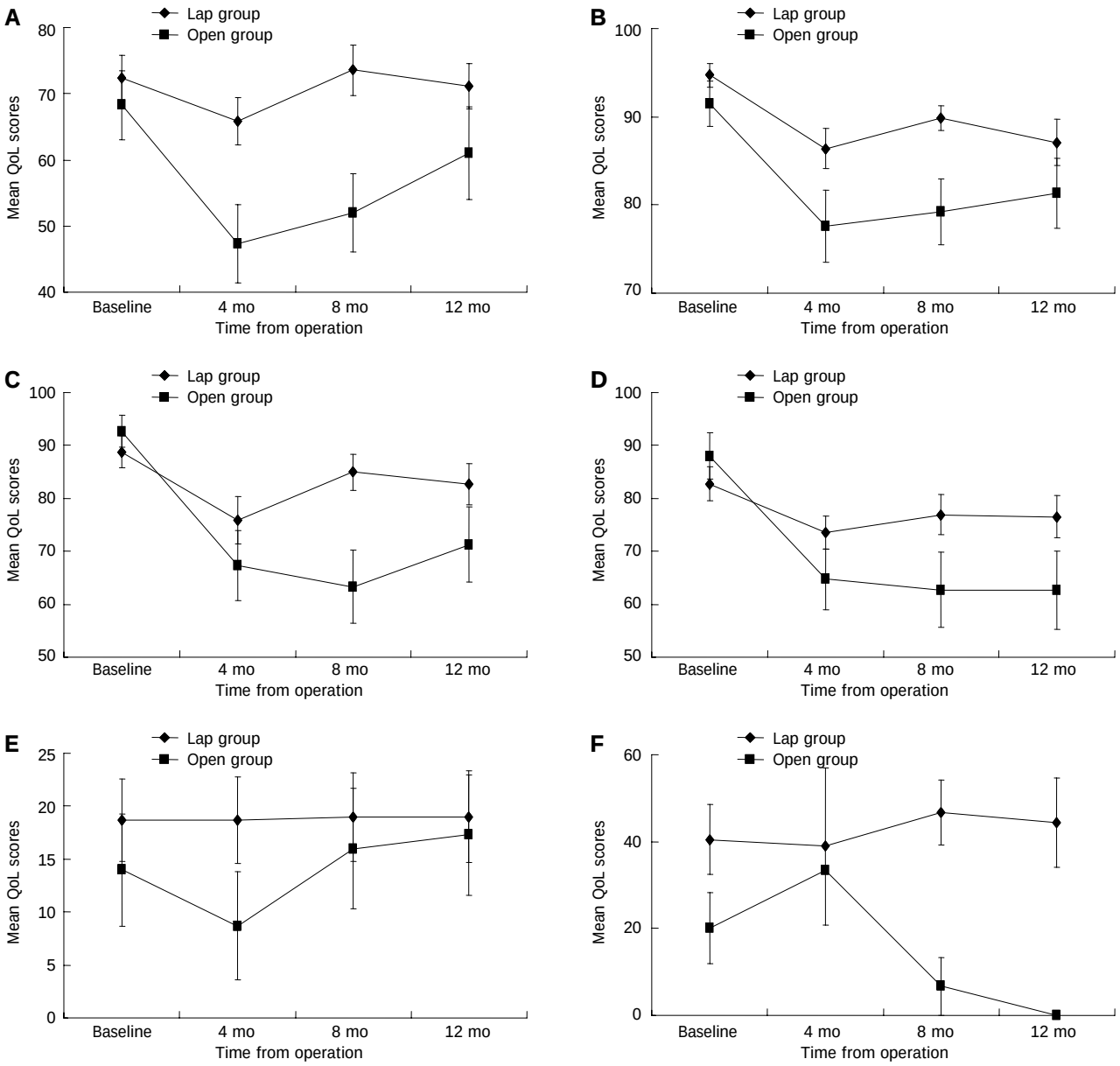

Figure 1 Longitudinal assessment of changes in quality of life scores over time for global quality of life and various functional scales. A: Global health status/quality of life (QoL); B: Physical functioning; C: Role functioning; D: Social functioning; E: Sexual functioning; F: Sexual enjoyment (only for men who have been sexually active). A higher score indicates better functioning. Error bars indicate the standard error of the mean. The Friedman test was not performed for sexual enjoyment, as the number of patients changed at different time points.

than the open approach only in the first year after surgery. Li *et al*[28] also reported transient QoL benefits in the early postoperative period after laparoscopic rectal surgery when compared with the open approach, but the overall QoL of the two groups was similar at a 1-year follow-up. Better QoL in the laparoscopic arm was also reported by the comparison of open *vs* laparoscopic surgery for mid and low rectal cancer after neoadjuvant chemoradiotherapy (COREAN) trial at 3 mo, but 1-year data were not provided[2].

On longitudinal assessment, the QoL scores of most of the functional and symptom scales of the laparoscopic group in our study remained relatively stable at different time points, whereas most of the QoL scores in the open group predominantly showed deterioration at 4-8 mo but gradually recovered by 1 year after surgery. This explains why significant differences in QoL scores between the laparoscopic and open groups in our study were primarily observed at 8 mo after surgery.

We have previously reported better short-term clinical outcomes and less long-term morbidity among patients undergoing laparoscopic surgery for rectal cancer when compared with the open approach[7,20,21]; this may partly account for the better short-term QoL associated with the laparoscopic arm in our study. However, in addition to the healthcare experience, patients' expectations also play an important role in the determination of QoL. According to the dynamic model proposed by Carr *et al*[30], QoL is typically impacted when the health experience falls short of expectations. The better preservation of

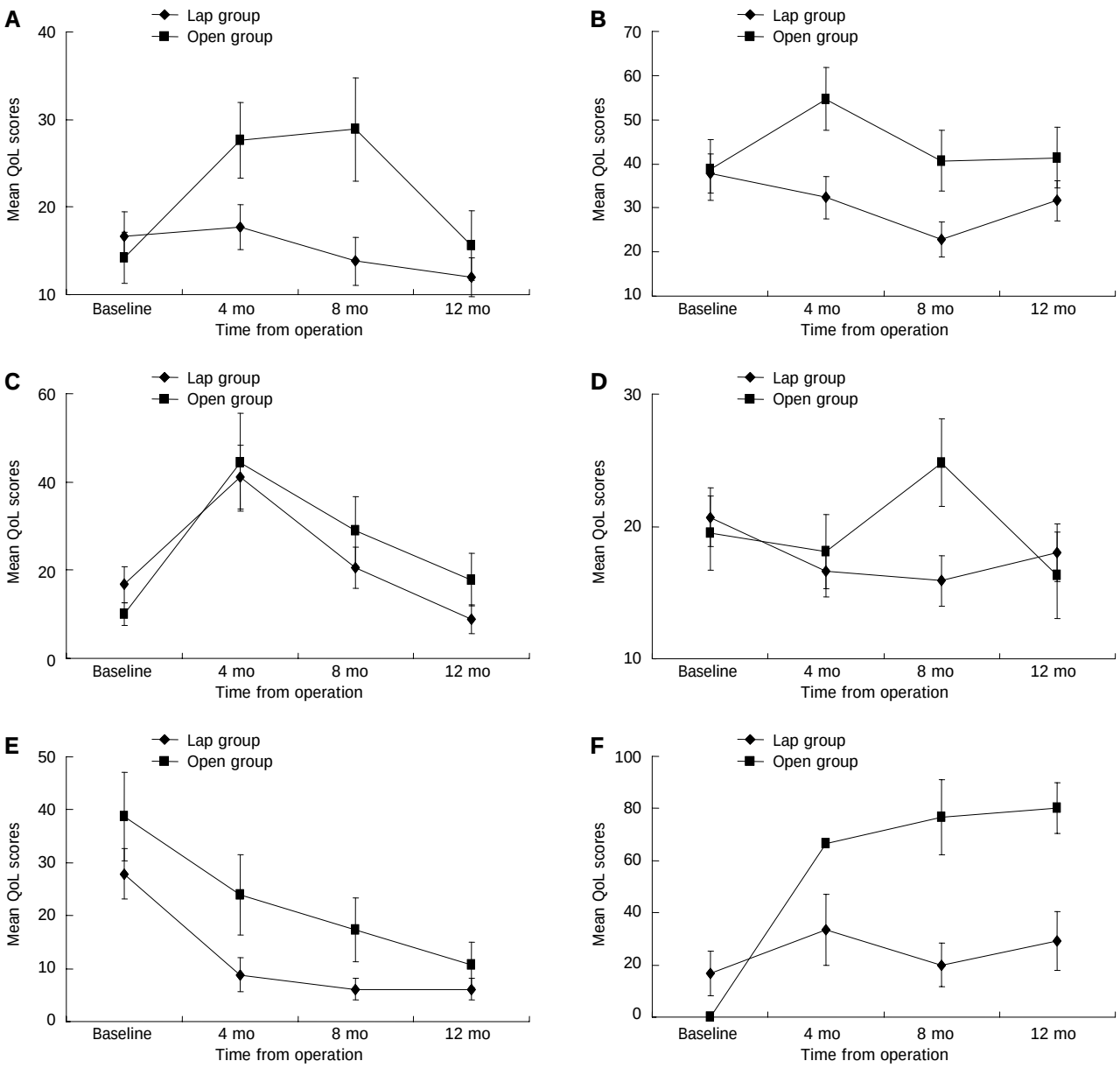

Figure 2 Longitudinal assessment of changes in quality of life scores over time for various symptom scales/items. A: Fatigue; B: Micturition problems; C: Chemotherapy side effects (only for patients who received chemotherapy); D: Gastrointestinal tract symptoms; E: Weight loss; F: Male sexual problems (only for men who have been sexually active). A higher score indicates a higher degree of symptoms. Error bars indicate the standard error of the mean. The Friedman test was not performed for male sexual problems, as the number of patients changed at different time points. QoL: Quality of life.

QoL in the laparoscopic group over time may imply that most of their initial positive expectations of laparoscopic surgery (the treatment that they had chosen) were met by their postoperative experience (the clinical benefits) and that the "expectation-experience homeostasis" remained unchanged throughout the entire assessment period. However, discrepancies between preoperative expectations and the postoperative experience may explain the initial deterioration of QoL in the open group, and a period of adaptation and alteration of expectations may have been needed to reestablish "homeostasis" by 1 year after surgery.

Interestingly, the same argument can also be used to explain the paradoxical finding of a worse functional scale of body image after laparoscopic surgery in our study. Patients in the laparoscopic group might have had high expectations regarding the initial cosmetic results. However, when they realized that the final cosmetic outcome (a 5-cm incisional wound over the left iliac fossa and an ileostomy over the right iliac fossa) did not meet their preoperative expectations, a significant impact on QoL with respect to body image occurred. Conversely, patients in the open group who did not have high expectations regarding the cosmetic results might not have experienced a significant change in the functional scale of body image after surgery.

Urinary and sexual dysfunctions are recognized complications after rectal cancer surgery, which may have a negative impact on QoL[11]. In our study, the laparoscopic group had significantly fewer micturition problems at 4-8

Table 3 Sexual enjoyment and sexual problems among men who have been sexually active: Laparoscopic *vs* open groups

	Baseline			4 mo			8 mo			12 mo		
	Lap	Open	*P* value	Lap	Open	*P* value	Lap	Open	*P* value	Lap	Open	*P* value
Number of men who have been sexually active	14	5	/	6	1	/	10	5	/	12	5	/
Sexual enjoyment (functional scale)	40.5 (8.0)	20.0 (8.2)	0.164	38.9 (18.1)	33.3 (/)	1.000	46.7 (7.4)	6.7 (6.7)	0.004	44.4 (10.3)	0 (0.0)	0.019
Male sexual problems (symptom scale)	16.7 (8.4)	0 (0)	0.194	33.3 (13.6)	66.7 (/)	0.309	20.0 (8.5)	76.7 (14.5)	0.013	29.2 (11.3)	80.0 (9.7)	0.026

Quality of life scores are presented as the mean (standard error of mean). Scores ranged from 0 to 100. A higher score on a functional scale indicates better functioning, whereas a higher score on a symptom scale indicates a higher degree of symptoms. Scores in the laparoscopic and open groups were compared by the Mann-Whitney *U* test.

mo after surgery when compared with the open group, but the benefit disappeared at 1 year. The COREAN trial also reported fewer micturition problems in the laparoscopic group when compared with the open group at 3 mo after surgery[2]. This benefit of less urinary dysfunction is believed to be the result of better preservation of the autonomic nerves and less traumatic surgery, attributable to the magnified view provided by laparoscopic surgery[2,4,5]. However, when the transient neuropraxia of the pelvic autonomic nerves in the open group has fully recovered, this benefit will disappear.

In our study, male sexual enjoyment and male sexual problems were the only two QoL scales that remained worse in the open group when compared with the laparoscopic group at 1 year after surgery. Yang *et al*[29] also reported fewer male sexual problems and better sexual functioning at 12-18 mo after laparoscopic total mesorectal excision for low rectal cancer when compared with open surgery; better sexual enjoyment in the laparoscopic group was even observed after 24 mo postoperatively. By contrast, a nonsignificant trend for worse sexual function in males after laparoscopic surgery for rectal cancer was reported by the United Kingdom Medical Research Council trial of conventional *vs* laparoscopic-assisted surgery in colorectal cancer (CLASICC)[16]. Interestingly, the design of the CLASICC trial required that every participating surgeon had undertaken at least 20 laparoscopic resections, and most of the surgeons were likely still on their learning curve[1,31]. Although the laparoscopic approach can provide a clear, magnified view in the deep pelvis, the risk of autonomic nerve injury will still be substantial if the rectal dissection is performed by an inexperienced surgeon.

Similar to the study by Yang *et al*[29], our study is limited by its nonrandomized design, and the risk of selection bias is inevitable. Furthermore, the number of sexually active men recruited and analyzed was small, and therefore, a very strong conclusion regarding sexual function after laparoscopic *vs* open surgery for rectal cancer could not be drawn. Nevertheless, based on our findings, we may still conclude that laparoscopic sphincter-preserving resection for rectal cancer is associated with better preservation of QoL and fewer male sexual problems when compared with the open approach in the first year after surgery. Further large-scale, multicenter, randomized trials, including the American College of Surgeons Oncology Group Z6051 trial and the European COLOR II trial[32,33], will more definitively evaluate whether laparoscopic surgery truly provides better QoL and reduces urosexual dysfunction in patients with rectal cancer.

In conclusion, this prospective nonrandomized study demonstrates that laparoscopic sphincter-preserving resection for rectal cancer is associated with better preservation of quality of life and fewer male sexual problems when compared with open surgery in Chinese patients. These findings, however, should be interpreted with caution because of the small sample size of the study.

COMMENTS

Background

Most colorectal surgeons are only concerned about the surgical and oncologic safety of laparoscopic surgery for rectal cancer in comparison with the open approach, and many have ignored the importance of functional outcomes and quality of life (QoL). Furthermore, few studies have evaluated QoL outcomes in Chinese patients after laparoscopic surgery for rectal cancer. Authors therefore conducted a prospective study to compare QoL outcomes in Chinese patients after curative laparoscopic *vs* open sphincter-preserving resection for rectal cancer.

Research frontiers

The magnified vision and less traumatic surgery offered by the laparoscopic approach may allow better preservation of the pelvic autonomic nerves, and presumably, functional outcomes following laparoscopic surgery for rectal cancer may be better compared to open surgery. However, conflicting results have been reported in the literature; some studies have even reported a higher incidence of sexual dysfunction after laparoscopic rectal surgery. Furthermore, it is also unclear whether the short-term and long-term clinical benefits associated with the laparoscopic approach will translate into better QoL outcomes for patients with rectal cancer.

Innovations and breakthroughs

This study has several strengths. First, although nonrandomized, the baseline characteristics and sociodemographic data of the two groups of patients were similar, and a fair comparison could therefore be made. Second, other studies have included metastatic cases and abdominoperineal resection in their QoL analysis, whereas the study only focused on Chinese patients undergoing curative sphincter-preserving rectal resection, thus minimizing the impact of other potential confounders on the QoL assessment. Third, all their questionnaires were administered by a single research assistant and were completed by the patients during clinic visits. As a result, authors achieved 100% compliance at different time points, a figure that was not achieved by other studies in which the questionnaires were collected by mail.

Applications

Their prospective nonrandomized study, albeit small in sample size, demonstrates that laparoscopic sphincter-preserving resection for rectal cancer is associated with better preservation of quality of life and fewer male sexual problems when compared with open surgery in Chinese patients. Further large-scale, multicenter, randomized trials, including the American College of Sur-

geons Oncology Group Z6051 trial and the European COLOR Ⅱ trial, will more definitively evaluate whether laparoscopic surgery truly provides better QoL and reduces urosexual dysfunction in patients with rectal cancer.

Terminology

Within the context of medical and healthcare research, QoL is the patient's subjective perception of the impact of his/her disease and its treatments on his/her physical, psychological, and social functioning and general well-being. Health-related QoL after cancer surgery can be assessed by standardized instruments such as the questionnaires developed by the European Organization for Research and Treatment of Cancer (EORTC), which contain multidimensional generic and disease-specific domains; the EORTC QLQ-C30 and QLQ-CR38 are the most commonly used questionnaires in colorectal cancer trials.

Peer review

The external validity of this study is limited by the fact that only Chinese patients with low body mass index were included, and patients with stage Ⅳ disease were excluded.

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BRIEF ARTICLE

Sonographic evaluation of proximal gastric accommodation in patients with functional dyspepsia

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Author contributions: Zhu Q designed the research and revised the manuscript; Fan XP and Wang L performed the experiments and wrote the paper; Ma T, Xia CX and Zhou YJ analyzed the data.

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Abstract

AIM: To assess the value of ultrasonography (US) in evaluation of proximal gastric accommodation disorder in patients with functional dyspepsia (FD).

METHODS: Between April 2011 and March 2012, 45 patients with FD and 27 healthy volunteers were enrolled in this study. Two-dimensional ultrasound (2DUS) and 3-dimensional ultrasound (3DUS) were performed sequentially to measure proximal gastric area (PGA), maximal proximal gastric diameter (MPGD), and proximal gastric volume (PGV). These values were measured separately in the two groups every other 5 min for a duration of 25 min after the beginning of ingestion of a test meal. Air pocket grading was done separately for images of 2DUS and blocks of 3DUS obtained at five scanning time points.

RESULTS: Both PGA and PGV of patients were significantly smaller than healthy controls (P = 0.000 and 0.002, respectively). Comparing the two parameters between the groups at each time point, the differences were also statistically significant (P = 0.000-0.013), except at 10 min for the PGV (P = 0.077). However, no overall difference was found between the groups in the MPGD measurements (P = 0.114), though it was statistically significant at a 20-minute examination point (P = 0.026). A total of 360 sets or blocks of images were obtained for both 2DUS and 3DUS. For the images analyzed by 2DUS, none were excluded because of gastric gas, and 50 (13.9%) and 310 (86.1%) sets were determined as air pockets grades 1 and 2, respectively. For the images analyzed by 3DUS, 23 (6.4%) blocks were excluded from the measurement due to presence of a large fundus air pocket (grade 3); fifty (13.9%) and 287 (79.7%) blocks were also graded as 1 and 2, respectively.

CONCLUSION: Measurement of both PGA and PGV by 2DUS and 3DUS could be useful for assessment of the proximal gastric accommodation.

Key words: Functional dyspepsia; Gastric accommodation; Ultrasonography; Diagnosis; 2-dimensional ultrasound; 3-dimensional ultrasound

Core tip: We adopted 2-dimensional and 3-dimensional ultrasonography to measure area and volume of the proximal stomach in patients with functional dyspepsia; a condition whereby patients can experience impaired gastric accommodation. Area and volume could be used to assess accommodation impairment, because both area and volume of the patients were smaller than the controls (P < 0.05). Therefore, the ultrasound measurement of gastric area and volume could help predict the functional dyspepsia.

Fan XP, Wang L, Zhu Q, Ma T, Xia CX, Zhou YJ. Sono-

graphic evaluation of proximal gastric accommodation in patients with functional dyspepsia. *World J Gastroenterol* 2013; 19(29): 4774-4780 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i29/4774.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i29.4774

INTRODUCTION

Functional dyspepsia (FD) is the presence of symptoms thought to originate from the gastro-duodenal region, in the absence of organic, systemic, or metabolic disease that is likely to explain the symptoms[1]. The prevalence of FD is 24.4% in Australia and 23.5% in China, based on the Rome Ⅱ criteria[2]. The pathogenesis of FD is still unknown, but several studies have indicated that the proximal stomach, which includes the fundus and the proximal one-third of the body, is the site of an accommodation disorder that is likely to substantially contribute to the pathogenesis of FD[3-5]. Cannon *et al*[6] first described gastric accommodation in 1911. It is thought to be a vagally mediated reflex that occurs postprandially and results in reduction of tone, providing a reservoir for the meal[7]. In patients with impaired gastric accommodation, the proximal stomach cannot relax and change its volume to the content following meal ingestion, and the subsequent increase of intragastric pressure contributes to postprandial discomfort[8]. The impairment of proximal gastric accommodation has been found in 40% of patients with FD[3]. Hence, it is likely that FD can be diagnosed through the recognition of impaired gastric accommodation.

There are two methods to measure proximal gastric accommodation. One method is the intragastric barostat technique, in which a polyethylene bag is directly placed into the proximal stomach *via* oral intubation. The intragastric barostat bag technique is regarded as the gold standard because it allows simultaneous acquisition of volume, pressure, and tone, and makes the user correlate these variables to sensory parameters[9]. The disadvantages of the method are its interventional and time-consuming nature, leading to discomfort of patients[10], and a likely interference with gastric physiology due to pressure caused by the bag[11]. The second method is imaging, such as magnetic resonance (MR) imaging, single photon emission computed tomography (SPECT), and ultrasonography (US). MR imaging and SPECT can estimate volumetric change of the stomach directly and accurately[12], but the equipment is not widely available and is costly, and the natural state of the stomach impacted by gravity is also neglected owing to the flat position of the examination. In addition, there is a problem of SPECT-associated radiation exposure[13].

US is widely available, inexpensive, non-radioactive, and can be performed repeatedly, even at the bedside, making it a much more attractive option for the measurement of proximal gastric accommodation. Moreover, because gravity plays a role in the propulsion of gastric contents, accommodation should be measured in a sitting or standing position that can be easily accomplished during the US examination[12,14,15]. However, published studies reviewing the feasibility of this method are limited, presumably due to the complex procedure of scanning the stomach with US. Therefore, the aim of the current study was to investigate the usefulness of US, including 2-dimensional US imaging (2DUS) and 3-dimensional US imaging (3DUS), in the measurement of proximal gastric accommodation disorders in patients with FD compared to healthy controls.

MATERIALS AND METHODS

Subject characteristics

Between April 2011 and March 2012, 46 consecutive patients with FD underwent US scanning. One patient was excluded from the study because of nephroptosis, which obscured the left kidney as a landmark in obtaining a sagittal plane of the proximal stomach. Thus, 45 enrolled patients consisted of 17 men and 28 women, with an age range of 19-64 years (mean: 33.70 ± 9.86 years) and a body mass index (BMI) of 16.33-25.95 kg/m^2 (mean: 20.67 ± 2.34 kg/m^2). None of the patients had a history of other abdominal diseases, abnormal hepatic function tests, organic changes on gastroendoscopy, and positive findings on routine abdominal US scanning.

The Rome Ⅲ classification system was the basis for the diagnostic criteria for inclusion of patients with FD[1]. According to these criteria, the patient must have one or more of the following symptoms: bothersome postprandial fullness, early satiation, epigastric pain, or epigastric burning. Further, the patient could have no evidence of structural gastrointestinal diseases on upper endoscopy likely to explain the symptoms, and the symptoms must have occurred 6 mo prior to diagnosis and be active for the last 3 mo. Of the 45 patients, 33 (73.3%), 20 (44.4%), 19 (42.2%), and 14 (31.1%) presented with postprandial fullness, epigastric pain, early satiation, and epigastric burning, respectively.

Twenty-seven healthy volunteers were examined by US. This sample included 14 men and 13 women with an age range of 19-75 years (mean: 38.07 ± 14.55 years) and a BMI of 18.02-24.21 kg/m^2 (mean: 21.10 ± 1.74 kg/m^2). Healthy controls had no symptoms and physical signs of gastrointestinal diseases in the past six months, history of other abdominal diseases, abnormal hepatic function tests, and positive findings on routine abdominal US examination.

There were no statistically significant differences between the patients with FD and control groups with respect to age and BMI. Informed consent was obtained from all of the subjects.

Test meal

A 500 mL esculent liquid was used as the test meal, and was prepared by mixing 200 mL of nutrient emulsion (Enteral Nutritional Emulsion; Sino-Swed Pharmaceutical

Corp, Beijing, China) with 300 mL of warm water. The emulsion contained 15 g of protein, 11.6 g of fat, and 34 g of carbohydrate (300 kcal). To decrease the presence of small bubbles in the nutridrink, the meal was allowed to sit stationary on a table for approximately 10 min before consumption.

US equipment

A Voluson 730 expert system with a RAB 2-5 type probe with 3DUS imaging function was employed (GE Medical Systems, Milwaukee, WI, United States).

Examination protocol

To avoid increscent gas within the stomach, the examination was performed before 10:00 am after an overnight fasting of > 8 h. Administration of medication affecting gastrointestinal motility was discontinued for at least 48 h prior to US. Smoking was not allowed on the day of examination. All the patients were examined within 7 d following gastric endoscopy.

The subjects were scanned in a half-sitting position, leaning back at an angle of approximately 80° on an examining couch. The antrum was observed 2-3 min before nutridrink ingestion to avoid antral contractions and emptying into duodenum, in which the elevation of proximal stomach tone is induced by an enterogastric reflex occurring in phase Ⅲ of the migrating motor complex[16]. Thereafter, in the other phases without the contraction, a 500 mL meal was ingested with a straw within 4 min. The proximal stomach of each subject was scanned every other 5 min during 25 min after beginning ingestion.

Air pocket grading

To assess image quality, a grading system based on the amount of air pockets in the proximal stomach was established as follows: grade 1 (absence of visible air within the stomach); grade 2 (some air within the stomach, but the following measurements still being able to be proceed); and grade 3 (a great amount of gastric air so that the image would be excluded from the measurement). Grading was done separately using 2DUS and 3DUS using five examinations for each subject.

US scanning and measurement

Subjects were instructed not to move and to hold their breath at the end of expiration to permit diaphragmatic rising and restoration of the gastric configuration.

For 2DUS imaging, a scanning probe was placed longitudinally under the left subcostal margin and tilted cranially in the long axial direction to show the top of the gastric fundus. In this way, a sagittal section of the proximal stomach was visualized, in which the left renal sinus, the left lobe of the liver, and the tail of the pancreas served as anatomic landmarks (Figure 1). Then, the probe was rotated 90° and tilted cranially in the short axial direction to obtain a maximal transverse section of the proximal stomach, in which the left diaphragm

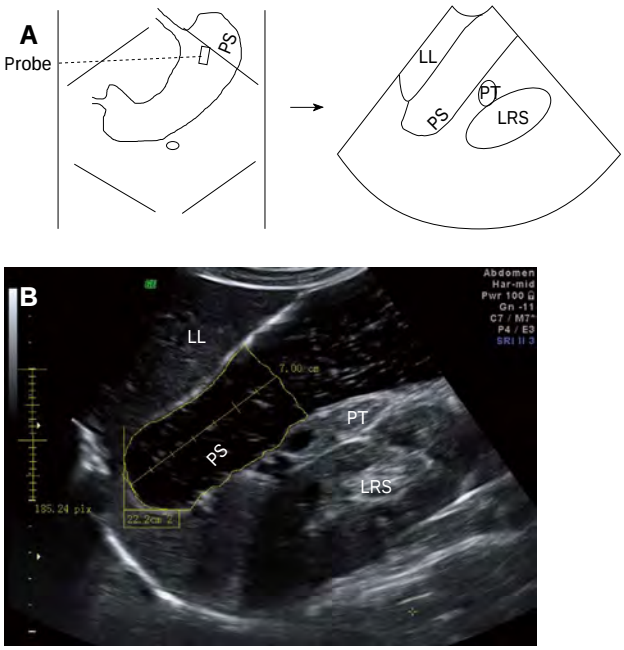

Figure 1 Sagittal section of the proximal stomach. A: To obtain the section, a probe is placed longitudinally under the left subcostal margin and tilted cranially in the long axial direction of proximal stomach (PS) to show the top of gastric fundus, in which left renal sinus (LRS), left liver (LL), and pancreatic tail (PT) are simultaneously displayed; B: Proximal gastric area (PGA) is measured by means of outlining along the echogenic mucosa surface of PS in the distance between the echoic inner surface of the fundus top down to 7 cm level (between cursors).

and the left liver were landmarks (Figure 2). Image post-processing was done using image-processing software (4D View, version 5.0; GE Medical Systems). On the sagittal section, the proximal gastric area (PGA) was outlined by tracing along the luminal echogenic surface corresponding to the interface between the liquid and mucosa of the gastric wall, from the top margin of the fundus to 7 cm level inferiorly (Figure 1). On the transverse section, a maximal proximal gastric diameter (MPGD) was measured between the inner echogenic surfaces of the lesser and greater curvatures (Figure 2).

For 3DUS analysis, volumetric image data was acquired immediately following 2DUS using similar placement of the probe to that of the above sagittal section. A sweeping angle of 85° was set. The proximal stomach was scanned *via* automated sweeping between the curvatures over 5-10 s. The block cut at a distance of 7 cm inferior to the top of the fundus was saved for further processing on the workstation. Using a virtual organ computer-aided analysis (VOCAL) technique of the 4D View, six sections of one block were separately outlined manually along the echoic interface, with each rotating 30° from the previous section. The proximal gastric volume (PGV) was automatically calculated from these six highlighted areas and displayed as a reconstructive volume (Figure 3).

One physician (Wang L), blinded to the subject (FD patient or healthy control), completed the measurement. The mean value was calculated after two measurements.

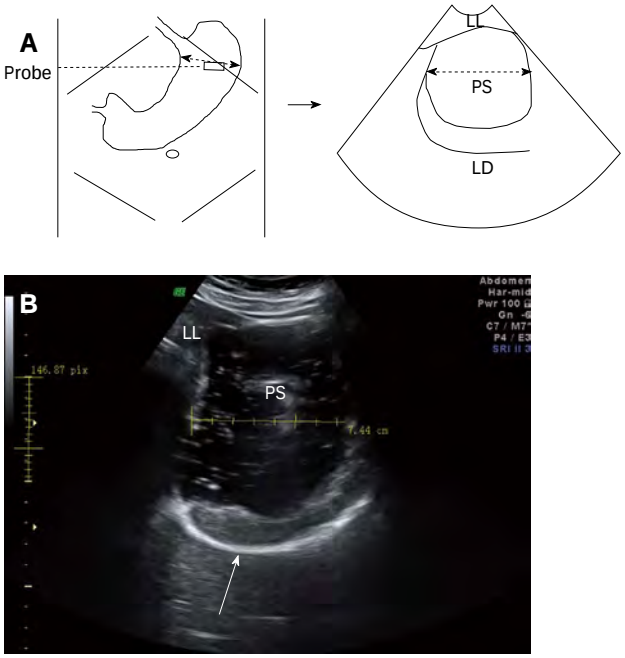

Figure 2 Maximal transverse section of the proximal stomach. A: After acquiring the previous sagittal section, the probe is rotated about 90° to show the maximal transverse diameter (dotted line with double arrow), in which the left diaphragm (LD) and left liver (LL) are simultaneously depicted; B: In measuring maximal proximal gastric diameter, the cursors are placed on the echogenic mucosal surfaces of the lesser and greater curvatures. LL and the arrow indicate left liver and left diaphragm, respectively. PS: Proximal stomach.

Statistical analysis

The measurement values are presented as the mean ± SD. With repeated measures analysis of variance (ANOVA), the values of PGA, MPGD, and PGV were compared between two groups, as a total and at each scanning time within 25 min. Statistical significance was accepted as $P < 0.05$.

RESULTS

Air pocket grading

Three-hundred-and-sixty sets of 2DUS images (one sagittal and one transverse section), and the same number of 3DUS blocks, were graded. Of these, 225 were obtained from patients and 135 from controls in five time examinations. The 2DUS imaging revealed 50 (13.9%) and 310 (86.1%) sets of the image were determined as grades 1 and 2, respectively, and none were excluded due to grade 3. In 3DUS, 50 (13.9%) and 287 (79.7%) blocks were graded as 1 and 2, and the other 23 (6.4%) were grade 3 and in turn excluded from the measurement. Of these excluded blocks, 13 (56.5%) appeared at 10 min, and the remaining four (17.4%), three (13.0%), one (4.3%) and two (8.7%) occurred at 5, 15, 20 and 25 min after the meal, respectively.

PGA, MPGD and PGV measurements

The PGA and PGV of patients were significantly smaller than those of healthy controls ($P = 0.000$ and 0.002, respectively). When the two parameters were compared at each time point separately, the differences were also statistically significant between the two groups ($P = 0.000$-0.013), except at 10 min of the PGV.

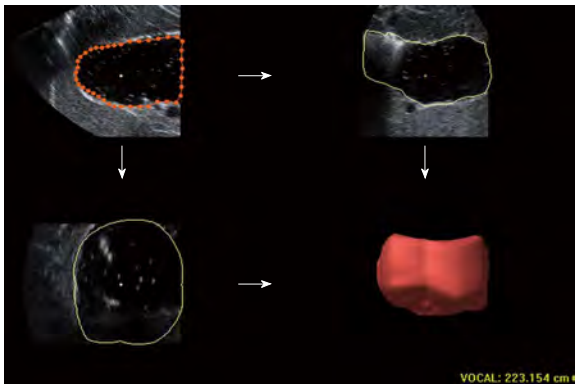

Figure 3 Three-dimensional ultrasound applied for measuring proximal gastric volume. The volume is measured similarly from the top inner margin of the fundus to 7 cm level inferiorly along the long axis of proximal stomach; six sections of the block from six 30° rotations are separately outlined along the echoic interface in the upper left view. A reconstructive volume is displayed in the lower right view.

The patients with FD revealed shorter MPGD than healthy controls postprandially; however, it was not statistically significant in the two groups, and the difference was significant ($P = 0.026$) only at 20 min when comparing each time point (Table 1).

DISCUSSION

Based on the theory that the impairment of proximal gastric accommodation is likely to lead to the pathogenesis of FD, both 2DUS and 3DUS imaging were utilized to measure the size of the proximal stomach. The data indicated that both PGA and PGV could help assess the proximal gastric accommodation.

US can provide an indirect evaluation of stomach relaxation and intragastric pressure by measuring the size of the stomach[17]. The 2DUS method of assessing gastric accommodation was developed first by Gilja *et al*[18]. According to their study, 2DUS could offer a geometric estimation of proximal gastric size by measuring PGA and MPGD. They also found that the patients with FD exhibited a smaller PGA and MPGD than controls ($P = 0.018$ and 0.046, respectively)[19]. Having adopted a similar method, our study showed that the PGA was significantly different between FD patients and healthy controls, but the MPGD was not different overall, being significant only at 20 min ($P = 0.026$). The reason why PGA was superior to MPGD could be related to an irregular shape of the proximal stomach. When estimating the size of an organ that had an irregular contour, using an area was likely more accurate than a diameter. The other reason might be that the left renal sinus, which served as a key landmark in a sagittal section used for PGA measurement, had a relatively narrow distance in a transverse di-

Table 1 Ultrasonography measurement of postprandial size of proximal stomach (mean ± SD)

Time in min	Patients (n = 45)	Controls (n = 27)	t	P
	PGA in cm^2			
5	22.78 ± 6.59	30.68 ± 6.97	4.819	0.000
10	23.13 ± 6.39	29.52 ± 7.46	3.853	0.000
15	22.32 ± 5.93	27.51 ± 7.13	3.332	0.001
20	21.34 ± 6.34	28.25 ± 7.76	4.110	0.000
25	21.51 ± 6.02	26.35 ± 7.23	3.062	0.003
	F = 17.499 P = 0.000			
	MPGD in cm			
5	6.77 ± 1.34	7.08 ± 1.10	1.035	0.304
10	6.92 ± 1.31	7.27 ± 1.00	1.185	0.240
15	6.66 ± 1.43	7.11 ± 1.03	1.432	0.157
20	6.33 ± 1.29	7.00 ± 1.06	2.275	0.026
25	6.31 ± 1.50	6.66 ± 1.24	1.032	0.305
	F = 2.562 P = 0.114			
	PGV in cm^3			
5	145.75 ± 60.40	185.08 ± 60.81	2.645	0.010
10	152.91 ± 52.10	177.13 ± 59.10	1.797	0.077
15	142.46 ± 49.50	184.16 ± 52.28	3.358	0.001
20	132.45 ± 46.70	169.12 ± 48.64	3.147	0.002
25	126.15 ± 50.23	157.46 ± 49.97	2.544	0.013
	F = 10.319 P = 0.002			

PGA: Proximal gastric area; MPGD: Maximal proximal gastric diameter; PGV: Proximal gastric volume.

rection and a constant relationship to the proximal stomach. Therefore, the measurement of PGA might tend to be of less operator variability in various examination time points. Besides, when MPGD was transversely measured, the right cursor was placed on or near the gastric lesser curvature with less expansive function[20], and hence the difference of the transverse dimension between the patient and the control would be decreased. This phenomenon also occurred in other previous studies[21,22]. Thus, the application value of MPGD was limited in assessment of gastric accommodation.

The feasibility of assessing the proximal gastric accommodation with PGA on 2DUS images has been confirmed by several studies[21-24]. The measuring of PGA was simple, with only one section being outlined in this process. It was less likely to be affected by gastric air, which was verified since no subject was excluded for this factor. However, it was difficult to find the landmarks in subjects with obese body types, nephroptosis, or renal ectopy. The volumetric estimation that was based on values of 2DUS, *i.e.*, $V = PGA \times MPGD$, and adopted by other studies before the advent of 3DUS could to some extent bring an error because of the irregular-shaped stomach[18].

In general, 3DUS has advantages over 2DUS, which can measure a volume directly and needs no landmarks. Using this technique, Gilja *et al*[25] obtained a good correlation (r = 0.997, $P < 0.05$) between the estimated volume of porcine stomach filled with water *in vitro* and the actual quantity of water injected. Another study demonstrated that 3DUS had a moderate correlation (r = 0.55, P = 0.002) with the barostat in measurement of proximal gastric volumes[26]. However, there were some drawbacks of the freehand 3DUS technique used in these studies[23,24]. An additional tracking device that consisted of a transmitter generating a pulse magnetic field and a position sensor attached to the probe was required. Therefore, the environment where the patient was examined requires magnetic shielding to avoid image distortion. The distortion also appeared while the data of both image and position could not be transformed simultaneously to a post-processing workstation. The time-consuming process of coordinating images with their spatial locations also restricted it. The previous method of 3DUS scanning was still limited by the complicated manipulation in which the operator had to scan several times to obtain an image with high quality, because the image was easily distorted in free-hand moving a probe on body surface at an appropriate speed. In the current study, a new type of 3DUS probe (RAB 2-5, Voluson 730) was used, in which a 1D transducer array moving mechanically through a designed trajectory was mounted together with integrated positioning system and sensor. Hence, the data acquisition was automatic rather than manual, could be done during a single breath-hold, and could be displayed on the monitor immediately after scanning. Using this type of transducer, PGV could be obtained in most (337/360, 93.6%; air pockets grading 1 and 2) of the 3DUS blocks at five examination time points. Consequently, the patients with FD showed smaller PGV than healthy controls (P = 0.002). Adopting the same transducer, Manini *et al*[27] measured the whole gastric volume accurately, with the results comparable to that from SPECT, establishing a reference standard for measuring gastric size.

An air grading system was designed to assess the image quality. Intragastric gas is a critical interference factor in US stomach examination, which may induce multiple reflection artifacts and limit the stomach outline. The accumulation of gas in the fundus was a gradually incremental process. Small bubbles could be seen in the entire stomach immediately after drinking a test meal, in addition to the usual existence of fundus air pockets, but these bubbles did not decline the image quality. Over time, they burst and mixed into the fundus air pockets, which to some degree obstructed the visualization of the gastric wall, especially the posterior wall. Within minutes, the gas reduced because of burping and the image quality was improved again. In our results, none of images were excluded from the 2DUS analysis but 23 (6.4%) 3DUS blocks could not be utilized due to the grade 3 air pockets. We hypothesize that the higher resolution of the single section 2DUS images[28], combined with fact that 3DUS was more subject to the intragastric gas explains this phenomenon. We also found that most of the grade 3 (13/23, 56.5%) appeared at 10 min after the beginning of ingestion. The decrease of sample size after excluding the 13 3DUS blocks might cause no significant difference in PGV measurement between two groups this time. These results suggested that 3DUS testing at the 10 min time point or with the appearance of fundus air pockets should be avoided.

There were some limitations in the current study. The

limited sample size did not allow us to divide patients into two subgroups of postprandial distress syndrome and epigastric pain syndrome according to the Rome Ⅲ criteria[1]. After fasting overnight, ingesting a 500 mL test meal in a short time would make subjects uncomfortable, and consequently the smaller amount of the meal should be tested. The observation duration of 25 min might not be enough to investigate into the gastric accommodation, which lasts in the entire process of postprandial digestion[29]. Because no reference method as the barostat procedure or SPECT was adopted in our study, the comparative study of 2DUS and 3DUS imaging could not be carried out to find out which one was more accurate.

In conclusion, we show that the impaired gastric accommodation to a test meal was present in patients with FD. Two parameters of PGA and PGV on 2DUS and 3DUS images could be used for assessing the proximal gastric accommodation in which 2DUS was simpler in manipulation and less likely to be degraded by gastric gas, and 3DUS had the merit of measuring volume directly, providing less gastric gas.

COMMENTS

Background

There is a high global incidence of functional dyspepsia (FD), with about half of the reported gastroenterological outpatients being in China. Several studies have indicated that impaired gastric accommodation is a major pathogenesis of the disease. Hence, assessment of proximal gastric accommodation disorder could help diagnose FD.

Research frontiers

Imaging can provide an indirect evaluation of gastric relaxation in accommodation reflex by measuring the size (*i.e.*, area and volume) of the proximal stomach. Ultrasonography is a preferable imaging method, which is accurate, easy to manipulate, non-invasive, and cost effective.

Innovations and breakthroughs

The proximal gastric accommodation was evaluated by measuring the proximal gastric area (PGA) and proximal gastric volume (PGV) in patients with FD using 2-dimensional and 3-dimensional ultrasound. PGA and PGV of the patients were significantly smaller than the controls. Therefore, the change of PGA or PGV could be pertinent to the impairment of gastric accommodation and utilized in predicting FD.

Applications

US is likely to be a convenient and accurate imaging modality to assess the gastric accommodation. It has the potential value of diagnosing FD and evaluating the effect of therapy.

Terminology

The proximal stomach is a compartment consisting of the fundus and the upper one-third of gastric body, which has the function of gastric accommodation. The boundary between the proximal and distal stomach has not been definitely determined anatomically and physiologically. Gastric accommodation is a vagally mediated reflex that occurs postprandially, results in reduction of gastric tone, and provides a reservoir for the meal.

Peer review

In this study, the authors found that both PGA and PGV on 2-dimensional ultrasound and 3-dimensional ultrasound images could be used in assessment of the proximal gastric accommodation. Very interesting study, it could give the readers some new information about the use of US in evaluation of proximal gastric accommodation disorder in patients with functional dyspepsia. The manuscript is well written.

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BRIEF ARTICLE

Influence of endoscopic submucosal dissection on esophageal motility

Bao-Guo Bu, En-Qiang Linghu, Hui-Kai Li, Xiao-Xiao Wang, Rong-Bin Guo, Li-Hua Peng

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Abstract

AIM: To assess esophageal motility after esophageal endoscopic submucosal dissection (ESD).

METHODS: Twelve patients (6 men and 6 women) aged 53-64 years (mean age, 58 years) who underwent regular examination 3-12 mo after esophageal ESD for neoplasms of the esophageal body were included in this study. The ESD procedure was performed under deep sedation using a combination of propofol and fentanyl, and involved a submucosal injection to lift the lesion and use of a dual-knife and an insulated-tip knife to create a circumferential incision around the lesion extending into the submucosa. Esophageal motility was examined using a high-resolution manometry system. Dysphagia was graded using a five-point scale according to the Mellow and Pinkas scoring system. Patient symptoms and the results of esophageal manometry were then analyzed.

RESULTS: Of the 12 patients enrolled, 1 patient had grade 2 dysphagia, 1 patient had grade 1 dysphagia, and 3 patients complained of sporadic dysphagia. Ineffective esophageal motility was observed in 5 of 6 patients with above semi-circumference of resection extension. Of these 5 patients, 1 patient complained of grade 2 dysphagia (with esophageal stricture), one patient complained of grade 1 dysphagia, and 3 patients complained of sporadic dysphagia. Normal esophageal body manometry was observed in all 6 patients with below semi-circumference of resection extension. The 6 patients with normal esophageal motility did not complain of dysphagia.

CONCLUSION: Extensive esophageal ESD may cause esophageal dysmotility in some patients, and might also have an influence on dysphagia although without esophageal stricture.

Key words: Esophageal neoplasm; Endoscopic submucosal dissection; Dysphagia; Ineffective esophageal motility; Esophageal manometry

Core tip: Endoscopic submucosal dissection (ESD) is widely used to treat esophageal epithelial neoplasms. ESD has the advantage over esophagectomy of being less invasive and having lower postoperative morbidity. ESD also has the advantage over endoscopic mucosal resection of enabling the removal of larger epithelial neoplasms in an *en bloc* manner for complete resection. It is not known whether esophageal ESD affects esophageal motility. Therefore, the present study aimed to evaluate the effects of ESD on esophageal motility.

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INTRODUCTION

Endoscopic submucosal dissection (ESD) is widely used to treat esophageal epithelial neoplasms[1-3]. ESD has the advantage over esophagectomy of being less invasive and having lower postoperative morbidity[4-6]. ESD also has the advantage over endoscopic mucosal resection (EMR) of enabling the removal of larger epithelial neoplasms in an *en bloc* manner for complete resection[7-9]. Some postoperative complications such as bleeding and perforation may occur, but can be minimized by the development of the endoscopist's skill and the use of advanced equipment[10,11]. Postoperative stricture is a major complication of ESD due to the increase in the scope of ESD resection, which can result in dysphagia and thus affect patients' quality of life. Some studies have found that more than 3/4 of the circumference of esophageal ESD has a high risk of esophageal stricture[12,13]. Post-ESD stricture can be prevented with repeated endoscopic balloon dilation and oral prednisolone[14,15].

In addition, dysphagia is not always related to esophageal stricture and motility abnormalities also play an important role[16-19]. Some patients undergoing esophageal ESD at our center complained of sporadic dysphagia without postoperative esophageal stricture, especially when swallowing a large mass of food. This observation has raised concerns that sporadic dysphagia in these patients may be due to postoperative esophageal dysmotility. Moreover, the healing of an ESD-induced iatrogenic esophageal ulcer is also involved in destruction and fibrosis of the muscularis propria[20]. It is not known whether esophageal ESD affects esophageal motility. Therefore, the present study aimed to evaluate the effects of ESD on esophageal motility.

MATERIALS AND METHOD

Patients

Twelve patients (6 men, 6 women; mean age, 58 years; range, 53-64 years) examined between 3 and 12 mo after ESD for neoplasms of the esophageal body at the Chinese PLA General Hospital were included in this study. Clinicopathological data of the patients are shown in Table 1. After written informed consent was obtained, data on dysphagia were collected by questionnaire and all patients underwent esophageal manometry.

ESD procedure

The ESD procedure was performed in a standardized way under deep sedation using a combination of propofol and fentanyl. Patients were continuously monitored by electrocardiography. The margins of the lesion were marked by electrocautery (30 W soft coagulation) to determine the resection border. A submucosal injection was then performed to lift the lesion. When the lesion was lifted sufficiently, a dual-knife and insulated-tip knife were used to create a circumferential incision around the lesion extending into the submucosa and a submucosal dissection was performed to remove the lesion in an *en bloc* fashion.

Grading of dysphagia

Dysphagia was graded on a five-point scale according to Mellow and Pinkas[21]: 0 = no dysphagia, 1 = dysphagia to normal solids, 2 = dysphagia to soft solids, 3 = dysphagia to solids and liquids, 4 = complete dysphagia, even to saliva.

Esophageal manometry

Assessment of esophageal motility was performed using a high-resolution manometry system with 36 channels spaced at 1 cm intervals (outer diameter 4.2 mm) (Sierra Scientific Instruments, Los Angeles, CA, United States). This system can automatically capture esophageal motor function from the pharynx to the stomach with a single placement of the catheter.

Esophageal manometry was performed after fasting for at least 6 h, and the high-resolution catheter was passed transnasally, positioned 2 cm below the lower esophageal sphincter (LES) and then fixed in place by taping it to the nose. The upper esophageal sphincter and LES were located using a real-time pressure monitor. The protocol included a 5-min period to assess basal sphincter pressure and then ten swallows of water (5 mL each swallow) in the supine position[22]. All swallows and pressure measurements were analyzed by Manoview Analysis Software (Sierra Scientific Instruments, United States).

RESULTS

Grading of dysphagia

Of the 12 patients, one patient complained of grade 2 dysphagia, one complained of grade 1 dysphagia and three patients complained of sporadic dysphagia (especially when swallowing a large mass of food) (Table 1).

Esophageal manometry

Esophageal manometry showed that of the 6 patients with above semi-circumference of resection extension, 5 patients had low esophageal body pressure, including four patients with low mean esophageal body pressure and one with low pressure at an interval of 3 cm below the LES (Table 1). Of these 5 patients, 1 patient (with post-ESD esophageal stricture) complained of grade 2 dysphagia, 1 patient (without post-ESD esophageal stricture) complained of grade 1 dysphagia and 3 patients complained of sporadic dysphagia. Segmental simultaneous wave was observed in ESD scar site in 4 of these 5 patients, and wave amplitude was significant decreased (Figure 1).

DISCUSSION

ESD is a feasible method for treating early esophageal epithelial neoplasms, and was developed for *en bloc* and

Table 1 Clinicopathological features and esophageal manometry of 12 patients with esophageal epithelial neoplasms treated by endoscopic submucosal dissection

Number of patient	Sex	Age (yr)	Post-ESD pathology	Circumferential extension	Grade of dysphagia	Pressure of esophageal body (mmHg)			Non-peristaltic contractions of esophageal body in ten water swallows
						7.0 cm above LES	3.0 cm above LES	mean	
1	F	64	T1 cancer	2/4-3/4	0	129.8	108.7	119.2	0%
2	M	64	LGIN	1/4-2/4	0	99.9	75.3	87.6	0%
3	M	53	HGIN	2/4-3/4	2	26.1	45.2	35.6	40%
4	F	55	HGIN	2/4-3/4	Sporadic	31.8	41.2	36.5	50%
5	M	55	HGIN/Tis	1/4-2/4	0	139.1	125.9	132.5	0%
6	M	55	LGIN	2/4-3/4	Sporadic	90.1	31.9	61.0	20%
7	M	58	T1 cancer	> 3/4	Sporadic	34.1	21.7	27.9	20%
8	F	64	HGIN/Tis	1/4-2/4	0	58.6	76.6	67.6	0%
9	F	55	LGIN	1/4-2/4	0	99.0	109.2	104.1	0%
10	F	57	HGIN	> 3/4	1	34.3	52.5	43.4	50%
11	F	58	HGIN	1/4-2/4	0	96.2	109.2	102.7	0%
12	M	57	LGIN	1/4-2/4	0	90.6	76.2	83.4	0%

HGIN: High grade intraepithelial neoplasia; LGIN: Low grade intraepithelial neoplasia; LES: Lower esophageal sphincter; ESD: Endoscopic submucosal dissection; Tis: Cancer *in situ*; M: Male; F: Female.

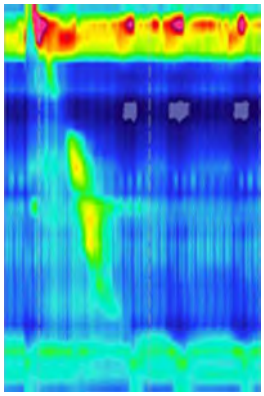

Figure 1 Segmental simultaneous wave and decreased amplitude in endoscopic submucosal dissection scar site.

complete resection of large esophageal epithelial neoplasms, to allow precise histological assessment of specimens excised in one piece with tumor-free lateral/basal margins and to reduce residual disease and local recurrence[1-3]. With the increase in the scope of esophageal ESD resection, postoperative esophageal stricture and dysphagia are major complications during long-term follow-up, and require repetitive endoscopic balloon dilatation which severely affects patient quality of life. More than 3/4 of the circumference of esophageal ESD is a significant cause of postoperative esophageal stricture[12,13]. Ono *et al*[10] reported that post-ESD stricture with dysphagia was successfully managed with repeated endoscopic balloon dilation in a median of 2 sessions.

Honda *et al*[23] found that in a dog model, mucosal defects after EMR were associated with inflammation and although the muscularis propria appeared not to have been damaged by the EMR procedure, myofiber atrophy was found to develop after the first postoperative week, eventually leading to fibrosis. Ota *et al*[20] found that an artificial ulcer after esophageal EMR/ESD in humans had a similar healing time to that in dogs. Kahrilas et *al*[24] reported an increased proportion of failed primary peristalsis and a reduction in peristalsis amplitude in peptic esophagitis. Singh *et al*[25] found a reduction in peristaltic amplitude after healing of esophagitis. Malhi-Chowla *et al*[19] found that photodynamic therapy may worsen esophageal motility in some patients with esophageal adenocarcinoma and Barrett's esophagus or Barrett's esophagus with high-grade dysplasia. Esophageal dysmotility resulting from photodynamic therapy or caustic esophageal burns is due to muscularis propria destruction. Bautista *et al*[16] found that caustic esophageal burns can also result in esophageal dysmotility without stricture. McDougall *et al*[26] found that there was no improvement in esophageal motility after healing of esophagitis, and they suggested that there was either a primary motility problem, or that reflux damage caused irreversible impairment of motility which failed to recover when the mucosa healed. Healing of post-ESD esophageal ulcer is a similar process to that in caustic esophageal burns and photodynamic therapy. It is not known whether esophageal ESD affects esophageal motility.

In the present study, ineffective esophageal body motility was observed in 5 of 6 patients with above semi-circumference of resection extension. Segmental simultaneous wave was observed in ESD scar site in 4 of these 5 patients, and wave amplitude was significant decreased. These results showed that extensive esophageal ESD might have an effect on esophageal motility. These were some observed phenomenon, and further research was needed to confirm.

Malhi-Chowla *et al*[19] found that some patients complained of dysphagia after photodynamic therapy without esophageal stricture, while esophageal dysmotility was observed in these patients. Therefore, they considered that dysphagia may be related to underlying esophageal dysmotility and may not always be caused by stricture or underlying carcinoma.

In the present study, 2 patients complained of dysphagia, and 3 patients complained of sporadic dysphagia. Of these 5 patients, only one had esophageal stricture.

However, ineffective esophageal motility was observed in all five patients. These results showed that besides esophageal stricture, ineffective esophageal motility might play an important role in dysphagia after esophageal ESD.

In summary, extensive esophageal ESD may cause ineffective esophageal motility, and ineffective esophageal motility may be a cause of dysphagia after esophageal ESD. Further studies should be encouraged to compare esophageal motility in patients before and after they undergo esophageal ESD.

COMMENTS

Background

Endoscopic submucosal dissection (ESD) is used to resect the esophagea epithelial neoplasms and has the advantage over endoscopic mucosal resection of enabling the removal of the larger epithelial neoplasms in an *en bloc* manner for complete resection. The previous study showed that esophageal dysmotility after caustic burn may cause dysphagia. However, esophageal dysmotility after esophageal ESD has not been studied. The present study therefore aimed to assess esophageal motility after esophageal ESD.

Research frontiers

The study involved testing the esophageal motility after esophageal ESD and analyzing the relation of esophageal dysmotility and extension of ESD.

Innovations and breakthroughs

Esophageal dysmotility after esophageal ESD has not been reported. In this study, the authors found that low esophageal body pressure was observed in 5 patients of 6 patients with above semi-circumference of resection extension, whereas normal esophageal body manometry was observed in all 6 patients with below semi-circumference of resection extension. The 5 patients with low esophageal body pressure complained of various degree of dysphagia.

Applications

The results suggest that extensive esophageal ESD may cause ineffective esophageal motility, and ineffective esophageal motility may be a cause of dysphagia after esophageal ESD.

Peer review

ESD is widely used to treat esophageal epithelial neoplasms. The authors evaluated the effects of ESD on esophageal motility. The authors suggest that extensive esophageal ESD may cause ineffective esophageal motility, and ineffective esophageal motility may be a cause of dysphagia after esophageal ESD The data in this study is very important and informative.

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BRIEF ARTICLE

Learning curve of transumbilical suture-suspension single-incision laparoscopic cholecystectomy

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Abstract

AIM: To investigate the learning curve of transumbilical suture-suspension single-incision laparoscopic cholecystectomy (SILC).

METHODS: The clinical data of 180 consecutive transumbilical suture-suspension SILCs performed by a team in our department during the period from August 2009 to March 2011 were retrospectively analyzed. Patients were divided into nine groups according to operation dates, and each group included 20 patients operated on consecutively in each time period. The surgical outcome was assessed by comparing operation time, blood loss during operation, and complications between groups in order to evaluate the improvement in technique.

RESULTS: A total of 180 SILCs were successfully performed by five doctors. The average operation time was 53.58 ± 30.08 min (range: 20.00-160.00 min) and average blood loss was 12.70 ± 11.60 mL (range: 0.00-100.00 mL). None of the patients were converted to laparotomy or multi-port laparoscopic cholecystectomy. There were no major complications such as hemorrhage or biliary system injury during surgery. Eight postoperative complications occurred mainly in the first three groups (n = 6), and included ecchymosis around the umbilical incision (n = 7) which resolved without special treatment, and one case of delayed bile leakage in group 8, which was treated by ultrasound-guided puncture and drainage. There were no differences in intraoperative blood loss, postoperative complications and length of postoperative hospital stay among the groups. Bonferroni's test showed that the operation time in group 1 was significantly longer than that in the other groups (F = 7.257, P = 0.000). The majority of patients in each group were discharged within 2 d, with an average postoperative hospital stay of 1.9 ± 1.2 d.

CONCLUSION: Following scientific principles and standard procedures, a team experienced in multi-port laparoscopic cholecystectomy can master the technique of SILC after 20 cases.

Key words: Single incision laparoscopic surgery; Cholecystectomy; Learning curve; Suture-suspension

Core tip: As a new technology, single-incision laparoscopic cholecystectomy (SILC) is more difficult to perform than multi-port laparoscopic cholecystectomy with higher technical demands. Thus, SILC may have a specific learning curve. The present study retrospectively analyzed the surgical outcomes of transumbilical suture-suspension SILC performed by the same team in our department to investigate the learning curve of this technology, thereby guiding the surgeons to pass the

initial learning period smoothly, safely, and quickly.

Pan MX, Liang ZW, Cheng Y, Jiang ZS, Xu XP, Wang KH, Liu HY, Gao Y. Learning curve of transumbilical suture-suspension single-incision laparoscopic cholecystectomy. *World J Gastroenterol* 2013; 19(29): 4786-4790 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i29/4786.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i29.4786

INTRODUCTION

Laparoscopic cholecystectomy has become widely accepted and is considered the gold standard for the treatment of benign gallbladder diseases. With continuous development in techniques and devices, laparoscopy has moved in the direction of minimally invasive surgery in order to meet patients' cosmetic requirements, *i.e.*, from a four-port conventional laparoscope to three-port, two-port, and the present transumbilical single-port. As a new technology, single-incision laparoscopic cholecystectomy (SILC) is more difficult to perform than multi-port laparoscopic cholecystectomy with higher technical demands. Thus, SILC may have a specific learning curve. The present study retrospectively analyzed the surgical outcomes of transumbilical suture-suspension SILC performed by the same team in our department to investigate the learning curve of this technology, thereby guiding the surgeons to pass the initial learning period smoothly, safely, and quickly.

MATERIALS AND METHODS

General information

During the period from August 2009 to March 2011, 180 patients received transumbilical SILCs in our department, including 78 males and 102 females, with an average age of 48.7 ± 13.0 years (range: 18.0-79.0 years). All patients had agreed to receive SILC, and for this study the records were retrospectively analyzed. Patients were eligible for SILC if they met the following criteria: (1) clinical diagnosis of symptomatic gallstones, gallbladder polyps, with acute cholecystitis (less than 72 h); and (2) ability to tolerate the procedure without a functional failure in important organs including the lungs, heart, liver and kidneys. Patients were considered ineligible if they had an American Society of Anesthesiologists score > 3 or were unable to tolerate general anesthesia due to other reasons, suspected gallbladder malignancy, and a history of recent upper abdominal surgery.

All operations were completed by the same surgical team. The principal surgeons were both hepatobiliary surgeons with 10 or more years of clinical experience, and had independently performed more than 500 consecutive cases of multiple-port laparoscopic cholecystectomy. The assistants were senior residents who had laparoscopic training on virtual or animal models in the training centre.

The 180 patients were divided evenly into 9 groups according to the time period they received surgery (20 patients in each period). Clinical data collected included patient name, gender, age, primary diagnosis, and previous abdominal surgery history. The difference in operation time, the amount of blood loss during the operation and postoperative complications in all groups were analyzed and compared.

Surgical procedure

The procedure has been described previously[1]. In summary, a single, curved 1.5-cm incision was made below the umbilicus and a Veress needle was inserted followed by the creation of a pneumoperitoneum which was maintained at 13 mmHg. An 11-mm trocar was inserted as the port for a 30° laparoscope at the left side of the incision. A 5-mm trocar was inserted as a manual port at the right side of the incision. The tissues between the two ports were kept to prevent gas leakage. Two size 7-0 silk threads were placed through the tissues on the bottom of the gallbladder and the muscular layer of the ampulla, respectively, and then pulled out through the abdominal wall in order to fully expose the cystic duct and the gallbladder triangle. After the gallbladder triangle was dissected using an ultrasonic scalpel, the cystic duct was clipped with titanium clips and excised. Next, the whole gallbladder was isolated, dissected, and extracted through the umbilical incision. Careful control of homeostasis and bile leakage was achieved. The abdominal cavity was then rinsed, the pneumoperitoneum was released and the incision was closed.

Statistical analysis

Quantitative data were expressed as mean ± SD. Comparisons between groups were performed using one-way ANOVA followed by Bonferroni's test, and quantitative data was analyzed by the χ^2 test. SPSS 15.0 software was used for statistical analysis, and a value of $P < 0.05$ was considered statistically significant.

RESULTS

Demographic data

Of the 180 patients enrolled, 78 were men and 102 were women. The average age was 48.7 ± 13.0 years (range: 18.0-79.0 years). The mean body mass index was 23.31 ± 2.83 kg/m^2 (range: 16.35-29.36 kg/m^2). Among these 180 patients, 17 had a history of abdominal surgery, 11 of whom had undergone open procedures, including appendectomy (n = 5), cesarean section (n = 4), total hysterectomy (n = 1) and bilateral tubal ligation (n = 1); the other six candidates had undergone laparoscopic appendectomy (n = 4) and laparoscopic herniorrhaphy (n = 2) (Table 1). The nine groups were similar with respect to sex, age, body mass index and previous history of abdominal surgery (Table 2)

Clinical outcomes

All patients underwent successful SILC, and no patient

Table 1 General data

SILCs (n = 180)		
Gender		
Male	78	
Female	102	
Age (yr)	48.7 ± 13.0	
BMI (kg/m^2)	23.31 ± 2.83	
Previous history of abdominal surgery		
Yes	5 appendectomy	4 laparoscopic appendectomy
	4 cesarean sections	2 laparoscopic herniorrhaphy
	1 total hysterectomy	
	1 bilateral tubal ligation	
No	163	

SILC: Single-incision laparoscopic cholecystectomy; BMI: Body mass index.

required conversion to standard LC or open surgery. The average operative time was 53.58 ± 23.44 min (range: 20-160 min) and the average blood loss during surgery was 12.7± 11.6 mL (range: 0-100 mL). There was no intraoperative abdominal organ damage or complications such as hemorrhage or biliary system injury during surgery. Eight postoperative complications occurred, mainly in the first three groups (n = 6), and included ecchymosis around the umbilical incision (n = 7) which resolved without special treatment, and one case of delayed bile leakage in group 8, which was treated by ultrasound-guided puncture and drainage. The majority of patients in each group were discharged within 2 d, with an average postoperative hospital stay of 1.9 ± 1.2 d. There were no differences in intraoperative blood loss and postoperative complications among the groups (Table 3).

Bonferroni's test showed that the operation time in group 1 was significantly longer than that in the other groups (F = 7.257, P = 0.000). No differences were found following comparisons within the other groups ($P \geqslant 0.05$). The standard deviation of operative time was 30.1 min in group 1, and gradually declined and stabilized from group 2 to group 9 (Figure 1). The blood loss in group 1 was 16.65 ± 9.96 mL, which was greater than that in the other groups, however, the difference was not statistically significant (F = 2.130, P = 0.082, Table 3).

DISCUSSION

Single-incision laparoscopy is a new technique, which has evolved due to the recent development of minimally invasive surgery. This technique has been applied in hepatobiliary surgery[1-8], general surgery[9-12], urological surgery[13-17], gynecology and obstetrics[18,19], and other areas. Of these, SILC is the most widely used and sophisticated technique. Some researchers believe that it has the potential to replace multi-port laparoscopic techniques and become the gold standard for cholecystectomy[20-22]. Compared with multi-port laparoscopy, single-incision laparoscopic surgery has unique features. First, it requires the operator to have a comprehensive knowledge of *in vivo* micro-anatomy and three-dimensional sensibility in order to compensate for limited surgical fields. Second, it requires skillful techniques and clear procedure understanding to replace the sense of hand touch. Finally, it requires good team work and appropriate surgical devices to compensate for interference between instruments. For these reasons, operators in the early stage of training will inevitably experience a process of exploration and skill acquisition, and this process is the learning curve of single-port laparoscopic cholecystectomy.

A learning curve is usually evaluated by the number of surgeries required to progress from an initial to a skilled state. After completing a certain number of operations, a surgeon's skill will be improved significantly and they will be competent in the surgical procedure and able to manage complications skillfully. At this stage, the learning curve is complete and the learning plateau is reached[23]. The criteria for evaluating the skillfulness of performing SILC are operative time, blood loss during the operation, and complications including bile leakage and wound infection.

The change in operation time is the most direct and accurate indicator reflecting the learning curve, because it reflects a surgeon's mastery of the technique. Operation time is influenced by four main factors, including operator skill, surgical concepts, the cooperation of the surgical team, and the choice of surgical devices. In order to use operation time as an indicator of skill, we chose a team with experience in conventional laparoscopic surgery and used the same set of surgical devices to complete the surgeries in this study. Therefore, the impact of different teams and surgical devices on operation time were minimized as much as possible.

Single-port laparoscopic cholecystectomy has been incorporated into national standards for the quality control of single disease, and the procedures are standardized. Once patients were recruited into this study, the operation was performed using a standard procedure, and the difference resulting from variation in surgical concepts was excluded. In this study, one-way ANOVA followed by Bonferroni's test were performed for multiple statistical comparisons. The advantage of Bonferroni's test is that it is a simple and conservative method to control type I error. We found that the decrease in operation time was significant from group 1 to group 2, and was relatively stable from group 2 to group 9; thus, the number of cases in group 1 reflects the learning curve. The fluctuation in operation time was greatest in group 1, indicating that in the first 20 cases the surgical technique was not standardized and the stability of technique was poor. As shown in Figure 1, the operation time continuously decreased and the shortest operation time was 20 min, suggesting that after continuous learning and training it is possible to improve the technique.

Patients recruited for SILC in the early period were carefully selected. Even so, blood loss in group 1 was greater than that in the other groups, but without statistical significance. After the first 20 cases, the average blood loss decreased rapidly and gradually stabilized. There

Table 2 Patient characteristics

Groups	1	2	3	4	5	6	7	8	9	*F*	*P*
Age (yr)[1,2]	47.7 ± 10.8	45.1 ± 13.8	50.4 ± 14.1	50.0 ± 11.7	49.4 ± 13.8	52.7 ± 14.5	52.9 ± 12.0	46.9 ± 14.3	43.7 ± 11.2	1.2082	0.297
Gender (M/F)[3]	11/9	7/13	9/11	6/14	5/15	11/9	9/11	7/13	13/7	11.343	0.183
Previous history of abdominal surgery[3]	0	2	1	2	1	2	3	2	4	6.333	0.610
BMI (kg/m^2)[1,2]	23.11 ± 2.86	23.96 ± 3.49	22.31 ± 2.99	23.83 ± 3.28	23.75 ± 2.28	22.96 ± 2.41	24.26 ± 2.76	22.80 ± 2.16	22.85 ± 2.89	1.0752	0.383

[1]Values are mean ± SD; [2]One-way ANOVA; [3]χ^2 test. BMI: Body mass index; M/F: Male/female.

Table 3 Surgical data

	Overall	Group 1[5]	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9
Surgery time[1,4]	53.58 ± 30.08	82.45 ± 23.44[2]	59.00 ± 25.47	58.50 ± 21.16	55.00 ± 17.77	49.50 ± 12.45	45.50 ± 20.22	44.75 ± 18.67	45.25 ± 17.95	42.25 ± 13.52
Blood loss[1]	12.70 ± 11.60	16.65 ± 9.96	12.55 ± 13.45	14.05 ± 14.55	13.70 ± 9.76	12.90 ± 12.01	11.60 ± 12.31	12.70 ± 12.23	11.60 ± 11.10	10.55 ± 9.83
Complications[3]	8	3	1	2	0	1	0	0	1	0

[1]One-way ANOVA; [2]Values are mean ± SD; [3]χ^2 test; [4]Comparisons between groups were performed using ANOVA followed by Bonferroni's test; [5]The operation time in group 1 was significantly longer than that in the other groups (F = 7.257, P = 0.000).

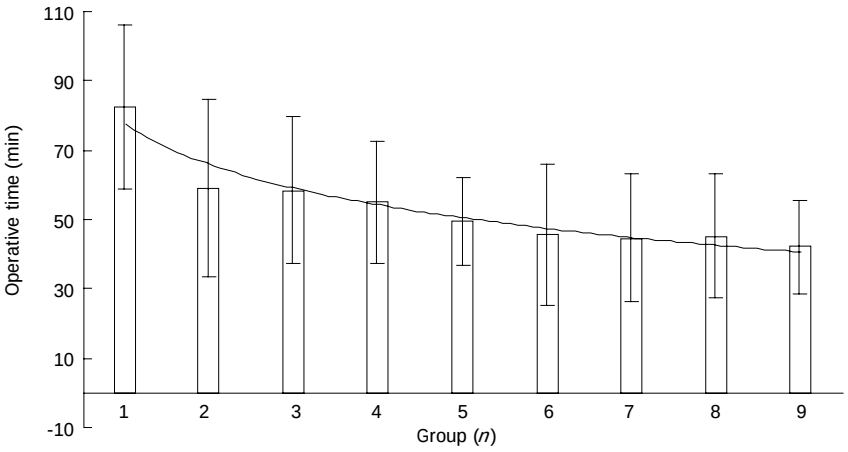

Figure 1 The changes in operation time.

were no significant differences between the groups in terms of complications and duration of hospitalization, suggesting that in the early period the operation was carried out very carefully and successfully. In all 180 patients, the outcomes were satisfactory and no patients required conversion to laparotomy or multi-port laparoscopy, and no patients required additional surgery. These results indicate that SILC is safe and feasible when performed by surgeons who are experienced in multi-port laparoscopic cholecystectomy.

The circumstances are different in different medical centers and endoscopy teams, thus, the surgical experience and concepts would also be different. As a result, the learning curves for SILC would be different[23-26]. Our results showed a rapid decline in operation time in the early period, and a slow stabilizing, declining curve in the late period, demonstrating that the learning curve was approximately 20 cases. That is, surgeons who are experienced in multi-port laparoscopic cholecystectomy are likely to pass the learning curve smoothly and safely after performing 20 cases of SILC.

COMMENTS

Background

The potential benefits of single-incision laparoscopic technology include reduced postoperative pain, improved cosmetic result and earlier return to normal life. Some investigators have predicted that single-incision laparoscopic cholecystectomy (SILC) may become an alternative standard approach for benign gallbladder diseases. However, there is still controversy with regard to its safety and reproducibility as SILC is more difficult to perform than multi-port laparoscopic cholecystectomy with higher technical demands.

Research frontiers

SILC is a rapidly advancing technique in laparoscopic surgery. However, there is limited evidence on the learning curve and practicality of performing this procedure. The present study retrospectively analyzed the surgical outcomes of transumbilical suture-suspension SILC performed by the same team in our department to investigate the learning curve of this technology, thereby guiding the surgeons to pass the initial learning period smoothly, safely, and quickly.

Innovations and breakthroughs

This is a large-scale retrospective study to explore the safety and reproducibility of SILC for the management of benign gallbladder diseases in selected patients.

Applications

The results confirmed that SILC was a safe and effective method for treating benign gallbladder diseases. There was a rapid decline in operation time in the early period, and a slow stabilizing, declining curve in the late period, demon-

strating that the learning curve was approximately 20 cases. That is, surgeons who are experienced in multi-port laparoscopic cholecystectomy are likely to pass the learning curve smoothly and safely after performing 20 cases of SILC.

Terminology

SILC is a minimally invasive surgical procedure in which cholecystectomy is accomplished exclusively through a single 15-25 mm incision in the patient's navel. Unlike the traditional multi-port laparoscopic approach, SILC leaves only a single small scar in the navel.

Peer review

This is an interesting article, which presents a series of SILC procedures. The study does demonstrate that there is learning curve for SILC.

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BRIEF ARTICLE

Stepwise sedation for elderly patients with mild/moderate COPD during upper gastrointestinal endoscopy

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Abstract

AIM: To investigate stepwise sedation for elderly patients with mild/moderate chronic obstructive pulmonary disease (COPD) during upper gastrointestinal (GI) endoscopy.

METHODS: Eighty-six elderly patients with mild/moderate COPD and 82 elderly patients without COPD scheduled for upper GI endoscopy were randomly assigned to receive one of the following two sedation methods: stepwise sedation involving three-stage administration of propofol combined with midazolam [COPD with stepwise sedation (group Cs), and non-COPD with stepwise sedation (group Ns)] or continuous sedation involving continuous administration of propofol combined with midazolam [COPD with continuous sedation (group Cc), and non-COPD with continuous sedation (group Nc)]. Saturation of peripheral oxygen (SpO_2), blood pressure, and pulse rate were monitored, and patient discomfort, adverse events, drugs dosage, and recovery time were recorded.

RESULTS: All endoscopies were completed successfully. The occurrences of hypoxemia in groups Cs, Cc, Ns, and Nc were 4 (9.3%), 12 (27.9%), 3 (7.3%), and 5 (12.2%), respectively. The occurrence of hypoxemia in group Cs was significantly lower than that in group Cc ($P < 0.05$). The average decreases in value of SpO_2, systolic blood pressure, and diastolic blood pressure in group Cs were significantly lower than those in group Cc. Additionally, propofol dosage and overall rate of adverse events in group Cs were lower than those in group Cc. Finally, the recovery time in group Cs was significantly shorter than that in group Cc, and that in group Ns was significantly shorter than that in group Nc ($P < 0.001$).

CONCLUSION: The stepwise sedation method is effective and safer than the continuous sedation method for elderly patients with mild/moderate COPD during upper GI endoscopy.

Key words: Upper gastrointestinal endoscopy; Adverse events; Sedation; Monitoring; Chronic obstructive pulmonary disease

Core tip: Many patients with chronic obstructive pulmonary disease (COPD) have to undergo upper gastroin-

testinal (GI) endoscopy because of digestive symptoms. A sedation method specially designed for elderly patients with COPD is urgently needed for use in clinical practice. In this study, we designed a new stepwise sedation method. Eighty-six elderly patients with COPD and 82 elderly patients without COPD scheduled for upper GI endoscopy were randomly assigned stepwise sedation or continuous sedation. The results indicate that the stepwise sedation method is effective and safer than the continuous sedation method for elderly patients with mild/moderate COPD during upper GI endoscopy.

Xu CX, Chen X, Jia Y, Xiao DH, Zou HF, Guo Q, Wang F, Wang XY, Shen SR, Tong LL, Cao K, Liu XM. Stepwise sedation for elderly patients with mild/moderate COPD during upper gastrointestinal endoscopy. *World J Gastroenterol* 2013; 19(29): 4791-4798 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i29/4791.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i29.4791

INTRODUCTION

Upper gastrointestinal (GI) endoscopy is a commonly used interventional examination method for reliable diagnosis of upper GI diseases. The incidence of chronic obstructive pulmonary disease (COPD) in Chinese urban populations over 40 years old is 8.2%[1]. Due to tobacco smoking, solid-fuel use, and other reasons, an estimated 65 million people will die of COPD in China between 2003 and 2033[2]. Many patients with COPD have to undergo upper GI endoscopy because of digestive symptoms. Due to inadequate experience with the application of sedation technology during endoscopy in past decades, patients typically underwent routine endoscopic procedures without sedatives, receiving only local pharyngeal lidocaine anesthesia before endoscopy. However, this methodology can lead to various adverse effects, including fear, nausea and vomiting, elevated blood pressure, angina, myocardial infarction, and even death. Moreover, the risk of adverse effects is even greater in elderly patients with cardiopulmonary diseases[3-6]. These issues give rise to patient reluctance to be examined and delay of diagnosis and treatment of alimentary system diseases.

In recent years endoscopy with sedation has become a popular option for both patients and gastroenterologists. Midazolam and propofol are generally used as sedatives during endoscopic procedures[7-10]. However, the use of sedatives during endoscopy in elderly patients with COPD remains controversial because of safety concerns[11]. Compared with younger patients, elderly patients exhibited a significant increase in risk of oxygen saturation below 90% and oxygen saturation decrease more than 5%[12]. Moreover, propofol is a potent depressant of airway reflexes at hypnotic concentrations[13]. Elderly patients with COPD usually have cough, phlegm and respiratory insufficiency, and are more likely to experience decreased saturation of peripheral oxygen (SpO_2) because of the dysfunction of the cough reflex and respiratory track blockage by phlegm during an endoscopic procedure with sedation. These symptoms commonly lead to a higher risk of adverse events during GI endoscopy. Therefore, a sedation method specially designed for elderly patients with COPD is urgently needed in clinical practice. Most episodes of hypoxemia under sedation occur in the five minute interval following medication administration and/or intubation, and less frequent administration of medications or diligent monitoring during this period might decrease hypoxemia[14].

In this study, we designed a stepwise sedation method that involves three-stage administration of propofol combined with midazolam so that the sedation depth is approached gradually. Through analysis of SpO_2, blood pressure, pulse rate, patient discomfort, adverse events, midazolam dosage, propofol dosage, and recovery time; we compared the efficacy and safety of this new method with the continuous sedation method during upper GI endoscopy in elderly patients with and without COPD.

MATERIALS AND METHODS

Patients

Eighty-six elderly patients with mild/moderate COPD and 82 elderly patients without COPD (> 70 years old) who underwent diagnostic upper GI endoscopic procedures using sedation at the endoscopic unit of Third Xiangya Hospital of Central South University between March 2011 and December 2012 were included in this study. COPD was diagnosed based on the guidelines for the diagnosis and treatment of COPD[15]. Two hundred sixty-one other patients were excluded because they did not consent to participate (n = 35) or because they had other diseases or excluding conditions (n = 226). These included hypertension (> 140/90 mmHg), hypotension (< 90/60 mmHg), sick sinus syndrome, neurologic or psychiatric disease, metabolic disease, liver/renal insufficiency, severe cough and sputum, SpO_2 < 90%, COPD class Ⅲ/Ⅳ, American Society of Anesthesiology (ASA) class Ⅳ/Ⅴ, and drug allergy. Thus, our study focused exclusively on mild/moderate COPD patients.

The eligible participants with and without COPD were randomized into the two treatment groups in equal numbers. Computer-generated randomization blocks were utilized. Sealed envelopes containing the treatment protocol were opened in the procedure room after enrollment in the study.

Figure 1 shows the flow of participants through the trial, and Table 1 lists data and clinical characteristics of the study population. The differences in COPD and ASA classification between COPD with stepwise sedation (group Cs) and COPD with continuous sedation (group Cc) were not significant, and ASA classification did not differ significantly between non-COPD with stepwise sedation (group Ns) and non-COPD with continuous sedation (group Nc). The differences in other factors among the four groups were not significant. Gastroenterologists

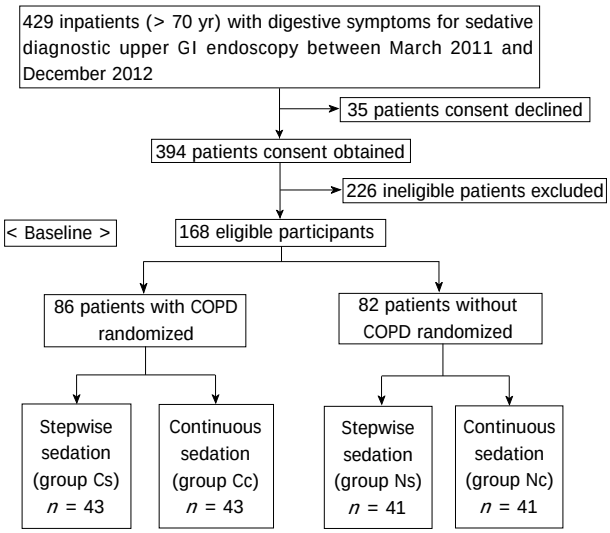

Figure 1 Flow of participants throughout the trial. GI: Gastrointestinal; COPD: Chronic obstructive pulmonary disease; Group Cs: COPD with stepwise sedation; Group Cc: COPD with continuous sedation; Group Ns: Non-COPD with stepwise sedation; Group Nc: Non-COPD with continuous sedation.

Table 1 Demographic data and clinical characteristics of the study population

Parameter	Group			
	Cs (*n* = 43)	Cc (*n* = 43)	Ns (*n* = 41)	Nc (*n* = 41)
Sex: male/female	35/8	34/9	30/11	30/11
Age: yr, mean ± SD	74.5 ± 2.5	74.2 ± 2.3	77.6 ± 4.6	76.7 ± 4.6
(range)	(71-80)	(71-80)	(71-88)	(71-90)
Weight: kg, mean ± SD	56.3 ± 6.4	56.8 ± 6.6	56.5 ± 7.0	57.2 ± 6.5
(range)	(45-70)	(48-71)	(46-73)	(47-71)
Alcohol consumption: Y/N	7/36	5/38	6/35	4/37
Smoking: Y/N	28/15	26/17	24/17	22/19
COPD classification				
I	28	30		
II	15	13		
ASA classification				
I	0	0	35	37
II	11	13	6	4
III	32	30	0	0
Major endoscopic findings				
Chronic superficial gastritis	14	11	13	15
Chronic atrophic gastritis	5	6	4	5
Gastric ulcer	6	5	7	4
Duodenal ulcer	8	7	9	6
Gastric cancer	3	4	2	3
Gastric polyp	2	3	2	4
Esophagus cancer	2	3	1	2
Reflux esophagitis	3	4	3	2

Group Cs: COPD with stepwise sedation; Group Cc: COPD with continuous sedation; Group Ns: Non-COPD with stepwise sedation; Group Nc: Non-COPD with continuous sedation; COPD: Chronic obstructive pulmonary disease; ASA: American Society of Anesthesiology; Y/N: Yes/no.

with specific expertise in GI endoscopy performed endoscopic procedures, and an anesthetist administered the sedatives. Before the procedure, the participants signed an informed consent form. Our institution's ethics committee approved this study.

Sedation procedure

All patients were given lidocaine *via* throat spray before the endoscopic procedure and received nasal oxygen insufflations at a rate of 2 L/min during the endoscopy. Both stepwise and continuous methods of sedation were used.

For the stepwise sedation (groups Cs and Ns), the following step-up sedation method was used: Step 1: Initial administration of an intravenous injection of midazolam (Nhwa Pharmaceutical Group Co. Ltd., Xuzhou, China; technical concentration of 5 g/L diluted to 0.25 g/L with normal saline) at 0.015 mg/kg (with a maximum dose of 1.0 mg). The Ramsay Sedation Scale score here was 1, and after a 3-5 min interval, a mouthpiece was placed in the patient's mouth for the start of step 2. Step 2: Administration of 15-40 mg propofol (AstraZeneca, Shanghai, China; technical concentration of 10 g/L diluted to 5 g/L with normal saline) *via* intravenous injection until the Ramsay Sedation Scale score was 2-3. The endoscope then was passed through the patient's throat. Step 3: Administration of an additional intravenous injection of propofol at 1.0 mg/kg per minute until the Ramsay Sedation Scale score was 5-6 (when retardation or loss of eyelash reflex was achieved)[16]. At this point, the endoscopic procedure was carried out.

For continuous sedation (groups Cc and Nc), the following technique was used: The patient was initially given an intravenous injection of midazolam at 0.015 mg/kg (with a maximum dose of 1.0 mg). Next, a mouthpiece was placed in the patient's mouth, and he/she received an intravenous injection of propofol at 1.0 mg/kg per minute that did not stop until the Ramsay Sedation Scale score reached 5-6, when the endoscopic procedure was carried out. If necessary, propofol was administered again to prevent the patient from experiencing discomfort during long-lasting endoscopic procedures.

Patient age, sex, weight, alcohol and cigarette consumption, COPD and ASA classification, major endoscopic findings, SpO_2, blood pressure, pulse rate, adverse events, dosage of midazolam and propofol, and recovery time were recorded. Recovery time was defined as the interval between the moment when propofol injection was stopped and when the patient could open his/her eyes in response to the doctor's call and answer questions.

Monitoring

Degree of pharyngeal malaise during the endoscopic procedure: The same anesthetist and endoscopist, each with more than 5 years of experience, and a registered nurse who had worked for 10 years in an endoscopy room, independently evaluated the degree of pharyngeal

Table 2 Pharyngeal malaise when passing the endoscope through the throat *n* (%)

Group	*n*	Scores of degree of pharyngeal malaise			
		0	1	2	3
Cs	43	33 (76.7)[a]	7 (16.3)	3 (7.0)	0
Cc	43	40 (93.0)	2 (4.7)	1 (2.3)	0
Ns	41	31 (75.6)[a]	6 (14.6)	4 (9.8)	0
Nc	41	38 (92.7)	2 (4.9)	1 (2.4)	0

Group Cs: Chronic obstructive pulmonary disease (COPD) with stepwise sedation; Group Cc: COPD with continuous sedation; Group Ns: Non-COPD with stepwise sedation; Group Nc: Non-COPD with continuous sedation. [a]$P < 0.05$ between Group Cs *vs* Group Cc and Group Ns *vs* Group Nc.

Table 3 SpO_2, blood pressure, and pulse rate during the endoscopy procedure (mean ± SD)

Group	Time	SpO_2 (%)	SBP (mmHg)	DBP (mmHg)	P (bpm)
Cs	Before	98.1 ± 1.6	126.0 ± 11.2	80.7 ± 6.7	74.4 ± 13.7
(*n* = 43)	During	95.8 ± 6.0[b]	115.6 ± 11.9[b]	72.8 ± 6.6[b]	73.8 ± 14.0
	After	98.0 ± 1.6	124.9 ± 11.3[b]	79.1 ± 6.5[b]	74.2 ± 14.1
Cc	Before	98.0 ± 1.8	128.5 ± 11.0	80.5 ± 5.6	77.2 ± 14.3
(*n* = 43)	During	90.7 ± 13.6[b]	113.5 ± 9.4[b]	71.0 ± 4.9[b]	75.0 ± 14.3[b]
	After	97.3 ± 2.6[b]	126.3 ± 11.0[b]	78.5 ± 5.8[b]	76.6 ± 14.5
Ns	Before	98.2 ± 1.8	130.2 ± 5.0	82.0 ± 4.1	73.7 ± 13.7
(*n* = 41)	During	96.5 ± 5.0[a]	120.0 ± 5.2[b]	74.3 ± 4.4[b]	73.0 ± 13.7
	After	98.1 ± 1.7	127.9 ± 5.0[b]	80.0 ± 4.3[b]	73.4 ± 13.6
Nc	Before	98.2 ± 1.8	127.8 ± 9.3	81.4 ± 2.6	70.4 ± 12.7
(*n* = 41)	During	95.6 ± 6.7[b]	117.1 ± 8.7[b]	73.5 ± 2.9[b]	69.0 ± 12.3[b]
	After	98.1 ± 1.7	125.5 ± 8.9[b]	79.0 ± 2.7[b]	70.4 ± 12.5

Group Cs: Chronic obstructive pulmonary disease (COPD) with stepwise sedation; Group Cc: COPD with continuous sedation; Group Ns: Non-COPD with stepwise sedation; Group Nc: Non-COPD with continuous sedation; SpO_2: Saturation of peripheral oxygen; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; P: Pulse rate. [a]$P < 0.05$, [b]$P < 0.01$ *vs* before the endoscopy.

malaise. Pharyngeal malaise was scored according to observations of the patient's discomfort and the effect of passing the endoscope through the throat, as follows: obvious nausea and vomiting, difficulty continuing the endoscopic procedure (3 points); nausea and vomiting, able to continue the endoscopic procedure (2 points); slight nausea and vomiting, no effect on the endoscopic procedure (1 point); no nausea or vomiting, easy to finish the endoscopic procedure (0 points).

SpO_2, blood pressure, and pulse rate: All patients were continuously monitored for SpO_2, blood pressure, and pulse rate using a multi-functional monitor. The SpO_2, blood pressure, and pulse rate were recorded before the procedure (1-2 min before the use of sedatives; this value, measured when the patient was resting on his/her side, was used as the base value), during the procedure (to identify the minimum value throughout the procedure), and after the procedure (1-2 min after the endoscopic procedure was completed). After the endoscopy, patients were continuously monitored in the recovery unit until mental ability and walking gait recovered to the normal level (usually within 30-60 min). Occurrences of adverse events were observed.

Statistical analysis

Data are presented as mean ± SD. Statistical analyses were performed using SPSS software (version 17.0 for Windows; SPSS Inc., Chicago, IL, United States). The measurement data were compared using the two-sample *t*-test for normally distributed data and the Mann-Whitney test for non-normally distributed data. The enumeration data were expressed as *n* (%) and compared using the χ^2 test. A two sided *P*-value < 0.05 was considered statistically significant.

RESULTS

Degree of pharyngeal malaise during the endoscopic procedure

All endoscopies were completed successfully. Degrees of pharyngeal malaise evaluated by the anesthetist, endoscopist, and nurse did not differ significantly. The degree of pharyngeal malaise when the endoscope was passing through the throat in group Cs was greater than that in group Cc, and that in group Ns also was greater than group Nc ($P < 0.05$) (Table 2). However, the discomfort disappeared after the endoscope passed through the throat and propofol was administered again. All patients had no pharyngeal malaise and no memory of this discomfort after endoscopy.

SpO_2, blood pressure, and pulse rate

The average SpO_2, systolic blood pressure (SBP), and diastolic blood pressure (DBP) in all four groups decreased during the endoscopic procedure, but the blood pressure in almost all patients remained within the normal range. Only one patient in group Cc exhibited hypotension, but it was transient, did not require any treatment, and returned to normal rapidly after the endoscopic procedure. The pulse rate changed significantly during the procedure in group Cc and group Nc (Table 3).

Adverse events

Table 4 lists the adverse events in the four treatment groups. The overall rate of adverse events in group Cs was lower than that in group Cc ($P < 0.01$), but the difference between group Ns and group Nc was not statistically significant.

Hypoxemia

Twenty-four patients (14.3%) in all four groups exhibited hypoxemia. The occurrence of hypoxemia in group Cs was significantly lower than that in group Cc (9.3% and 27.9%, respectively, $P < 0.05$). The average decrease in value of SpO_2 during the procedure in group Cs was significantly lower than that in group Cc ($P < 0.05$). For 22 patients with slight hypoxemia ($SpO_2 \geqslant 60\%$), SpO_2 quickly returned to normal after holding the mandible with two hands, patting him/her on the back, and increasing oxygen flow through the nasal catheter. For

Table 4 Adverse events in the four treatment groups *n* (%)

Adverse events	Group			
	Cs (*n* = 43)	Cc (*n* = 43)	Ns (*n* = 41)	Nc (*n* = 41)
Hypoxemia (SpO_2 < 90% for ⩾ 15 s)	4 (9.3)[a]	12 (27.9)	3 (7.3)	5 (12.2)
SpO_2 89%-80%	2 (4.7)	5 (11.2)	2 (4.9)	3 (7.3)
SpO_2 79%-60%	2 (4.7)	5 (11.2)	1 (2.4)	2 (4.9)
SpO_2 59%-40%	0 (0.0)	1 (2.3)	0 (0.0)	0 (0.0)
SpO_2 < 40%	0 (0.0)	1 (2.3)	0 (0.0)	0 (0.0)
Average decrease in value of SpO_2	2.3 ± 5.4[a]	7.3 ± 12.6	1.7 ± 4.3	2.6 ± 5.8
Hypotension (SBP < 90 mmHg or DBP < 60 mmHg)	0 (0.0)	1 (2.3)	0 (0.0)	0 (0.0)
Extent of SBP decrease				
⩽ 10	28 (65.1)	17 (39.5)	34 (82.9)	28 (68.3)
11-20	15 (34.9)[a]	26 (60.5)	7 (17.1)	13 (31.7)
> 20	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Average decrease in value of SBP	10.4 ± 3.3[b]	15.1 ± 6.8	10.2 ± 3.3	10.6 ± 4.5
Extent of DBP decrease				
⩽ 10	40 (93.0)	34 (79.1)	37 (90.2)	38 (92.7)
11-20	3 (7.0)	9 (20.9)	4 (9.8)	3 (7.3)
> 20	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Average decrease in value of DBP	7.9 ± 2.2[b]	9.5 ± 1.8	7.6 ± 2.6	8.0 ± 1.7
Tachycardia (> 100 bpm)	3 (7.0)	2 (4.7)	2 (4.9)	1 (2.4)
Bradycardia (< 60 bpm)	3 (7.0)	6 (14.0)	3 (7.3)	4 (9.8)
Decrease of pulse rate	33 (76.7)	36 (83.7)	29 (70.7)	28 (68.3)
Increase of pulse rate	8 (18.6)	6 (14.0)	8 (19.5)	8 (19.5)
No change of pulse rate	2 (4.7)	1 (2.3)	4 (9.8)	5 (12.2)
Average change in value of pulse rate	0.6 ± 2.8[a]	2.2 ± 2.8	0.7 ± 2.4	1.4 ± 2.5
Arrhythmias	2 (4.7)	1 (2.3)	1 (2.4)	0 (0.0)
Other adverse events	3 (7.0)	5 (11.6)	2 (4.9)	3 (7.3)
Extrapyramidal reactions	0 (0.0)	1 (2.3)	0 (0.0)	0 (0.0)
Somnolence	1 (2.3)	2 (4.7)	1 (2.4)	1 (2.4)
Dizziness	2 (4.7)	2 (4.7)	1 (2.4)	2 (4.9)
Overall rate of adverse events	15 (34.9)[b]	27 (62.8)	11 (26.8)	13 (31.7)

Group Cs: Chronic obstructive pulmonary disease (COPD) with stepwise sedation; Group Cc: COPD with continuous sedation; Group Ns: Non-COPD with stepwise sedation; Group Nc: Non-COPD with continuous sedation; SpO_2: Saturation of peripheral oxygen; SBP: Systolic blood pressure; DBP: Diastolic blood pressure. Overall rate of adverse events included hypoxemia, hypotension, tachycardia, bradycardia, arrhythmias and other adverse events. [a]$P < 0.05$, [b]$P < 0.01$ *vs* Group Cc.

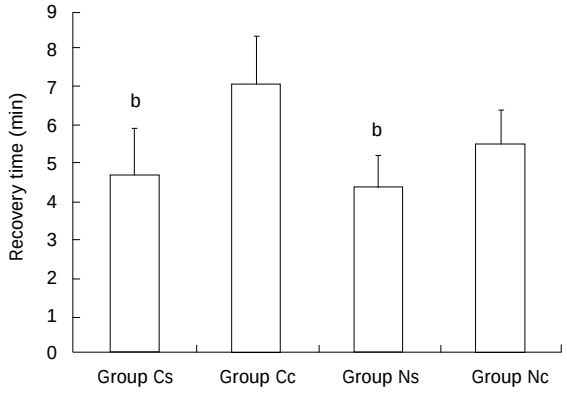

Figure 2 Recovery times of patients in the four groups. Group Cs: Chronic obstructive pulmonary disease (COPD) with stepwise sedation; Group Cc: COPD with continuous sedation; Group Ns: Non-COPD with stepwise sedation; Group Nc: Non-COPD with continuous sedation. [b]$P < 0.01$ between Group Cs *vs* Group Cc and Group Ns *vs* Group Nc.

these patients, the endoscopic procedure was continued and completed. For two patients with serious hypoxemia (SpO_2 < 60%), SpO_2 returned to normal after removing the endoscope, holding the mandible with two hands, pressing the chest, sucking out sputum, giving oxygen by mask, and intravenously injecting the benzodiazepine antagonist flumazenil (0.5 mg). The endoscopic procedure in these patients was restarted and completed after awakening, and with no obvious signs of discomfort during the endoscopic procedure.

Hypotension

Only one patient in group Cc had hypotension (88/60 mmHg). The average decreases in values of SBP and DBP in group Cs were significantly lower than those in group Cc ($P < 0.001$ for SBP and $P < 0.005$ for DBP).

Change of pulse rates

The pulse rates of patients in the four groups showed a slight increase (1-6 bpm), no change, or a slight decrease (1-7 bpm) during the procedure. The average change in value of pulse rate in group Cs was lower than that in group Cc ($P < 0.05$).

Other adverse events

During the injection with propofol, one patient in group Cc exhibited extrapyramidal signs (abnormal involuntary movement of limbs and opisthotonus), which disappeared after 30-60 s. Incidence of somnolence and dizziness did not differ significantly among the four groups,

and disappeared within 10-50 min after the endoscopic procedure ended.

Dosage of midazolam and propofol

Midazolam dosages in groups Cs, Cc, Ns and Nc were 0.6-1.0 mg (0.90 ± 0.12 mg, 0.90 ± 0.11 mg, 0.90 ± 0.11 mg, and 0.92 ± 0.10 mg, respectively), and differences in dosages were not statistically significant among the four groups. Propofol dosages in groups Cs, Cc, Ns and Nc were 53.3 ± 9.4 mg (30-80 mg), 58.9 ± 11.6 mg (40-80 mg), 64.8 ± 10.1 mg (40-80 mg), and 70.7 ± 9.6 mg (50 -88 mg), respectively. Propofol dosage in group Cs was significantly lower than that in group Cc ($P < 0.05$), and dosage in group Ns also was significantly lower than group Nc ($P < 0.01$).

Recovery time

The recovery times in groups Cs, Cc, Ns and Nc were 4.7 ± 1.2 min (2.5-8.0 min), 7.1 ± 1.2 min (3.5-10.0 min), 4.4 ± 0.8 min (2.5-6.0 min), and 5.5 ± 0.9 min (3.0-7.0 min), respectively. The recovery time in group Cs was significantly shorter than that in group Cc, and that in group Ns also was significantly shorter than group Nc ($P < 0.001$) (Figure 2). All patients were fully conscious and able to answer questions accurately within 10 min after endoscopy. They were able to walk normally when they left the endoscopic unit 30-60 min after endoscopy. The majority of patients reported no discomfort; only 12 patients had slight dizziness and somnolence, which disappeared within 10-50 min after the endoscopic procedure ended.

DISCUSSION

Both the number and complexity of endoscopic procedures have increased considerably due to the wide availability and application of sedation, but the best methods for sedation during endoscopy are still being debated[17]. Providing an adequate regimen of sedation is an art, as it influences the quality of the examination and patient and physician satisfaction with the sedation process[18]. Conscious sedation, a type of sedation in which the individual can respond to verbal directions, is used for GI endoscopy. Even with this sedation, patients can experience discomforts such as nausea and vomiting, which in some cases precludes completing the endoscopic procedure. Deep sedation may be preferred for procedures in which it is important for patients to remain immobile[19]. However, selection of the most suitable drug or combination of drugs for use and the safety of the sedation method for special patient groups, such as elderly individuals and patients with co-morbidities, are important issues that need to be resolved[11,20-22].

Martínez *et al*[23] reported that continuous propofol sedation during endoscopic procedures is as safe in elderly patients > 80 years as it is in younger patients. Our results also showed that differences in hypoxemia, hypotension, and all adverse events in non-COPD patients with continuous sedation were not statistically different from those in non-COPD patients with stepwise sedation. We have used continuous propofol combined with midazolam for deep sedation during upper GI endoscopy since 1999[24], but sedation for patients with respiratory diseases accompanied by cough, phlegm, and snoring has been limited, as these patients are prone to experience respiratory track blockage by phlegm and lingual root fall back under sedation, resulting in decreased SpO_2 and respiratory inhibition. Most episodes of hypoxemia during sedation for gastrointestinal endoscopy occur within 5 min of the administration of medication and/or intubation, and less frequent administration of medications or diligent monitoring during this period might decrease hypoxemia[14]. For this class of patients, we proposed using a new sedation method that would allow patients to gradually reach the appropriate sedation depth *via* administration of propofol combined with midazolam in three stages. When the Ramsay Sedation scale was 2-3, the endoscope was passed through the patient's throat. At this moment, the patients were conscious, and the procedure usually did not lead to cough reflex disappearance, lingual root fall back, or respiratory track blockage by phlegm. Therefore, this method reduced the decline of SpO_2 during the endoscopy.

In this study, the patients who underwent the stepwise sedation method also exhibited a smaller decrease in SBP, DBP, and pulse rate than patients who received continuous sedation. The new method, moreover, reduced the propofol dosage and the overall rate of adverse events. Elderly patients with COPD have much greater risk of experiencing cardiopulmonary abnormality during upper GI endoscopic procedures than elderly patients without COPD, thus use of the stepwise sedation method is safer for these patients. This method will contribute to the wider use of upper GI endoscopy in diagnosis and treatment of alimentary system diseases.

Midazolam and propofol are commonly used in sedative endoscopic procedures at doses of 0.05-0.1 mg/kg and 1-3 mg/kg, respectively. In our study, propofol combined with midazolam was used, and the midazolam dosage was decreased to less than 0.015 mg/kg (the range was 0.6-1.0 mg). Moreover, the required propofol dosage in the stepwise sedation was significantly lower than that in the continuous sedation (53.3 ± 9.4 mg and 58.9 ± 11.6 mg, respectively, for elderly patients with COPD). In addition, the concentration of midazolam was diluted 20 times and that of propofol was diluted two times, which resulted in reduced drug dosage, consistent with previous reports[25]. Drug dosage (which allows for control of degree of sedation) is one of the key factors for a successful upper GI endoscopy procedure. Generally, drug dosage is proportional to its adverse effects. In our study, the dosage of sedative was lower and the incidence of hypoxemia and the extent of decreased blood pressure were lower than those reported in the literature[26]. The overall rate of adverse events when the stepwise sedation was used for elderly patients with mild/moderate COPD was significantly lower than that when the continuous sedation was

used. The recovery time also was shorter for the stepwise sedation than the continuous sedation. There was no mortality in our study. Agostoni *et al*[27] reported that the endoscopic procedure resulted in 3 deaths in a total of 17999 patients (mortality rate = 0.017%).

In conclusion, the findings of this study showed that the stepwise sedation method reduced the propofol dosage and the extent of the drop in SpO_2 and blood pressure, and also decreased the incidence of hypoxemia and the overall rate of adverse events. Thus, this method was shown to be safer than the continuous sedation method in elderly patients with mild/moderate COPD during upper GI endoscopy.

COMMENTS

Background

The incidence of chronic obstructive pulmonary disease (COPD) in Chinese urban populations over 40 years old is 8.2%. Many patients with COPD have to undergo upper gastrointestinal (GI) endoscopy because of digestive symptoms. However, elderly patients with COPD usually have cough, phlegm, and respiratory insufficiency, and are more likely to experience decreased saturation of peripheral oxygen (SpO_2) because of dysfunction of the cough reflex and respiratory track blockage by phlegm during an endoscopic procedure with sedation. This oftentimes leads to a higher risk of adverse events during routine GI endoscopy in these patients. Therefore, a sedation method specially designed for elderly patients with COPD is urgently needed for use in clinical practice.

Research frontiers

In recent years endoscopy with sedation has become a popular option for both patients and gastroenterologists. Midazolam and propofol are generally used as sedatives during endoscopic procedures. However, propofol is a potent depressant of airway reflexes at hypnotic concentrations. It is critical to properly determine how to improve the safety of GI endoscopy by lowering the doses of sedatives.

Innovations and breakthroughs

Due to inadequate experience with the application of sedation technology during endoscopy in past decades, patients typically underwent routine endoscopic procedures without sedatives. This methodology can lead to various adverse effects, including fear, nausea and vomiting, elevated blood pressure, angina, myocardial infarction, and even death. These issues give rise to patient reluctance to be examined and delay of diagnosis and treatment of alimentary system diseases. We took the lead in using sedatives during endoscopic procedures in China in 1998. In the current study, we designed a new stepwise sedation method that involves three-stage administration of propofol combined with midazolam so that sedation depth is approached gradually. The results indicate that the stepwise sedation method is effective and safer than the continuous sedation method for elderly patients with mild/moderate COPD during upper GI endoscopy.

Applications

This stepwise sedation method can reduce drug dosage and the overall rate of adverse events in elderly patients with mild/moderate COPD during upper GI endoscopy with sedation. This will contribute to the wider use of upper GI endoscopy in diagnosis and treatment of alimentary system diseases.

Peer review

This is an interesting study and deserves attention, as the results are relevant to specific patients with COPD who need to undergo endoscopy examination. This manuscript presents new concepts and ideas. The authors analyzed the efficacy and safety of stepwise sedation for elderly patients with mild/moderate COPD during GI endoscopy. The results suggest that stepwise sedation is an effective and safe sedation method that can be used for elderly patients with mild/moderate COPD.

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META-ANALYSIS

Meta-analysis of stapled hemorrhoidopexy *vs* LigaSure hemorrhoidectomy

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Abstract

AIM: To compare outcome of stapled hemorrhoidopexy (SH) *vs* LigaSure hemorrhoidectomy (LH) by a meta-analysis of available randomized controlled trials (RCTs).

METHODS: Databases, including PubMed, EMBASE, the Cochrane Library, and the Science Citation Index updated to December 2012, were searched. The main outcomes measured were operating time, early postoperative pain, postoperative urinary retention and bleeding, wound problems, gas or fecal incontinence, anal stenosis, length of hospital stay, residual skin tags, prolapse, and recurrence. The meta-analysis was performed using the free software Review Manager. Differences observed between the two groups were expressed as the odds ratio (OR) with 95%CI. A fixed-effects model was used to pool data when statistical heterogeneity was not present. If statistical heterogeneity was present ($P < 0.05$), a random-effects model was used.

RESULTS: The initial search identified 10 publications. After screening, five RCTs published as full articles were included in this meta-analysis. Among the five studies, all described a comparison of the patient baseline characteristics and showed that there was no statistically significant difference between the two groups. Although most of the analyzed outcomes were similar between the two operative techniques, the operating time for SH was significantly longer than for LH ($P < 0.00001$; OR= -6.39, 95%CI: -7.68 - -5.10). The incidence of residual skin tags and prolapse was significantly lower in the LH group than in the SH group [2/111 (1.8%) *vs* 16/105 (15.2%); $P = 0.0004$; OR= 0.17, 95%CI: 0.06-0.45). The incidence of recurrence after the procedures was significantly lower in the LH group than in the SH group [2/173 (1.2%) *vs* 13/174 (7.5%); $P = 0.003$; OR= 0.21, 95%CI: 0.07-0.59].

CONCLUSION: Both SH and LH are probably equally valuable techniques in modern hemorrhoid surgery. However, LigaSure might have slightly favorable immediate postoperative results and technical advantages.

Key words: Stapled hemorrhoidopexy; Ligasure hemorrhoidectomy; Hemorrhoids; Meta-analysis

Core tip: Stapled hemorrhoidopexy (SH) and Ligasure hemorrhoidectomy are probably equally valuable techniques in modern hemorrhoid surgery. However, appropriate surgical techniques are important in SH, especially the placement of the purse-string suture. Its misplacement may cause operative and postoperative complications.

Yang J, Cui PJ, Han HZ, Tong DN. Meta-analysis of stapled hemorrhoidopexy *vs* LigaSure hemorrhoidectomy. *World J Gastroenterol* 2013; 19(29): 4799-4807 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i29/4799.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i29.4799

INTRODUCTION

Around 5% of the general population has hemorrhoidal disease to some extent, especially those aged > 40 years[1,2]. There is a vast number of available therapeutic methods, and hemorrhoidectomy is well established as the most effective and definitive treatment for grades 3 and 4 symptomatic hemorrhoidal disease[3]. Two well-established methods of hemorrhoidectomy, the open (Milligan-Morgan)[4] and closed (Ferguson)[5] techniques are especially popular. However, despite the relatively minor surgical trauma of these two methods, the intraoperative pain and protracted postoperative course are major concerns[6]. Thus, continuing efforts have been made to develop new techniques and modifications that promise a less painful course and faster recovery. Stapled hemorrhoidopexy [SH, also known as procedure for prolapse and hemorrhoids (PPH)] was introduced by Longo in 1998, and uses a specially designed circular stapling instrument to excise a ring of redundant rectal mucosa or expanded internal hemorrhoids[7]. Although some SH-related complications have been reported[8], its advantages, such as shorter operating time, less postoperative pain, and a quicker return to normal activity have been confirmed by several controlled studies[9-11]. Another new method, LigaSure hemorrhoidectomy (LH), uses the Ligasure vessel sealing system, which consists of a bipolar electrothermal hemostatic device that allows complete coagulation of vessels up to 7 mm in diameter with minimal surrounding thermal spread and limited tissue charring. The advantages of this method include simple and easy learning, excellent hemostatic control, minimal tissue trauma, lower postoperative pain, and shorter wound healing time[12-14].

Although meta-analysis of clinical trials has shown that SH and LH have some advantages over conventional hemorrhoidectomy[15], there is still a lack of evidence about the operative and postoperative outcomes of SH and LH. Therefore, we performed a meta-analysis of randomized controlled trials (RCTs) that compared the efficiency of SH and LH in treating hemorrhoidal disease.

MATERIALS AND METHODS

Literature search

Electronic databases, including PubMed (1966 to December 2012), EMBASE (1980 to December 2012), the Cochrane library (Issue 12, December 2012) and Science Citation Index (1975 to December 2012), were searched. Literature reference lists were hand-searched for the same time period. The search terms used were "Stapled hemorrhoidopexy or PPH and LigaSure hemorrhoidectomy".

Study selection

The initial inclusion criteria were as follows: (1) all originally published RCTs; (2) the treatment group underwent SH for hemorrhoidal disease; and (3) a parallel control group underwent LH for hemorrhoidal disease. Studies that met the initial inclusion criteria were then further examined. Those with duplicate publications, unbalanced matching procedures or incomplete data were excluded, in addition to abstracts without accompanying full texts.

Table 1 Quality analysis of included trials

Ref.	Randomization method	Allocation concealment	Blinding	Withdraws	Jadad score
Arslani *et al*[1]	Not mentioned	Adequate	No	Described	4
Basdanis *et al*[18]	Not mentioned	Adequate	No	Not mentioned	3
Chen *et al*[19]	Not mentioned	Adequate	No	Not mentioned	3
Kraemer *et al*[20]	Computer-generated	Adequate	No	Described	5
Sakr *et al*[21]	Computer-generated	Adequate	Single-blind	Described	5

Data extraction

Data were extracted independently by two reviewers (Yang J and Cui PJ) according to the prescribed selection criteria. Any disagreements were resolved by discussion between the two reviewers. The following data were extracted: baseline trial data (*e.g.*, sample size, mean age, gender, study protocol, grade of hemorrhoids, and follow-up time); operative and postoperative outcomes (operating time, early postoperative pain, postoperative urinary retention and bleeding, wound problems, gas or fecal incontinence, anal stenosis, length of hospital stay, residual skin tags, prolapse, and recurrence). When necessary, the corresponding authors were contacted to obtain supplementary information.

Study quality

The quality of the included trials was assessed using the Jadad composite scale[16] in addition to a description of an adequate method for allocation concealment[17]. Study quality was assessed independently by two authors (Yang J and Cui PJ), and any discrepancies in interpretation were resolved by consensus (Table 1).

Statistical analysis

The meta-analysis was performed using the free software Review Manager (Version 4.2.10, Cochrane Collaboration, Oxford, United Kingdom). Differences observed between the two groups were expressed as the OR with the 95%CI. A fixed-effects model was used to pool data when statistical heterogeneity was not present. If statistical heterogeneity was present ($P < 0.05$), a random-effects model was used.

RESULTS

The initial search identified 10 publications (Figure 1). After screening, seven RCTs were identified. Consequently, two trials were excluded from the pooled meta-analysis. We compared the conventional Ferguson technique with SH and LH and the other study was a duplicate publi-

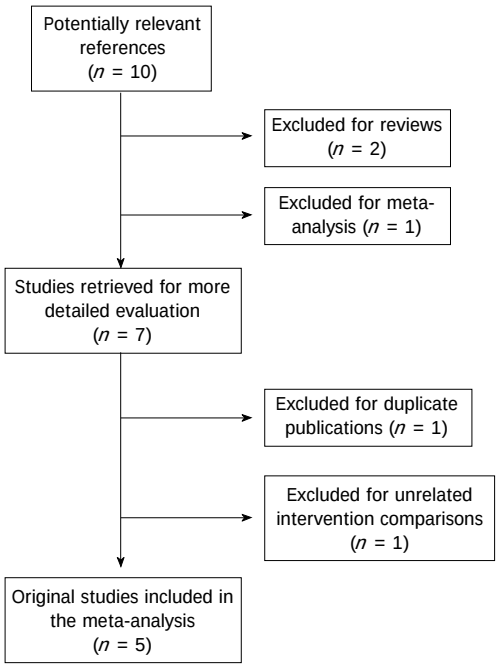


Figure 1 Search protocol for the meta-analysis.

cation. Five RCTs[1,18-21] published as full articles were included in this meta-analysis. All five studies described a comparison of the patient baseline characteristics and showed that there was no statistically significant difference between the two groups. The principal characteristics of the included studies are shown in Tables 2 and 3. The outcomes were measured as follows.

Operating time

Four trials reported the operating time during hemorrhoidectomy[18-21]. However, two of them only reported the average operating time[18,20]. The combined data showed that the operating time of SH was significantly longer than that of LH ($P < 0.00001$; OR = -6.39, 95%CI: -7.68 - -5.10) (Figure 2A).

Early postoperative pain

All five trials reported early postoperative pain at varied time points after hemorrhoidectomy[1,18-21] with a Visual Analog scale (VAS) score (0 indicating no pain and 10 severe pain). Two trials reported average VAS scores[1,18] and only one showed the trend in postoperative VAS scores[20]. Combined data from the other two trials showed that there was no difference between LH and SH ($P = 0.23$; OR = 1.24, 95%CI: -0.78 - -3.26) (Figure 2B).

Postoperative urinary retention

Four trials reported urinary retention[1,18,20,21] after the procedure and there was no significant difference between the LH and SH groups [11/156 (7.1%) *vs* 13/155 (8.4%); $P = 0.74$; OR = 0.87, 95%CI: 0.37-2.01) (Figure 2C).

Postoperative bleeding

All five trials reported postoperative bleeding[1,18-21]. There was no significant difference between the LH and SH groups [5/198 (2.5%) *vs* 12/199 (6%); $P = 0.08$; OR = 0.42, 95%CI: 0.16-1.11) (Figure 2D).

Wound problems

Four trials reported procedure-related wound problems, including irritation, itching and moisture[18-21]. There was no significant difference between the LH and SH groups [46/146 (31.5%) *vs* 12/153 (7.8%); $P = 0.3$; OR = 3.49, 95%CI: 0.33-37.32) (Figure 2E).

Postoperative gas or fecal incontinence

Four trials reported the incidence of postoperative gas or fecal incontinence[1,18,20,21]. There was no significant difference between the LH and SH groups [5/156 (3.2%) *vs* 7/155 (4.5%); $P = 0.55$; OR = 0.70, 95%CI: 0.22-2.24] (Figure 2F).

Postoperative anal stenosis

Three trials reported postoperative anal stenosis[1,20,21]. There was no significant difference between the LH and SH groups [3/111 (2.7%) *vs* 4/105 (3.8%); $P = 0.65$; OR = 0.71, 95%CI: 0.16-3.17] (Figure 2G).

Hospitalization

Four trials reported the length of hospital stay after hemorrhoidectomy[18-21]. However, two of them only reported the average time[18,20]. Combined data from the other two trials showed that there was no difference between LH and SH ($P = 0.44$; OR = 0.82, 95%CI: -1.27-2.91) (Figure 2H).

Residual skin tags and prolapse

Three trials reported residual skin tags and prolapse[1,20,21]. The data showed that the incidence of residual skin tags and prolapse was significantly lower in the LH group than in the SH group [2/111 (1.8%) *vs* 16/105 (15.2%); $P = 0.0004$; OR = 0.17, 95%CI: 0.06-0.45] (Figure 2I).

Recurrence

Four trials reported the incidence of recurrence after the procedures[1,18,19,21]. The data showed that the incidence of recurrence was significantly lower in the LH group than in the SH group [2/173 (1.2%) *vs* 13/174 (7.5%); $P = 0.003$; OR = 0.21, 95%CI: 0.07-0.59] (Figure 2J).

DISCUSSION

Hemorrhoid is one of the most common anorectal disorders[2]. Although accepted as the gold standard for surgical treatment of hemorrhoids, conventional hemorrhoidectomy has some unavoidable drawbacks. Two recent techniques, SH and LH, provide some advantages over conventional hemorrhoidectomy. However, there is still a lack of evidence focusing on outcomes of SH and LH.

Our meta-analysis showed that LH took significantly less time to complete compared with SH. For SH, a

Table 2 Baseline characteristics of included trials in the meta-analysis

Ref.	Year	Technique (*n*)	Study protocol	Mean age (yr)	Sex (M/F)	Grade of hemorrhoids	Follow-up time (mo)
Arslani *et al*[1]	2012	SH (46)	RUT	52 (17-72)	21/25	3	24
		LH (52)		50 (18-78)	23/29		
Basdanis *et al*[18]	2005	SH (50)	RUT	46 (25–72)	29/21	3 and 4	6-clinical, 18 (12–24)- telephone
		LH (45)		44 (22–69)	25/20		
Chen *et al*[19]	2007	SH (44)	RUT	25-81 (48)	26/18	3	6
		LH (42)		23-85 (46)	24/18		
Kraemer *et al*[20]	2005	SH (25)	RUT	58 (40–71)	14/11	3 and 4	1.5
		LH (25)		48 (28–82)	13/12		
Sakr *et al*[21]	2010	SH (34)	RBT	43.7 ± 4.66 (29–56)	21/13	3 and 4	18
		LH (34)		39.3 ± 4.68 (33–52)	19/15		

RUT: Randomized unblinded trial; RBT: Randomized blinded trial; SH: Stapled hemorrhoidopexy; LH: LigaSure hemorrhoidectomy.

special anal dilator was used to set an interrupted purse-string suture above the dentate line. Then the suture was tightened around the anvil of the circular stapler. In some patients with significant prolapse of the anal mucosa, two circular interrupted sutures were used. After removal of the stapler, interrupted stitches were usually inserted to control bleeding points. With regard to LH, the procedure was more convenient. The LigaSure instrument was used to grasp the base of the hemorrhoid and activated. After coagulation, the hemorrhoid skin was excised with scissors. The reduced operating time was related to better hemostatic control and lack of any need to ligate the pedicles. Our meta-analysis was in accordance with the results of a study showing that LH was comparatively simple and easy to learn[20]. However, the median value and standard deviation (SD) were reported only in two studies, so this variable should be investigated in further studies.

Another significant difference between the SH and LH groups in our meta-analysis was a higher frequency of postoperative residual skin tags, prolapse and recurrence with SH. This might have been because SH does not excise the hemorrhoids but rather a circumferential column of mucosa and submucosa 2-3 cm above the dentate line and then staples the defect. Besides, it does not deal with external hemorrhoids or associated anal canal problems[22-24]. However, patients with the third or fourth degree hemorrhoids usually present with large unequally sized prolapsing piles or circumferential hemorrhoids. Chen *et al*[25] proposed one modified method with one to four additional traction sutures placed at sites about 1 cm below the level of the purse-string suture for those prominent hemorrhoidal positions. This helped to incorporate more distal components of internal hemorrhoids into the "stapler housing" and facilitated further resection. It was also able to pull the external components or skin tags into the anal canal and made the anal surface smoother. An alternative is to remove the residual prolapsing hemorrhoidal tissue or skin tags during the operation or at the postoperative stage.

Long-term risk of recurrence, which is usually defined as recurring symptoms or new prolapse (but not residual prolapse or skin tags)[1], is the main concern of patients and surgeons. Some studies found that the residual prolapsed piles could cause recurrent symptoms[26,27], so it is understandable that recurrence was higher in the SH group. Our meta-analysis was in accordance with the findings of several studies that reported a high recurrence rate of 10%-53%[11,28,29]. SH is therefore considered by some authors to be unsuitable for grade 4 hemorrhoids[22,29]. On the contrary, LH is more appropriate for treating anatomical deformities such as skin tags and prolapse. Using LH, concomitant external hemorrhoid components and skin tags can be addressed, ensuring complete removal of the hemorrhoid tissues[30]. When severe external piles are dominant or large skin tags accompany hemorrhoid prolapse, LH will be a good choice[12-14]. Considering that the surgical principle in LH is more similar to that of conventional hemorrhoidectomy, it would be expected that LH would have lower recurrence rates[30]. However, the follow-up time did not exceed two years in our included trials, therefore, further studies with longer follow-up are needed.

One study showed that SH caused severe postoperative pain[31]. However, the results were challenged by several other studies[26,32,33]. To the best of our knowledge, the rectal wall is innervated by the sympathetic and parasympathetic nerves, thus, excising the rectal mucosa should be painless. So, it is inexplicable why pain is a common immediate complication of SH. Pain is usually caused by anal dilatation, which leads to internal sphincter fragmentation in some patients[34], and the inclusion of smooth muscle in the doughnut[31]. It is conceivable that SH is more technically demanding and operator dependent. If the purse-string suture is not at an inadequate level or depth, serious postoperative pain may be avoided[28]. VAS scores in the SH group were always lower in patients with no fibers included in the excised piles and doughnuts[18]. Considering the surgical similarity between LH and conventional hemorrhoidectomy, it would be expected that patients receiving LH would present with greater postoperative pain compared with SH, as is found with conventional hemorrhoidectomy. However in our meta-analysis, there was no difference between LH and SH regarding average VAS scores. The low level of postoperative pain with LH may result from the fact that LH has no need for anal dilatation, which reduces the possibility of anal spasm[35] and temporary third degree burn

Table 3 Characteristics of randomized comparisons of stapled hemorrhoidopexy and LigaSure hemorrhoidectomy reported in the literature

Ref.	Technique	Operation time (min)	Hospitalization (d)	Postoperative pain (Visual Analog score)	Parenteral analgesic use	Postoperative urinary retention	Postoperative bleeding	Return to normal activity or work	Incontinence for gas or stool after the operation	Postoperative anal stenosis	Residual skin tags and prolapse	Wound Problems	Recurrence
Arslani *et al*[1]	SH	NR	NR	3 (1-5)	36	1	3	3-4 wk	2	2	6	NR	5
	LH			3 (1-6)	41	2	1	2-4 wk	1	1	0		1
Basdanis *et al*[18]	SH	15 (8-17)	4 (2-10)	3 (1-6)	1	7	0	NR	1	NR	NR	6	3
	LH	13 (9.2-16.1)	5 (2-10)	6 (3-7)	0	5	1		2			39	0
Chen *et al*[19]	SH	19.0 ± 6.4	3.3 ± 1.1	3.1 ± 1.3	23	NR	4	NR	NR	NR	NR	4	1
	LH	12.0 ± 4.1	5.2 ± 1.4	5.4 ± 2.4	35		1					3	0
Kraemer *et al*[20]	SH	21 (6-54)	1.6 (1-2)	Only showed the trend	3.8 (2-12)	4	3	6.3 (1.5) d	0	0	0	2	NR
	LH	26 (10-80)	2.1 (2-3)	Only showed the trend	3.2 (1-8)	2	1	9.8 (1.9) d	0	1	0	1	
Sakr *et al*[21]	SH	26.9 ± 3.26	2.44 ± 0.504	5.29 ± 0.914	5.7 ± 0.855	1	2	8.65 ± 0.485 d	4	2	8	0	4
	LH	20.8 ± 3.35	2.21 ± 0.410	5.53 ± 1.02	5.0 ± 0.776	2	1	7.68 ± 0.638 d	2	1	2	1	1

NR: Not reported; LH: LigaSure hemorrhoidectomy SH: Stapled hemorrhoidopexy.

injury to nerve endings at the site of the wound[36]. However, there were some limitations to our data. The median value and SD were only reported in two studies, and oral and parenteral analgesia requirements were reported too inconsistently for quantitative analysis.

In our meta-analysis the occurrence of postoperative bleeding was equivalent in the two groups. LigaSure is a diathermy system and allows complete coagulation of blood vessels up to 7 mm in diameter, using a precise amount of bipolar energy and pressure that permanently changes collagen and elastin within the vessel wall. However, for SH, the expected frequency of bleeding was at least 50%[37]. In some circumstances, too much folded mucosa in the stapled line will increase the occurrence of inefficient hemostasis[38]. Therefore, interrupted stitches were needed to control bleeding points after removal of the stapler in almost all patients.

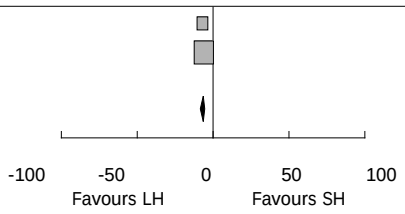
Early postoperative partial incontinence may be explained by pain that hinders voluntary sphincter contraction[39]. However potential anal sphincter injury may cause impairment of fecal continence. During SH, intraoperative sphincter stretching may play a role in postoperative fecal incontinence. The procedure requires insertion of a relatively large anal dilator, usually 33 mm, and placement of the circular stapler can lead to further sphincter injury[37], especially when excessive mucosal prolapse hampers the instrument encompassing all of the redundant tissue. Thus, in patients with pre-existing sphincter injury or with a narrow anal canal, modified techniques introduced by Ho *et al*[40] may minimize the risk of stretching the internal anal sphincter. They used the smaller Eisenheimer anal retractor instead of the circular anal dilator. Furthermore, if the deeper layers of the rectal wall are not included into the purse-string, making a mucosal instead of an all wall rectal layer anastomosis may reduced the incidence of SH-related postoperative incontinence and stenosis[38].Theoretically, intraoperative sphincter stretching was minimized when using the LigaSure system[41]. The system also had an effect on preservation of internal sphincter pressure[42]. However, in our meta-analysis, five cases of temporary gas incontinence and three of anal stenosis were encountered after LH, and one patient remained incontinent to gas for 1 mo[18]. Ramcharan *et al*[35] reported that after LH, the perianal skin, including the skin bridges, appeared scalded. At 3 mo follow-up, there was some mild circumferential fibrosis in the skin of the anal margin, which produced symptomatic anal stenosis that required once daily anal dilation with a 12-15-mm dilator for 3 mo. Therefore, occurrence of incontinence and stenosis with LH may be related to the device and technique. The LigaSure clamp may grasp the internal anal sphincter as well as the hemorrhoidal tissue above it. Thermal energy causes scalding that can contribute to anal stenosis. Similar mechanisms may result in injury to the anal sphincter, which may account for the fecal incontinence. To avoid this phenomenon, it is important: (1) to cut the anorectal margin with the cold knife before hemorrhoidectomy; (2) on the mucosal margin rather than on the cutaneous margin; and (3) to retract the cutaneous margin from bipolar blades before the sealing cycle begins[30,43]. When stenosis occurs, an early conservative approach with dilators will successfully treat this condition[43].

The prolonged hospital stays and delayed recovery usually related to the postoperative pain and wound problems. Our previous data showed that SH and LH have an equal effect in reducing postoperative pain. Although Basdanis *et al*[18] reported high occurrence of pruritus with LH immediately after the operation, our meta-analysis showed that

A

Study or subgroup	LH Mean	LH SD	LH Total	SH Mean	SH SD	SH Total	Weight	Mean difference IV, fixed, 95%CI
Chen 2007	12	4.1	42	19	6.4	44	32.6%	-7.00 (-9.26, -4.74)
Sakr 2010	20.8	3.35	34	26.9	3.26	34	67.4%	-6.10 (-7.67, -4.53)
Total (95%CI)			76			78	100.0%	-6.39 (-7.68, -5.10)

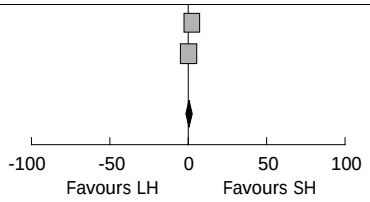
Heterogeneity: χ^2 = 0.41, *df* = 1 (*P* = 0.52); I^2 = 0%
Test for overall effect: *Z* = 9.71 (*P* < 0.00001)



B

Study or subgroup	LH Mean	LH SD	LH Total	SH Mean	SH SD	SH Total	Weight	Mean difference IV, random, 95%CI
Chen 2007	5.4	2.4	42	3.1	1.3	44	48.6%	2.30 (1.48, 3.12)
Sakr 2010	5.53	1.02	34	5.29	0.914	34	51.4%	0.24 (-0.22, 0.70)
Total (95%CI)			76			78	100.0%	1.24 (-0.78, 3.26)

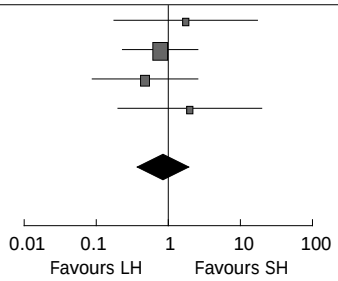
Heterogeneity: Tau^2 = 2.01; χ^2 = 18.39, *df* = 1 (*P* < 0.0001); I^2 = 95%
Test for overall effect: *Z* = 1.21 (*P* = 0.23)



C

Study or subgroup	LH Events	LH Total	SH Events	SH Total	Weight	Peto odds ratio Peto, fixed, 95%CI
Arslani 2012	2	52	1	46	13.4%	1.75 (0.18, 17.27)
Basdanis 2005	5	45	7	50	48.5%	0.77 (0.23, 2.58)
Kraemer 2005	2	25	4	25	24.7%	0.48 (0.09, 2.58)
Sakr 2010	2	34	1	34	13.4%	1.99 (0.20, 19.78)
Total (95%CI)		156		155	100.0%	0.87 (0.37, 2.01)
Total events	11		13			

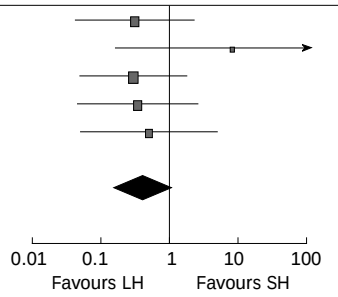
Heterogeneity: χ^2 = 1.38, *df* = 3 (*P* = 0.71); I^2 = 0%
Test for overall effect: *Z* = 0.33 (*P* = 0.74)



D

Study or subgroup	LH Events	LH Total	SH Events	SH Total	Weight	Peto odds ratio Peto, fixed, 95%CI
Arslani 2012	1	52	3	46	23.7%	0.31 (0.04, 2.30)
Basdanis 2005	1	45	0	50	6.1%	8.26 (0.16, 418.42)
Chen 2007	1	42	4	44	29.2%	0.30 (0.05, 1.80)
Kraemer 2005	1	25	3	25	23.1%	0.34 (0.05, 2.61)
Sakr 2010	1	34	2	34	17.9%	0.50 (0.05, 5.01)
Total (95%CI)		198		199	100.0%	0.42 (0.16, 1.11)
Total events	5		12			

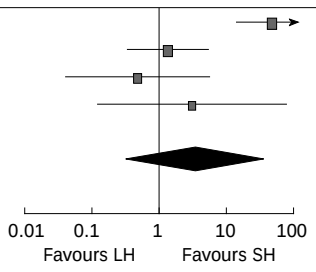
Heterogeneity: χ^2 = 2.50, *df* = 4 (*P* = 0.65); I^2 = 0%
Test for overall effect: *Z* = 1.75 (*P* = 0.08)



E

Ref.	LH Events	LH Total	SH Events	SH Total	Weight	Odds ratio M-H, random, 95%CI
Basdanis 2005	39	45	6	50	28.8%	47.67 (14.20, 159.99)
Chen 2007	5	42	4	44	28.2%	1.35 (0.34, 5.42)
Kraemer 2005	1	25	2	25	23.3%	0.48 (0.04, 5.65)
Sakr 2010	1	34	0	34	19.7%	3.09 (0.12, 78.55)
Total (95%CI)		146		153	100.0%	3.49 (0.33, 37.32)
Total events	46		12			

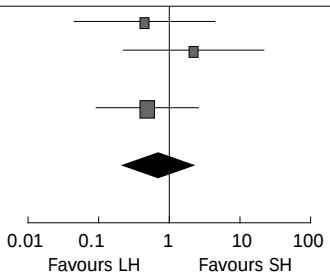
Heterogeneity: Tau^2 = 4.69; χ^2 = 19.80, *df* = 3 (*P* = 0.0002); I^2 = 85%
Test for overall effect: *Z* = 1.03 (*P* = 0.30)



F

Study or subgroup	LH Events	LH Total	SH Events	SH Total	Weight	Peto odds ratio Peto, fixed, 95%CI
Arslani 2012	1	52	2	46	25.7%	0.45 (0.05, 4.40)
Basdanis 2005	2	45	1	50	25.7%	2.21 (0.22, 21.80)
Kraemer 2005	0	25	0	25		Not estimable
Sakr 2010	2	34	4	34	48.7%	0.49 (0.09, 2.57)
Total (95%CI)		156		155	100.0%	0.70 (0.22, 2.24)
Total events	5		7			

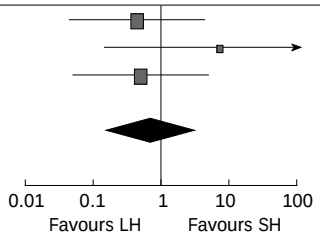
Heterogeneity: χ^2 = 1.30, *df* = 2 (*P* = 0.52); I^2 = 0%
Test for overall effect: *Z* = 0.60 (*P* = 0.55)



G

Study or subgroup	LH Events	LH Total	SH Events	SH Total	Weight	Peto odds ratio Peto, fixed, 95%CI
Arslani 2012	1	52	2	46	42.8%	0.45 (0.05, 4.40)
Kraemer 2005	1	25	0	25	14.6%	7.39 (0.15, 372.38)
Sakr 2010	1	34	2	34	42.6%	0.50 (0.05, 5.01)
Total (95%CI)		111		105	100.0%	0.71 (0.16, 3.17)
Total events	3		4			

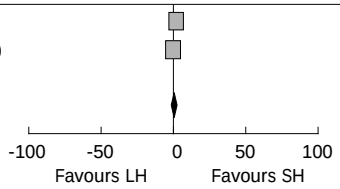
Heterogeneity: χ^2 = 1.62, *df* = 2 (*P* = 0.45); I^2 = 0%
Test for overall effect: *Z* = 0.45 (*P* = 0.65)



H

Study or subgroup	LH Mean	LH SD	LH Total	SH Mean	SH SD	SH Total	Weight	Mean difference IV, random, 95%CI
Chen 2007	5.2	1.4	42	3.3	1.1	44	49.3%	1.90 (1.37, 2.43)
Sakr 2010	2.21	0.41	34	2.44	0.504	34	50.7%	-0.23 (-0.45, -0.01)
Total (95%CI)			76			78	100.0%	0.82 (-1.27, 2.91)

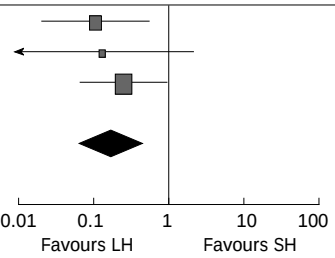
Heterogeneity: Tau^2 = 2.23; χ^2 = 52.40, *df* = 1 (*P* < 0.00001); I^2 = 98%
Test for overall effect: *Z* = 0.77 (*P* = 0.44)



I

Study or subgroup	LH Events	LH Total	SH Events	SH Total	Weight	Peto odds ratio Peto, fixed, 95%CI
Arslani 2012	0	52	6	46	34.8%	0.11 (0.02, 0.55)
Kraemer 2005	0	25	2	25	12.0%	0.13 (0.01, 2.14)
Sakr 2010	2	34	8	34	53.2%	0.25 (0.07, 0.95)
Total (95%CI)		111		105	100.0%	0.17 (0.06, 0.45)
Total events	2		16			

Heterogeneity: χ^2 = 0.68, *df* = 2 (*P* = 0.71); I^2 = 0%
Test for overall effect: *Z* = 3.56 (*P* = 0.0004)



J

Study or subgroup	LH Events	LH Total	SH Events	SH Total	Weight	Peto odds ratio Peto, fixed, 95%CI
Arslani 2012	1	52	5	46	39.7%	0.21 (0.04, 1.11)
Basdanis 2005	0	45	3	50	20.5%	0.14 (0.01, 1.42)
Chen 2007	0	42	1	44	7.0%	0.14 (0.00, 7.15)
Sakr 2010	1	34	4	34	32.9%	0.28 (0.05, 1.70)
Total (95%CI)		173		174	100.0%	0.21 (0.07, 0.59)
Total events	2		13			

Heterogeneity: χ^2 = 0.24, *df* = 3 (*P* = 0.97); I^2 = 0%
Test for overall effect: *Z* = 2.96 (*P* = 0.003)

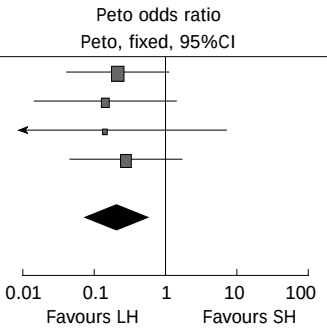


Figure 2 Comparison of outcome between LigaSure hemorrhoidectomy and stapled hemorrhoidopexy. A: Operating time; B: Early postoperative pain; C: Postoperative urinary; D: Postoperative bleeding; E: Wound problems; F: Postoperative gas or fecal incontinence; G: Postoperative anal stenosis; H: Hospitalization; I: Residual skin tags and prolapse; J: Recurrence. LH: LigaSure hemorrhoidectomy SH: Stapled hemorrhoidopexy.

wound problems did not differ significantly between the LH and SH groups. Therefore, as a consequence of reduced postoperative pain and tissue injury, it is understandable that there is no significant difference regarding the length of hospital stay between the two procedures. However, statistical heterogeneity was present, which may be a reflection of differences in hospital discharge protocols and the way in which the length of hospital stay was determined in these studies.

Our meta-analysis had several limitations. The small number of studies and the restricted sample size of most trials implied that the quantitative analysis was not very powerful. Moreover, the limited follow-up time of the included studies and different outcome measures considered may also have led to biased results. Large multicenter studies based on commonly accepted endpoints with long-term follow-up are warranted to compare better the results of these two different techniques of hemorrhoidectomy.

In conclusion, our meta-analysis supports that both SH and LH are probably equally valuable techniques in modern hemorrhoid surgery. However, LH might have slightly favorable immediate postoperative results and technical advantages.

COMMENTS

Background

Many clinical trials have shown that stapled hemorrhoidopexy (SH) and LigaSure hemorrhoidectomy (LH) have some advantages over conventional hemorrhoidectomy. However, there is still a lack of evidence comparing the clinical outcomes between SH and LH.

Research frontiers

Around 5% of the general population has hemorrhoidal disease to some extent, especially those > 40 years of age. There is a vast number of available therapeutic methods, but hemorrhoidectomy is well established as the most effective and definitive treatment for grades 3 and 4 symptomatic hemorrhoidal disease. SH and LH are new techniques that promise a less painful course and faster recovery.

Innovations and breakthroughs

Meta-analyses of clinical trials have shown that SH and LH have some advantages over conventional hemorrhoidectomy. There is still a lack of evidence focusing on the operative and postoperative outcomes of SH and LH. The present meta-analysis suggested that the operating time of SH was significantly longer than that of LH. The incidence of residual skin tags, prolapse and recurrence were significantly lower in LH than in SH.

Applications

The present meta-analysis showed that LH was more favorable than SH for patients with concomitant external hemorrhoid components and skin tags due to its slightly favorable technical advantages and immediate postoperative results, such as shorter operating time and lower occurrence of residual skin tags, prolapse and postoperative recurrence.

Terminology

SH (also known as PPH) was introduced by Longo in 1998, and uses a specially designed circular stapling instrument to excise a ring of redundant rectal mucosa or expanded internal hemorrhoids. LH uses the LigaSure vessel sealing system that consists of a bipolar electrothermal hemostatic device that allows complete coagulation of vessels up to 7 mm in diameter with minimal surrounding thermal spread and limited tissue charring.

Peer review

this study is very important meta-analysis for recently invented methods of treatment of hemorrhoid. This report is worthy for publication.

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META-ANALYSIS

FDG-PET in diagnosis, staging and prognosis of pancreatic carcinoma: A meta-analysis

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Author contributions: Wang Z, Chen JQ designed this study; Liu JL and Qin XG performed literature search; Liu JL and Huang Y extracted the data; Wang Z and Huang Y analyzed the data; Chen JQ and Qin XG interpreted the results; Wang Z drafted the manuscript; Chen JQ, Liu JL and Qin XG revised the paper; all authors read and approved the final manuscript to be published.

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Abstract

AIM: To investigate the potential role of positron emission tomography (PET) in the diagnosis, staging and prognosis predicting of pancreatic carcinoma (PC).

METHODS: A systematic review of relevant literatures in PubMed, Embase and Cochrane Library was performed. The sensitivity and specificity of diagnostic and staging studies, and HRs for prognosis predicting studies were pooled. The bivariate model was used for diagnostic studies and the random-effect model for prognostic studies. Heterogeneity between included studies was tested using χ^2 test, and subgroup analysis was performed to explain the heterogeneities. All of the calculations were performed using Stata version 11.0.

RESULTS: A total of 39 studies were included. The pooled sensitivity of PET in diagnosing PC (30 studies, 1582 patients), evaluating N stating (4 studies, 101 patients) and liver metastasis (7 studies, 316 patients) were 0.91 (95%CI: 0.88-0.93), 0.64 (95%CI: 0.50-0.76), and 0.67 (95%CI: 0.52-0.79), respectively; and the corresponding specificity was 0.81 (95%CI: 0.75-0.85), 0.81 (95%CI: 0.25-0.85), and 0.96 (95%CI: 0.89-0.98), respectively. In prognosis analysis (6 studies, 198 patients), significant difference of overall survival was observed between high and low standardized uptake value groups (HR = 2.39, 95%CI: 1.57-3.63). Subgroup analysis showed that PET/CT was more sensitive than PET alone in evaluating liver metastasis of PC, 0.82 (95%CI: 0.48-0.98) and 0.67 (95%CI: 0.52-0.79), respectively.

CONCLUSION: PET can be used as a valuable diagnostic and predictive tool for PC, but its effect in the staging of PC remains indeterminate.

Key words: Pancreatic carcinoma; Positron emission tomography; Diagnosis; Staging; Prognosis; Meta-analysis

Core tip: Positron emission tomography (PET) is an important tool for the diagnosis, staging and prognosis predicting of tumors. However, no consensus has been reached with regard to the role of PET in pancreatic carcinoma (PC) diagnosis. We performed meta-analysis of 39 included studies. The pooled results showed that PET can be used as a valuable diagnostic and predictive tool for PC, but its effect in the staging remains indeterminate. New tracers and PET scanning technology, as well as other parameters besides of standardized uptake value should be noticed in order to improve the diagnostic and predictive accuracy of PET in PC.

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INTRODUCTION

Pancreatic carcinoma (PC) is one of the leading causes of cancer death worldwide and is steadily increasing in incidence in most countries[1]. In industrialized countries, the incidence of PC ranks second after colorectal cancer among all gastrointestinal malignancies[1]. Despite recent significant advances in cancer diagnosis and treatment, the prognosis of PC remains extremely unfavorable with a reported 5-year survival rate of only 1%-10%[2,3]. For PC, surgery remains the only curative treatment, and the success depends on the stage of disease at diagnosis, but not the histological type[4]. Unfortunately, only 10%-15% of cancers are found to be resectable at the time of diagnosis for the late onset of the symptoms[5]. Therefore, to choose the most appropriate treatment and to avoid unnecessary surgical risk, timely diagnosis and staging is essential in the evaluation of patients with PC.

Although significant advances have been achieved in diagnostic technologies such as computed tomography (CT), endoscopic ultrasonography (EUS) and magnetic resonance imaging (MRI), the preoperative diagnosis and staging of PC remains suboptimal, which restricts the treatment planning of this disease[5]. The discrimination between inflammatory processes and PC, and the assessment of local resectability and distant metastases of the PC are still challenging with different imaging modalities[6]. Over the years, positron emission tomography (PET) has played an important role in oncology, especially for diagnosis, staging, and for evaluating the response to treatment and the prognosis of tumors[7]. However, there has been no consensus with regard to the role of PET in PC now. Some researchers held that PET could be used as a valuable measure in the diagnosis, staging and prognosis predicting of PC[8]; but others did not find enough evidences to justify the use of PET in PC[9]. Therefore, a systemic review aimed to evaluate the effect of PET in the diagnosis, staging and prognosis predicting of PC is urgently needed.

In this study, we assessed the pertinent literatures and conducted a meta-analysis to further investigate the potential role of PET in PC.

MATERIALS AND METHODS

Literature search

A systematic literature search was performed to identify studies assessing the effect of PET in the diagnosis, staging and prognosis predicting of PC. The PubMed, Embase and Cochrane Library databases were searched with the MeSH headings ("pancreatic neoplasms" and "tomography emission computed") and keywords ("pancreas or pancreatic neoplasms" or "pancreatic tumor/tumour" or "pancreatic cancer" or "PC" or "cancer of the pancreas") and (PET or "diffusion" or "weighted imaging"). The upper limit of search date was not limited, and the lower limit was December 2012. The language was not limited. In addition, reference lists from the included studies were hand searched.

Inclusion and exclusion criteria

Inclusion criteria for this meta-analysis were: (1) Studies assessing the effect of PET in the diagnosis, staging and prognosis predicting of PC. The participants were clinically suspected of PC, and diagnosed with PC by histology or follow-up exceeding 6 mo; (2) For diagnosis and staging, the results were judged with histopathology or clinical follow-up exceeding 6 mo; (3) For diagnosis and staging, true-positive (TP), false-positive (FP), true-negative (TN), and false-negative (FN) results of imaging methods could be calculated for per-patient; for prognosis, HRs and their 95%CIs for overall survival (OS) data were available or able to be calculated from original articles; (4) For eligible studies with data published more than once, we only included the articles with the largest sample size of patients; and (5) PET was performed with intravenous administration of ^{18}F-FDG.

Exclusion criteria for this meta-analysis were: (1) studies included patients with non-primary PC in staging or prognosis analysis (*e.g.*, metastatic cancer); (2) primary data were confounding and not able to be analyzed; (3) for staging, studies included patients who received radiotherapy or chemotherapy preoperatively, which may cause downstaging because neo-adjuvant protocols can lead to tumor downstaging and affect the diagnostic accuracy of imaging; (4) vitro studies and animal experiments; (5) Studies with a sample size less than 10; and (6) papers were not original research in type (*e.g.*, review articles).

Data extraction and quality assessment

Two authors extracted data using pre-defined tables, which included the following items: authors and publication time, country, study design, participants, sample size, quality score, and outcomes (TP, FP, TN and FN for diagnosis and staging analysis; HRs and their 95%CIs of OS for prognosis analysis). Follow-up period was recorded for prognosis analysis.

For diagnosis and staging, nine items of QUADAS closely related to this study were used to assess the methodological quality of eligible studies (the other five items of QUADAS were not related to this test)[10]. For prognosis, four items (closely related to this study) from previous literatures were selected as the quality standard[11]. Each item was described as Yes (high quality), Unclear, or No (low quality).

Statistical analysis

For diagnosis and staging analysis, the calculation was

based on max standardized uptake value (SUV), and pooled estimates of sensitivity and specificity of PET (with corresponding 95%CIs) were analyzed using the bivariate model[12], which was considered as a more valid statistical model for diagnostic meta-analysis[13,14]. The bivariate model uses a random effect approach for both sensitivity and specificity, which allows for heterogeneity beyond chance as a result of clinical and methodological differences between studies. To graphically present the results, we plotted the hierarchical summary receiver operating characteristic (HSROC) curves[13]. As a concern for meta-analysis of diagnostic trials, publication bias was tested using the funnel plot and Deeks test[15], which was conducted by a regression of diagnostic log OR against 1/sqrt (effective sample size), weighting by effective sample size, with $P < 0.1$ for the slope coefficient indicating significant asymmetry.

For prognosis analysis, HRs and their CIs for OS were retrieved from each primary study. In case they were not directly reported in primary literatures, we derived them from the survival curves using published method[16,17]. Kaplan-Meier curves of included studies were read by Engauge Digitizer version 2.11 (free software downloaded from http://sourceforge.net). HR calculation spreadsheet was freely downloaded from http://www.trialsjournal.com/content/supplementary/1745-6215-8-16-s1.xls. HRs for OS were pooled using a random-effect model.

Heterogeneity between included studies was tested using χ^2 test ($P < 0.1$ was considered significant). If heterogeneities were present, subgroup analysis was attempted to explain them.

All of the calculations were performed using Stata version 11.0. All *P* values were two-sided and all CIs had a two-sided probability coverage of 95%.

RESULTS

Study selection and description

According to the search strategy, a total of 629 papers were selected: 362 in PubMed, 216 in EMBASE, 37 in Cochrane Library and 14 by hand search. After browsing the titles and abstracts, we found that many studies were irrelevant and some were identified in more than one database; and 103 articles remained for potential inclusion and full texts were obtained. After screening the full text, 64 articles were excluded. The main reasons for exclusion were: nonclinical trials (such as review articles), repetitive publication, incomplete data, and inappropriate reference standard. At last, 39 studies were eligible for inclusion[18-56]. The number of studies evaluating primary tumor diagnosis, N staging, liver metastasis and prognosis was 30[18-21,25,32-56], 4[18-21], 7[19-25] and 6[26-31], respectively. The study selection process is summarized in Figure 1. The characteristics of included studies are listed in Tables 1-4. And the quality of included studies is shown in Figure 2.

Meta-analysis

In the diagnosis of primary tumors, 30 studies (1582 patients) were eligible for meta-analysis[18-21,25,32-56]. The pooled sensitivity and specificity of PET in the diagnosis of PC were 0.91 (95%CI: 0.88-0.93) and 0.81 (95%CI: 0.75-0.85), respectively.

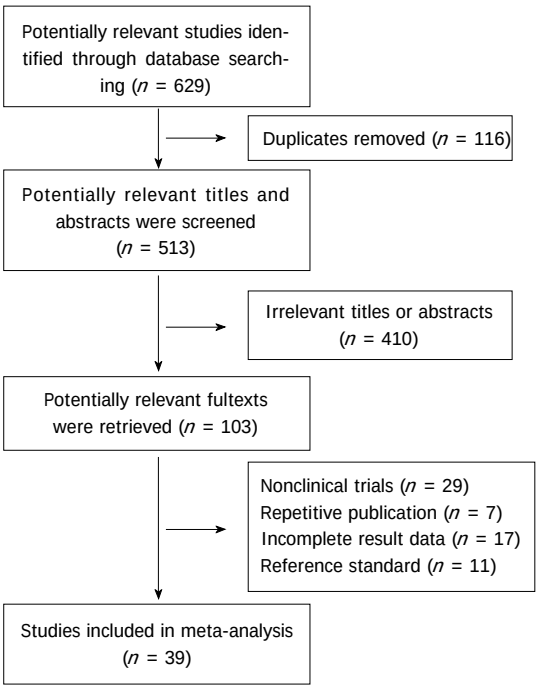

Figure 1 QUORUM flow chart for studies.

For lymph node metastasis, 4 studies (101 patients) were eligible for meta-analysis[18-21]. The pooled sensitivity, specificity and negative predictive value of PET in the diagnosis of N staging were 0.64 (95%CI: 0.50-0.76), 0.81 (95%CI: 0.25-0.85), and 0.65 (95%CI: 0.28-0.90), respectively.

For liver metastasis, 7 studies (316 patients) were eligible for meta-analysis[19-25]. The pooled sensitivity and specificity of PET in the diagnosis of liver metastasis were 0.67 (95%CI: 0.52-0.79) and 0.96 (95%CI: 0.89-0.98), respectively.

For predicting the prognosis, 6 studies (198 patients) were eligible for meta-analysis[26-31]. In the study by Nakata *et al*[29], the data about resectable and unresectable tumors were reported separately. The pooled HR for OS was 2.39 (95%CI: 1.57-3.63), which suggested that patients in low SUV group had a significant longer OS than patients in high SUV group (Figure 3).

Subgroup analysis

The *P* values of heterogeneity test for the meta-analysis were all less than 0.1. Considering that the results might be influenced by the study design and imaging method, we performed subgroup analysis according to the design and imaging method of included studies. The results of subgroup analysis are listed in Table 5.

HSROC curves

We plotted HSROC curves to graphically present the results of diagnosis and staging (Figure 4). In HSROC curves, the index test's sensitivity (TP rate) was plotted on the *y* axis against 1-specificity (FN rate) on the *x* axis.

Table 1 Characteristics of included studies for diagnosis

Ref.	Study design	Imaging	Population	*n* (M/F)	Results			
					TP	FP	FN	TN
Stollfuss *et al*[32]	NR	PET	Suspected PC or CP	73 (54/19)	41	3	2	27
Wang *et al*[33]	NR	PET	Pancreatic mass	40 (27/13)	26	3	1	10
Rose *et al*[34]	R	PET	Suspected PC	65 (NR)	48	2	4	11
Kauhanen *et al*[18]	P	PET	Suspected PC	38 (19/19)	17	3	1	17
Herrmann *et al*[35]	P	PET	Suspected PC or CP	41 (27/14)	30	4	3	4
		PET/CT		31 (NR)	24	5	1	1
Nakamoto *et al*[36]	P	PET	Suspected PC	47 (31/16)	22	3	5	17
Friess *et al*[37]	P	PET	Suspected PC or CP	74 (57/17)	41	4	1	28
Keogan *et al*[38]	P	PET	Suspected PC	37 (22/15)	22	2	3	10
Koyama *et al*[39]	NR	PET	Suspected PC	86 (50/36)	53	4	12	17
Nishiyama *et al*[19]	NR	PET	Suspected PC	86 (64/22)	49	11	6	20
Inokuma *et al*[40]	P	PET	Suspected PC	46 (25/21)	33	2	2	9
Bares *et al*[20]	P	PET	Suspected PC	40 (25/15)	25	2	2	11
Van *et al*[41]	NR	PET	Suspected PC or CP	109 (65/44)	29	10	3	67
Zimny *et al*[42]	P	PET	Suspected PC	106 (NR)	63	5	11	27
Kato *et al*[43]	NR	PET	Patients with PC or CP	24 (20/4)	14	2	1	7
Ruf *et al*[21]	R	PET	Suspected PC	32 (20/12)	14	10	1	7
Rasmussen *et al*[44]	P	PET	Suspected PC	20 (12/8)	9	1	3	7
Delbeke *et al*[45]	R	PET	Suspected PC	65 (33/32)	52	3	0	10
Farma *et al*[46]	R	PET/CT	Suspected PC	82 (43/39)	58	2	7	15
Borbath[25]	R	PET	Suspected PC	59 (29/30)	42	5	6	6
Sendler *et al*[47]	P	PET	Suspected PC	42 (21/21)	22	4	9	7
Bang *et al*l[48]	NR	PET	Suspected PC	102 (76/26)	90	2	3	7
Papós *et al*[49]	NR	PET	Suspected PC	22 (13/9)	6	2	0	14
Rajput *et al*[50]	R	PET	Suspected PC	11 (NR)	8	0	1	2
Ho *et al*[51]	NR	PET	Suspected PC	14 (7/7)	8	2	0	4
Mertz *et al*[52]	P	PET	Suspected PC	35 (NR)	27	2	4	2
Takanami *et al*[53]	R	PET/CT	Suspected PC	16 (13/3)	7	0	2	7
Sperti *et al*[54]	P	PET	Suspected PC	64 (33/31)	24	1	2	37
Tann *et al*[55]	R	PET	Suspected PC	30 (16/14)	4	8	3	15
		PET/CT		30 (16/14)	6	2	1	21
Bares *et al*[56]	NR	PET	Suspected PC	15 (11/4)	12	0	1	2

M: Male; F: Female; NR: Not report; R: Retrospective study; P: Prospective; PC: Pancreatic carcinoma; CP: Chronic pancreatitis; TP: True-positive; FP: False-positive; FN: False-negative; TN: True-negative; PET: Positron emission tomography; CT: Computed tomography.

Table 2 Characteristics of included studies for N staging

Ref.	Study design	Imaging method	Population	*n* (M/F)	Results			
					TP	FP	FN	TN
Kauhanen *et al*[18]	P	PET	Histologically proved PC	8 (NR)	2	0	5	1
Nishiyama *et al*[19]	NR	PET	PC diagnosed by histology or follow-up	55 (NR)	14	1	6	34
Bares *et al*[20]	P	PET	Histologically proved PC	23 (NR)	10	2	3	8
Ruf *et al*[21]	R	PET	PC diagnosed by histology or follow-up	15 (9/6)	8	2	5	0

M: Male; F: Female; NR: Not report; R: Retrospective study; P: Prospective; PC: Pancreatic carcinoma; TP: True-positive; FP: False-positive; FN: False-negative; TN: True-negative; PET: Positron emission tomography.

Additionally, the 95%CI and a 95% prediction region around the pooled estimates were plotted to illustrate the precision with which the pooled values were estimated (confidence ellipse of a mean) and to show the between-study variation (prediction ellipse; the likely range of values for a new study)[13].

Publication bias

Because the included studies for staging and prognosis were too few (less than 10), we explored publication bias using the data of PET/CT in the diagnosis of primary tumors, which included 30 studies. As a result, the funnel plot seemed symmetrical with a *P* value of 0.11, which suggested a low risk of publication bias (Figure 5).

DISCUSSION

In recent years, PET imaging has been increasingly used to identify and stage PC, and also utilized as a prognostic indicator. However, the value of PET in the management

Table 3 Characteristics of included studies for liver metastasis

Ref.	Study design	Imaging	Population	*n* (M/F)	Results			
					TP	FP	FN	TN
Strobel *et al*[22]	R	PET	Histologically proved PC	50 (25/25)	5	0	6	39
		PET/CT		50 (25/25)	9	1	2	38
Nakamoto *et al*[23]	NR	PET	Histologically proved PC	34 (22/12)	11	2	1	20
Nishiyama *et al*[24]	NR	PET	Histologically proved PC	42 (26/16)	10	3	3	26
Nishiyama *et al*[19]	NR	PET	PC diagnosed by histology or follow-up	55 (NR)	11	0	7	37
Bares *et al*[20]	P	PET	Histologically proved PC	23 (NR)	4	1	3	15
Ruf *et al*[21]	R	PET	PC diagnosed by histology or follow-up	15 (9/6)	3	2	5	5
Borbath *et al*[25]	R	PET	PC diagnosed by histology or follow-up	47 (NR)	10	1	2	34

M: Male; F: Female; NR: Not report; R: Retrospective study; P: Prospective; PC: Pancreatic carcinoma; TP: True-positive; FP: False-positive; FN: False-negative; TN: True-negative; PET: Positron emission tomography.

Table 4 Characteristics of included studies for prognosis

Ref.	Study design	Imaging method	Population	*n* (M/F)	Follow-up period	HR (95%CI)
Sperti *et al*[26]	R	PET	Histologically proved PC	60 (34/26)	NR	3.96 (1.92-8.17)
Maisey *et al*[27]	P	PET	Histologically proved PC	11 (7/4)	NR	3.4 (2.01-5.73)
Zimny *et al*[28]	NR	PET	Histologically proved PC	52 (33/19)	NR	2.27 (1.69-3.05)
Nakata *et al*[29]	NR	PET	Histologically proved PC	37 (21/16)	NR	0.93 (0.70, 1.25)[1] 4.9 (1.19-20.2)[2]
Maemura *et al*[30]	NR	PET	PC diagnosed by histology or follow-up	24 (NR)	NR	2.1 (1.5-2.92)
Nakata *et al*[31]	NR	PET	Histologically proved PC	14 (NR)	6-17 mo	2.99 (2.25-3.97)

[1]Patients received operation; [2]Patients did not receive operation. M: Male; F: Female; NR: Not report; R: Retrospective study; P: Prospective; PC: Pancreatic carcinoma; PET: Positron emission tomography.

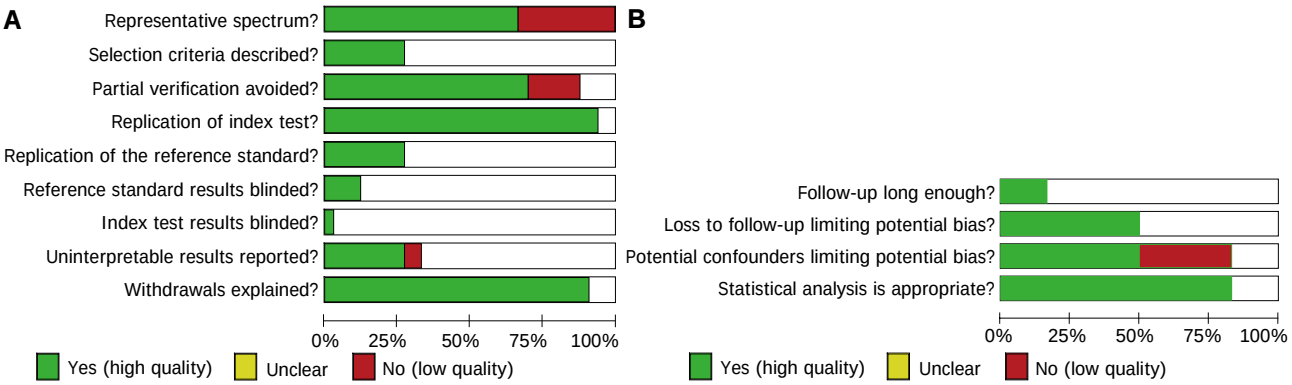

Figure 2 Methodological quality graph. A: Diagnosis and staging studies; B: Prognosis studies. Authors' judgments about each methodological quality item presented as percentages across all included studies.

of PC remains indeterminate. In our study, we collected existing data to assess the value of PET in the diagnosis, staging and prognosis predicting of PC. We found that PET could be used as a valuable diagnostic and predictive tool for PC; but for staging, PET has a moderate sensitivity and a relatively high specificity (Table 5).

Clinically, the diagnostic pathway for detection and staging of PC usually starts with abdominal ultrasound (US) followed by CT or MRI of the upper abdomen. However, even combined diagnostic approaches are limited by a low sensitivity for the detection of small lesions (a diameter of less than 2 cm) and for differentia-

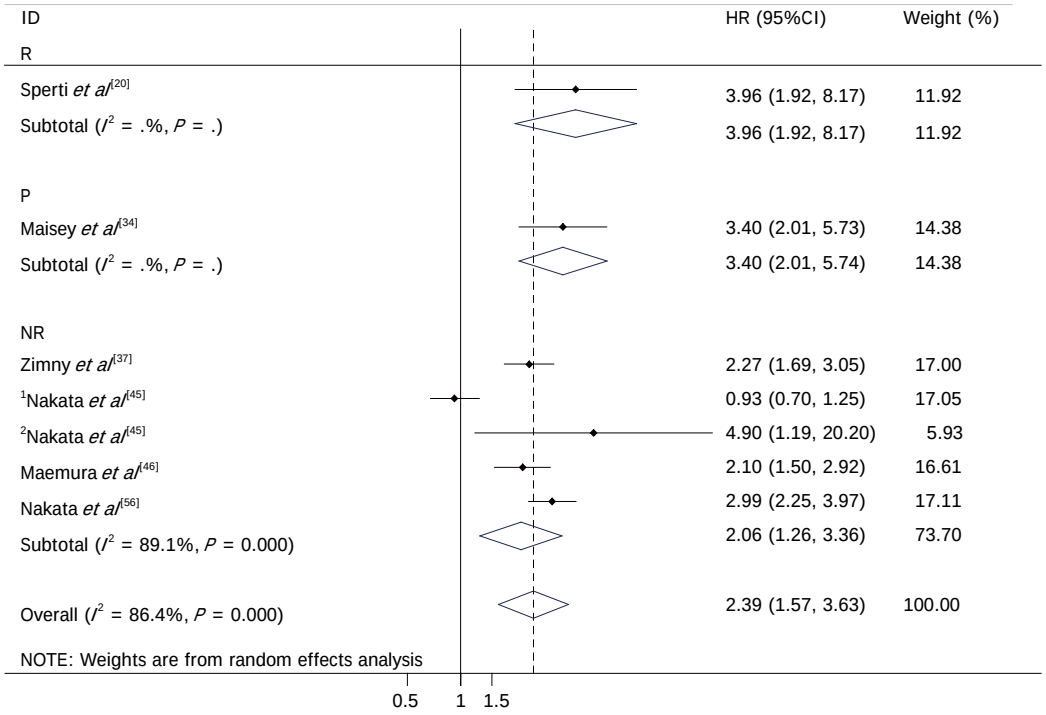

Figure 3 Forrest plot for the prognosis of pancreatic carcinoma. [1]Patients received operation; [2]Patients did not receive operation.

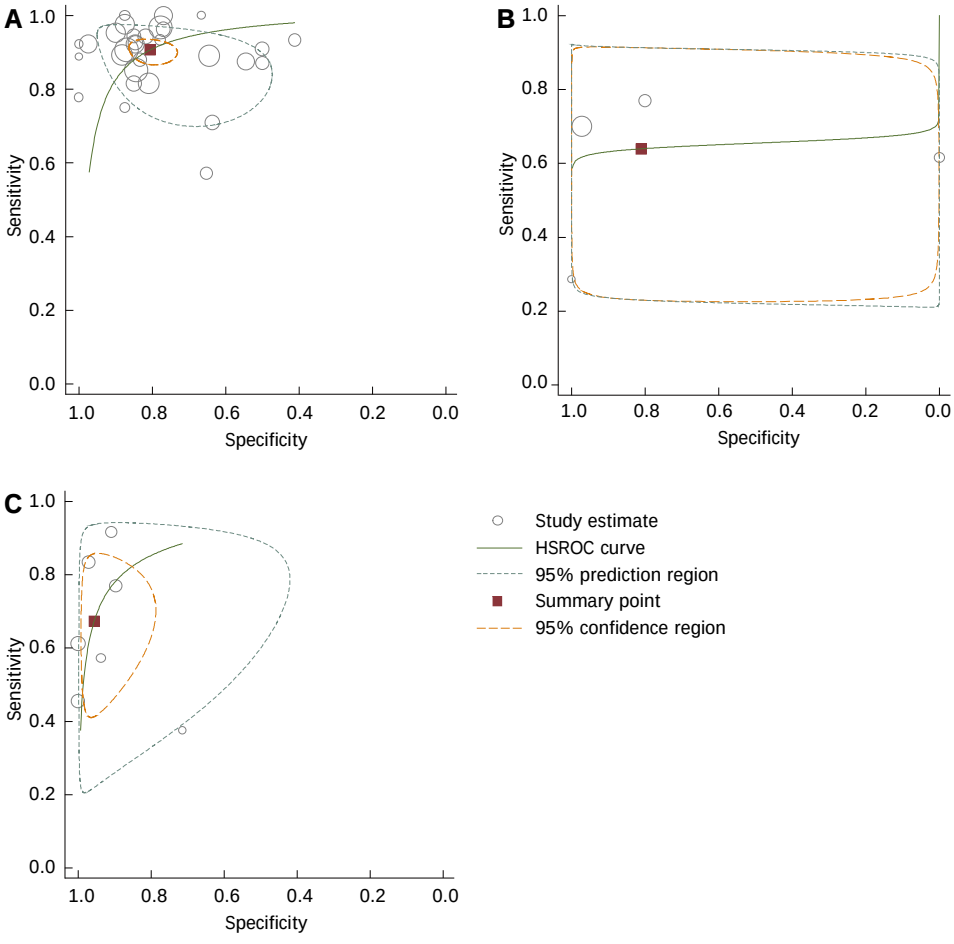

Figure 4 Hierarchical summary receiver operating characteristic curve. A: For the diagnosis of pancreatic carcinoma; B: For N staging of pancreatic carcinoma; C: For liver metastasis of pancreatic carcinoma. HSROC: Hierarchical summary receiver operating characteristic.

Table 5 Results of meta-analysis

Groups	Diagnosis		N staging			Liver metastasis		Prognosis
	Sen (95%CI)	Spe (95%CI)	Sen (95%CI)	Spe (95%CI)	Pv- (95%CI)	Sen (95%CI)	Spe (95%CI)	HR (95%CI)
Overall	0.91 (0.88-0.93)	0.81 (0.75-0.85)	0.64 (0.50-0.76)	0.81 (0.25-0.85)	0.65 (0.28-0.90)	0.67 (0.52-0.79)	0.96 (0.89-0.98)	
P subgroup	0.89 (0.84-0.92)	0.84 (0.76-0.89)	0.56 (0.15-0.90)	0.79 (0.48-0.94)	-	0.57 (0.21-0.88)	0.94 (0.68-0.99)	2.39 (1.57-3.63)
R subgroup	0.90 (0.83-0.95)	0.75 (0.58-0.87)	0.61 (0.32-0.85)	0.17 (0.04-0.81)	-	0.56 (0.28-0.81)	0.94 (0.65-0.99)	3.40 (2.01-5.74)
NR subgroup	0.93 (0.88-0.96)	0.82 (0.74-0.87)	0.70 (0.46-0.87)	0.97 (0.84-0.99)	-	0.74 (0.52-0.88)	0.92 (0.83-0.96)	3.96 (1.92-8.17)
PET subgroup	0.91 (0.88-0.93)	0.80 (0.74-0.85)	-	-	-	0.67 (0.52-0.79)	0.96 (0.89-0.98)	2.06 (1.26-3.36)
PET/CT subgroup	0.90 (0.79-0.95)	0.85 (0.38-0.98)	-	-	-	0.82 (0.48-0.98)	0.97 (0.87-1.00)	-

N: Lymph node; P: Prospective; R: Retrospective; NR: Not reporting; Sen: Sensitivity; Spe: Specificity; Pv-: Negative predictive value; PET: Positron emission tomography; CT: Computed tomography.

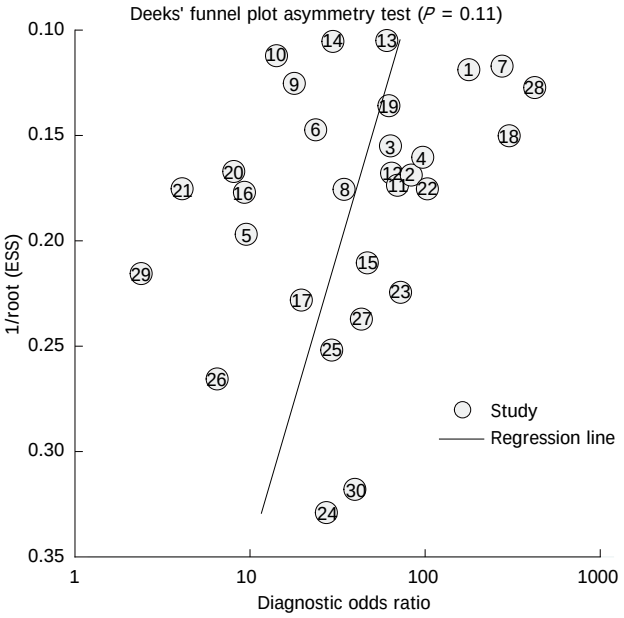

Figure 5 Funnel plot based on the data of positron emission tomography/computed tomography for the diagnosis of pancreatic carcinoma.

tion between malignant and benign lesions[57]. Recently, promising results in the diagnostic value of PET as a diagnostic and staging tool in PC have been reported[19-21]. In our review, we found PET had an acceptable sensitivity and specificity [sensitivity: 0.91 (95%CI: 0.88-0.93); specificity: 0.81 (95%CI: 0.75-0.85)] in the diagnosis of PC, which demonstrated that PET was valuable in the diagnosis of PC. This result was consistent with previous reports[18,22,32,33]. Considering that diagnostic accuracy might be influenced by study design and the usage of CT, we conducted subgroup analysis. However, the other confounding factors (such as tumor diameter, serum glucose and C-reactive protein levels) were not considered because of incomplete data in included studies. This reduced the reliability of our results to some extent, although the impact of these factors on diagnostic accuracy is indeterminate [19,34].

Because the only curative treatment for PC is surgery, accurate staging is necessary to properly select patients (surgical resection benefits only those patients with localized disease). Previous studies reported that both PET and CT were poor for N staging, although the diagnostic accuracy of PET was a little higher than CT[58]. This is consistent with our study (Table 5). As for liver metastasis, the value of PET is still controversial[7]. In our study, we found a sensitivity of 0.67 (95%CI: 0.52-0.79) for PET in detecting liver metastasis of PC. This suggests that the value of PET in assessing liver metastasis of PC remains indeterminate, although it has a relatively high specificity [0.96 (95%CI: 0.89-0.98)]. Recently, studies found that combined PET/CT could improve detection rates in the staging of PC[3]. In our study, we found that combined PET/CT was more sensitive than PET alone in assessing liver metastasis (82% *vs* 67%, Table 5), this confirmed the previous findings. Efforts have been made to to improve the diagnostic accuracy of PET in PC. It has been found that delayed PET scanning helped differentiate malignant lesions from benign ones, and new tracers such as ^{18}F fluorothymidine (FLT) could improve the diagnostic accuracy[19,59]. However, these findings need to be further validated.

Patients with PC usually have extremely poor prognosis among gastrointestinal malignancies. With conventional imaging modalities, it is often difficult to predict the prognosis of patients with PC preoperatively. Recently, studies found that the metabolic activity of the pancreas tumor, measured by PET usually through SUV, seemed to be useful in evaluating the prognosis of PC[29]. This result was consistent with ours, which suggested that patients with a higher SUV were associated with worse prognosis (HR = 2.39, 95%CI: 1.57-3.63). Additionally, the result did not change in the subgroup analysis (Table 5). This demonstrated that what we found was reliable. However, some researchers considered that the usage of SUV for prognostic assessment had some serious limitations (besides tumor characteristics, absolute value of SUV can also be influenced by several institution-dependent factors)[60]. And they found that SUVmax difference (between pre- and post-treatment scans) or the usage of relative values (such as the retention index) allowed more accurate prognostic evaluation[60,61]. Of course, more studies are needed to confirm these findings in the future.

In this study, we designed a systematic search strategy, selected studies according to the strict inclusion criteria, assessed the methodological quality using uniform criteria, and performed subgroup analysis in the presence of heterogeneity. Thirty-nine studies were included. These increased the reliability of the results to some extent.

However, several concerns must also be addressed when interpreting the pooled results. First, clinical follow-up was used as the reference standard in most of the included studies. Although the follow-up period was long enough, it might not correctly classify the target condition in some cases, which would affect the accuracy of the results. Second, some parameters (such as tumor diameter, glucose and C-reactive protein levels) which would affect the accuracy of the results were not considered in our study because of incomplete data, we failed to perform subgroup analysis or meta-regression, which might find out other possible causes of heterogeneity. Finally, publication bias was not tested because the few number of included studies in evaluating the staging and prognosis of PC may induce potential bias.

In conclusion, PET can be used as a valuable diagnostic and predictive tool for PC, but its effect in the staging of PC remains unclear. New tracers and PET scanning technology, as well as other parameters of PET besides SUV, should be noticed in order to improve the diagnostic and predictive accuracy of PET in PC.

COMMENTS

Background

Pancreatic carcinoma (PC) is one of the leading causes of cancer death worldwide and is steadily increasing in incidence in most countries. In industrialized countries, the incidence of PC ranks second after colorectal cancer among all gastrointestinal malignancies. Although significant advances have been achieved in diagnostic technologies, the preoperative diagnosis and staging of PC remains suboptimal.

Research frontiers

Over the years, positron emission tomography (PET) has played an important role in oncology, especially in the diagnosis, staging and prognosis prediction of tumors. However, there is no consensus with regard to the role of PET in PC now.

Innovations and breakthroughs

PET had an acceptable sensitivity and specificity [sensitivity: 0.91 (95%CI: 0.88-0.93); specificity: 0.81 (95%CI: 0.75-0.85)] in the diagnosis of PC. And higher standard uptake value measured by PET was associated with worse prognosis of PC patients (HR = 2.39, 95%CI: 1.57-3.63). However, the accuracy of PET in evaluating N staging and liver metastasis of PC was unsatisfied. This article gives them a comprehensive update based on previous studies.

Applications

PET can be used as a valuable diagnostic and predictive tool for PC, but its effect in the staging of PC remains indeterminate.

Peer review

Based on previous studies, this study evaluated the comprehensive role of PET in PC, including the diagnosis, staging and prognosis prediction. The authors found that PET can be used as a valuable diagnostic and predictive tool for PC, but its effect in the staging of PC remains indeterminate. The study is well designed, methodologically correct, elaborately prepared and full of significance in the field.

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CASE REPORT

Intrahepatic endometriosis as differential diagnosis: Case report and literature review

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Abstract

Intrahepatic endometriosis is one of the rarest forms of atypical endometriosis; only eighteen cases have been reported in the English literature. We describe the case of a 32-year-old woman, who presented with persistent, non-cyclical upper right quadrant abdominal pain, a central liver cyst, and no history of endometriosis. Three years previous, she was diagnosed with an intrahepatic cyst. The lesion progressed and two laparoscopic deroofing-operations were performed, yet the diagnosis of intrahepatic endometriosis was never reached. She presented in our clinic with further progress of the cyst as well as obstruction of the intrahepatic biliary system. The magnetic resonance imaging showed a 9.5 cm × 12 cm, lobulated intrahepatic cyst. We performed an ultrasonic pericystectomy. Immunostaining confirmed intrahepatic endometriosis. Only one of the previously described eighteen patients with intrahepatic endometriosis presented with cyclical pain in the upper right abdominal quadrant accompanying menstruation. This lack of a "typical" clinic makes it challenging to diagnose extragonadal endometriosis without histopathology. A previous history of endometriosis was described in only twelve cases, thus the diagnosis of this condition should not be limited to patients with a known history of endometriosis. Six of 18 patients were postmenopausal, demonstrating this condition is not limited to women of reproductive age. A preoperative diagnosis was only reached in seven of the previously described cases, highlighting the importance of preoperative biopsies. Yet due to the potential adverse effects, a transhepatic biopsy must be discussed individually. Although rare, intrahepatic endometriosis should always be considered as a differential diagnosis in women with recurrent hepatic cysts, regardless of age or previous medical history. In such cases, histology is essential and a pericystectomy should be performed as standard of care.

Key words: Intrahepatic endometriosis; Atypical endometriosis; Extrapelvic endometriosis; Hepatic cysts

Core tip: We describe the case of a 32-year-old woman who presented with non-cyclical upper right quadrant abdominal pain and a central liver cyst. Upon ultrasonic pericystectomy, the patient was diagnosed with intrahepatic endometriosis. The lack of "typical" clinical symptoms makes it challenging to diagnose extragonadal endometriosis without histopathology. Only eighteen cases of intrahepatic endometriosis have been reported in the literature, with only one reporting cyclical pain. Six of the eighteen patients were postmenopausal, and in twelve cases a previous history of endometriosis was described. Intrahepatic endometriosis should always be considered as a differential diagnosis in women of any age.

Fluegen G, Jankowiak F, Zacarias Foehrding L, Kroepil F, Knoefel WT, Topp SA. Intrahepatic endometriosis as differential diagnosis: Case report and literature review. *World J Gastroenterol* 2013; 19(29): 4818-4822 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i29/4818.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i29.4818

INTRODUCTION

Endometriosis, a common clinical condition most commonly noted in the pelvis, is found in approximately 6%-10% of women of reproductive age[1] and approximately 2.5% of postmenopausal women[2]. Atypical endometriosis, when the condition is found in extrapelvic regions[3], is rare. Endometrial tissue deposits have been described in almost all organs of the human body, and even very rarely in males[4]. Intrahepatic endometriosis is one of the rarest forms of atypical endometriosis. Since the first description of intrahepatic endometriosis in 1986[5], only 18 cases have been reported in the English literature[5-20]. We describe the case of a 32-year-old woman, who presented with persistent upper right quadrant abdominal pain and a central liver cyst. We discuss the occurrence of intrahepatic endometriosis in review of the previously published eighteen cases and summarize the proposed pathogenesis of this rare condition.

CASE REPORT

A 32-year-old woman, nulligravida, nullipara, consulted our hospital with constant right upper quadrant abdominal tenderness. She had no history of endometriosis. Three years previous, she was diagnosed with an intrahepatic cyst in segment Ⅳ. The lesion was closely monitored and upon progression two laparoscopic deroofing-operations, one in combination with a cholecystectomy, were performed. Due to further progression of the cyst with obstruction of the intrahepatic biliary system and resulting jaundice, multiple endoscopic retrograde cholangio pancreaticography were performed and on three occasions transhepatic drains were applied. As the patient presented at our clinic, the preoperative magnetic resonance imaging showed a 9.5 cm × 12 cm, lobulated cyst in segments Ⅳ, Ⅴ and Ⅷ (Figure 1). The serology for echinococcal disease and the tumor markers (carcinoembryonic antigen, carbohydrate antigen 19-9, α-fetoprotein) were normal, as was the routine lab work. We performed an ultrasonic pericystectomy and were able to remove the cyst with minimal damage to the surrounding liver tissue. We did not detect any other abnormalities during the operation. The patient was discharged on the eleventh postoperative day.

Histopathology analysis revealed a lobulated cyst, filled with old blood and detritus. While the surrounding liver tissue was normal, immunostaining of the cyst showed strong coloring for estrogen and progesterone receptor as well as CK7, which proved the epithelial origin of the cyst. Since no atypical cells were detected, the diagnosis of a benign intrahepatic endometriosis was confirmed (Figure 2).

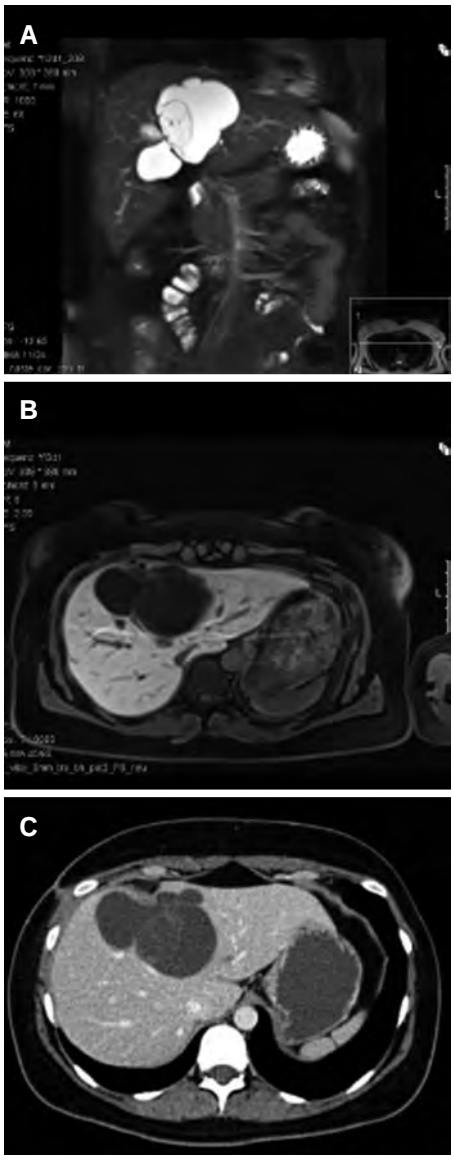

Figure 1 Radiographic images of the central liver cyst. Magnetic resonance imaging (MRI) demonstrated a well-defined lobulated cystic lesion without enhancement after administration of liver-specific contrast material (gadoxetic acid) in the arterial, venous or delayed phase. A: MRI, T2, coronary reconstruction; B: MRI, T1, transversal; C: Contrast-enhanced computed tomography scan, transversal.

DISCUSSION

Only 18 cases of this rare form of endometriosis are reported in the English literature (Table 1). Further, only one (5.5%) of these patients presented with cyclical pain in the upper right abdominal quadrant accompanying menstruation. In all other cases, patients presented with tenderness or pain, even jaundice, but no obvious connection to the menstrual cycle. This lack of a "typical" clinic makes it difficult to diagnose extragonadal endo-

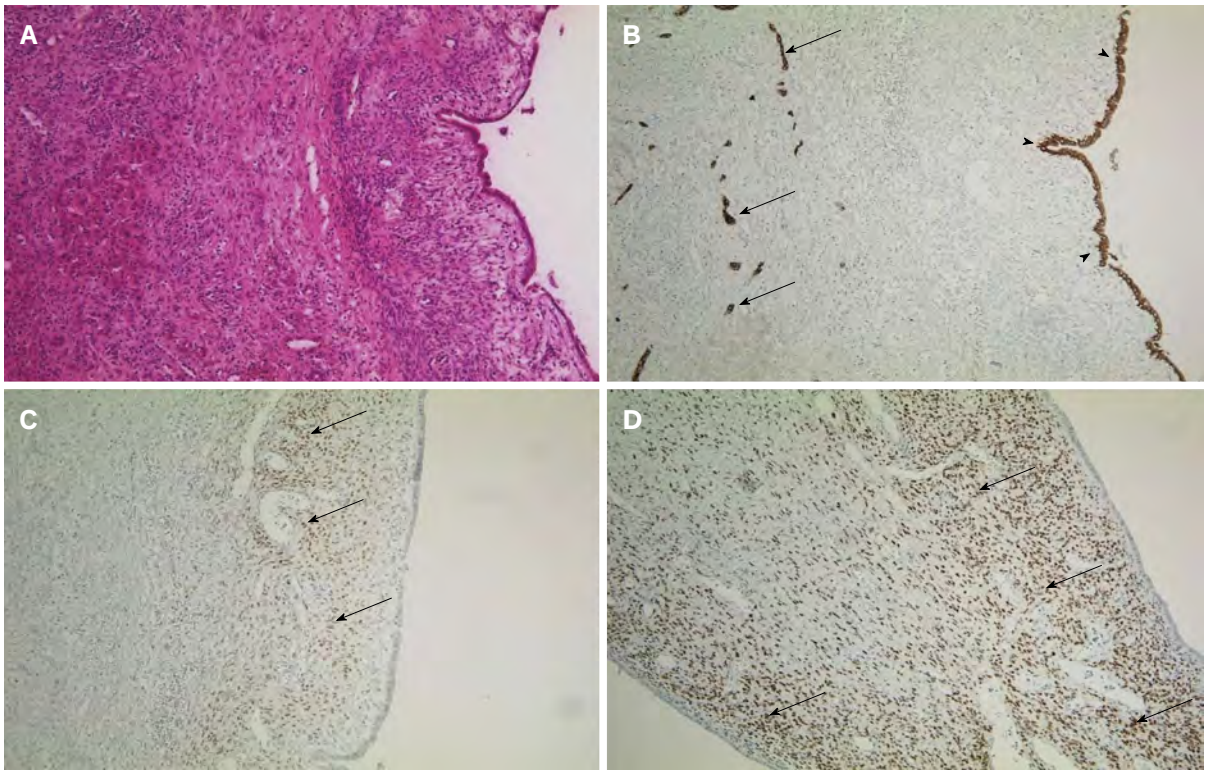

Figure 2 Immunohistochemistry of the cyst. A: Cyst and normal liver tissue in HE staining, × 100; B: Immunohistochemistry for CK7, an epithelial marker, × 100. The intrahepatic vessels (arrows, endothelium) and the cystic wall (arrowheads) are indicated; C and D: Estrogen receptor and progesterone receptor staining of the cystic wall (respectively), × 100. Arrows indicate positive cells.

metriosis without histopathology. Twelve cases (67%) reported a previous history of endometriosis, mostly in a typical pelvic location, while 6 patients (33%) had no history of endometriosis at all. Thus the diagnosis of this condition should not be limited to patients with a known history of endometriosis. Also, 6 of 18 patients (33%) were postmenopausal, thus showing this condition is not limited to women of reproductive age. Due to the difficulty of diagnosing this condition by preoperative radiography[6], only seven (39%) of the previously described eighteen cases were diagnosed preoperatively. In these cases, a transhepatic biopsy was obtained and the diagnosis verified histologically. Although our patient underwent several invasive procedures previous to the operation at our hospital, unfortunately no histological sample was obtained. This prolonged the suffering of the patient and highlights the importance of both histological and microbiological samples in any recurrent cystic formation.

The cause of endometriosis, first described by Rokitansky in 1860 in the pelvis, is still largely uncertain. Two major theories involve either the implantation of endometrial cells (implantation theory)[21] or the metaplasia of the peritoneal epithelium (coelomic metaplasia theory)[22] in the region of occurrence. Each theory, individually, fails to explain all cases of endometriosis. The frequent occurrence of retrograde menstruation is a strong argument for the implantation theory[23]. Yet how these endometrial cells may reach atypical locations such as the brain, the heart[24,25] or, and in our case, the parenchyma of the liver, is hard to explain with this theory. The transport of endometrial cells *via* blood and lymph vessels, similar to the metastasis of cancerous cells, may be a possible explanation for these atypical locations[9,21,26]. Keichel *et al*[26] found endometriosis-like cells in lymphatic vessels as well as endometriosis in locoregional lymph nodes in patients with deep infiltrating endometriosis.

The metaplasia of (peritoneal) epithelium due to chronic inflammation or an unknown signaling cascade[22] may be better suited to explain the occurrence of endometriosis in obscure locations, such as in the heart or even the male. Yet the observation that atypical endometriosis usually occurs in only one location in each individual is hard to resolve. If, as postulated by this theory, some signaling cascade prompts coelomic cells to develop into endometrial tissue, why does this usually happen in only one atypical location in a prone individual? Also, the high number of extragonadal, pelvic cases of endometriosis seems to favor a model in which proximity to the uterus may play an important role.

In conclusion, intrahepatic endometriosis is a rare condition in women. Due to the difficulties of radiographic diagnosis and the "atypical" clinic (no cyclical pain and lack of dysmenorrhea), the diagnosis may only be reached preoperatively by transhepatic biopsy[6]. Due to the potential adverse effects (risk of bleeding, possible dissemination of cells, biliary leakage) this procedure should be discussed individually. The operation of problematic cysts is a proven cure of the tenderness and

Table 1 Case reports of intrahepatic endometriosis

Ref.	Age (yr)	Meno pause	Cyclical pain	History of endo metriosis	Pre-operation diagnosis	Previous operations
Finkel *et al*[5]	21	No	No	No	No	Yes
Grabb *et al*[10]	21	No	No	No	No	Yes
Rovati *et al*[17]	37	No	No	Yes	Yes	No
Cravello *et al*[8]	34	No	Yes	Yes	Yes	No
Verbeke *et al*[19]	62	Yes	No	No	No	Yes
Verbeke *et al*[19]	34	No	No	No	No	No
Chung *et al*[7]	40	No	No	Yes	No	Yes
Weinfeld *et al*[20]	60	Yes	No	Yes	No	Yes
Inal *et al*[12]	25	No	No	Yes	No	No
N´Senda *et al*[15]	54	Yes	No	No	Yes	Yes
Huang *et al*[11]	56	Yes	No	Yes	No	Yes
Jeanes *et al*[13]	31	No	No	Yes	Yes	Yes
Khan *et al*[14]	31	No	No	Yes	Yes	Yes
Khan *et al*[14]	59	Yes	No	Yes	Yes	Yes
Tuech *et al*[18]	42	No	No	No	No	No
Reid *et al*[16]	46	No	No	Yes	No	Yes
Goldsmith *et al*[9]	48	No	No	Yes	No	Yes
Asran *et al*[6]	61	Yes	No	Yes	Yes	Yes
Fluegen *et al*, this study	32	No	No	No	No	Yes

pain mostly reported with this condition[27]. A selective operation should be considered top priority, even if a malignant progression is very rare[28]. Lifelong hormone therapy may also reduce the symptoms, as described in the case of Inal *et al*[12], but carries the risk of side effects and long-term dependence on medication. Rare conditions like intrahepatic endometriosis should always be considered as a differential diagnosis in women with recurrent hepatic cysts after surgical deroofing, regardless of age or previous medical history. In such cases, histology is essential and a pericystectomy should be performed.

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CASE REPORT

Bleeding Dieulafoy's-like lesions of the gut identified by capsule endoscopy

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Author contributions: Ciobanu L analyzed the capsule endoscopy records and wrote the paper; Pascu O analyzed the capsule endoscopy records; Matei D, Diaconu B and Pojoga C attended the patients; Tanţău M performed the enteroscopies.

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Core tip: This case series emphasizes the role of capsule endoscopy in active obscure gastrointestinal bleeding, even in hemodynamically unstable patients, because it is able to identify the site of bleeding and to guide therapeutic procedures. Emergency capsule endoscopy reduces the number of diagnostic enteroscopies in Dieulafoy's-like lesions of the small bowel and colon.

Ciobanu L, Pascu O, Diaconu B, Matei D, Pojoga C, Tanţău M. Bleeding Dieulafoy's-like lesions of the gut identified by capsule endoscopy. *World J Gastroenterol* 2013; 19(29): 4823-4826 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i29/4823.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i29.4823

Abstract

Dieulafoy's-like lesions (DLs-like) represent a cause of obscure gastrointestinal bleeding, enteroscopy being the main diagnostic and therapeutic procedure. Frequently, more than one enteroscopy is needed to identify the bleeding vessel. In our practice, video capsule endoscopy (VCE) identified and guided therapy in four cases of DLs-like; three of them were localized on the small bowel. We report, for the first time, a diagnosis of colonic DL-like performed by colon capsule endoscopy. Two patients presented with severe cardiovascular disorders, being hemodynamically unstable during VCE examination. Based on the VCE findings, only one invasive therapeutic procedure per patient was necessary to achieve hemostasis. VCE and enteroscopy may be regarded as complementary procedures in patients with gut DLs-like.

Key words: Obscure gastrointestinal bleeding; Dieulafoy's-like lesion; Video capsule endoscopy; Enteroscopy; Small bowel

INTRODUCTION

Dieulafoy's lesion (DL) is characterized by a small mucosal erosion involving an unusually large submucosal artery in an otherwise normal mucosa[1]. Even a predilection for proximal stomach was considered previously; no region of the gut is immune[1-3]. Characteristic histological lesions consist of a normal artery with an abnormally large diameter, maintaining a constant width of 1-3 mm, that runs a tortuous course within the submucosa and protrudes through a small mucosal defect, varying from 2 to 5 mm, which fibrinoid necrosis at its base[4]. The term DL-like is used to describe bleeding when angiographic, endoscopic ultrasound or pathological data are unavailable, the diagnosis being based on clinical and endoscopic features[4]. DLs are responsible for 3.5% of mid gastrointestinal bleeding[2]. The diagnosis is challenging, not only because of the small size of the lesion and the normal surrounding mucosa, but also because of the intermittent nature of bleeding; frequently more than one enteroscopic procedure being required for small bowel DLs-like.

In recent years, the extensive use of video capsule endoscopy (VCE) proved to be a useful procedure with high diagnostic yield in obscure gastrointestinal bleeding (OGIB)[5]. Recent reports also document the utility of this procedure in active, severe OGIB[5-8]. Some case reports describe DLs-like diagnosis on capsule endoscopy[9-11], but none of the larger series gives specific details on this topic. Colon capsule endoscopy, initially designed as a non-invasive procedure for colorectal cancer screening[12], with many technical improvements, can assess the entire length of the gut to identify sources of bleeding.

During 2006 to 2012, in our capsule endoscopy unit, 227 VCE procedures were performed, 70 patients being investigated for overt OGIB. Four DLs-like were diagnosed by capsule endoscopy (three localized on the small bowel and one in the colon) in active gastrointestinal bleeding and successful guided therapeutic procedures were performed. Capsule endoscopy findings suggestive of DLs-like were: active blood streaming from a minute mucosa defect or through surrounding mucosa, visualization of a protruding vessel or the appearance of a fresh, densely adherent clot with a narrow point of attachment to a minute mucosal defect or to normal appearing mucosa, as described in conventional endoscopy[1].

CASE REPORT

Case 1

A 69-year-old female, receiving long-term warfarin therapy for a prosthetic mitral valve was referred to our department for intermittent melena in the last four weeks. No bleeding source was identified after two upper and lower gastrointestinal endoscopies. She did not use nonsteroidal anti-inflammatory drugs (NSAIDs). At the time of VCE, her blood pressure was 90/60 mmHg, her heart rate was 110 beats/min and her hemoglobin level was 6.8 g/dL. Capsule endoscopy revealed significant amounts of fresh blood in the first jejunal loop (Figure 1A), without an identifiable lesion, highly suggestive of DL-like. On the same day, an emergency push enteroscopy was performed, with therapeutic hemoclipping of jejunal bleeding mucosal break. Her clinical status significantly improved, and she was discharged a few days later, with a hemoglobin level of 10 g/dL. She did not experience any gastrointestinal bleeding during a 5-year follow-up period.

Case 2

A 58-year-old female who experienced four episodes of melena in the last five years, without an identifiable source of bleeding at multiple upper and lower endoscopies. At the time of VCE she had melena and a hemoglobin level of 5.8 g/dL. Capsule endoscopy revealed a very small mucosal break suggestive of DL-like (Figure 1B), with a significant amount of fresh blood in the jejunum. Based on these findings, 12 h later, an emergency enteroscopy was performed that was able to stop the bleeding by hemoclipping. She remained in excellent clinical condition 1 year later.

Case 3

A 68-year-old female presented two severe episodes of melena and hematochezia with hemorrhagic shock in the last five months. She was not using NSAIDs and no other symptoms were present. She was investigated with upper and lower gastrointestinal endoscopies on the first episode without an identifiable source of bleeding. On the second episode of bleeding, another gastroscopy did not find any bleeding source, and she was referred for capsule endoscopy. After a standard preparation we used a colon capsule endoscopy to examine the entire gut mucosa. Capsule endoscopy found fresh blood only in the colon with a mucosal break (Figure 1C). Colonoscopy was performed the same night: a bleeding vessel was found on the transverse colon and hemoclips were placed. The patient was released from hospital five days later in good clinical condition. She did not experience other episodes of gastrointestinal bleeding during a 2 years follow up period.

Case 4

Case 4 was an 80-year-old female patient with known cardiovascular disorders that required both antiagregants (low doses of aspirin) and anticoagulants (warfarin). She was admitted for a severe episode of hematochezia, with hemorrhagic shock, with a 5.8-g/dL hemoglobin level. Upper and lower endoscopies did not find the source of the bleeding; therefore, a capsule endoscopy was performed. The patient being closely monitored because she was hemodynamically unstable. VCE detected active bleeding in the proximal jejunum and a small mucosal break (Figure 1D). An emergency push enteroscopy was performed the next morning, which was able to place metal clips on the bleeding jejunum mucosal vessel. Her clinical condition improved and she left the hospital five days later. During a 6-mo follow up period she did not experience another episode of gastrointestinal bleeding.

DISCUSSION

DLs account for 1%-2% of all gastrointestinal bleeding episodes[4]. From 1884, when it was first described by Gallard[13], until today, the management of DLs-like has represented a challenge. The patients present with acute onset of bleeding, frequently without identifiable risk factors, sometimes life-threatening. Identifying the site of bleeding by endoscopy procedures may be difficult, as the dimension of the mucosal break is minute and is not easy to visualize, in the context of large amounts of blood or if the bleeding stops[4]. These aspects are even more relevant for small bowel localization, as the bleeding lesions are more difficult to reach. Small bowel DLs-like account for 3.5% of OGIB, as assessed recently by Dulic-Lakovic *et al*[2] in a large retrospective study based on enteroscopy findings in patients with OGIB. Enteroscopy, even if it is invasive, requires sedation and sometimes may be technically difficult, has proved to

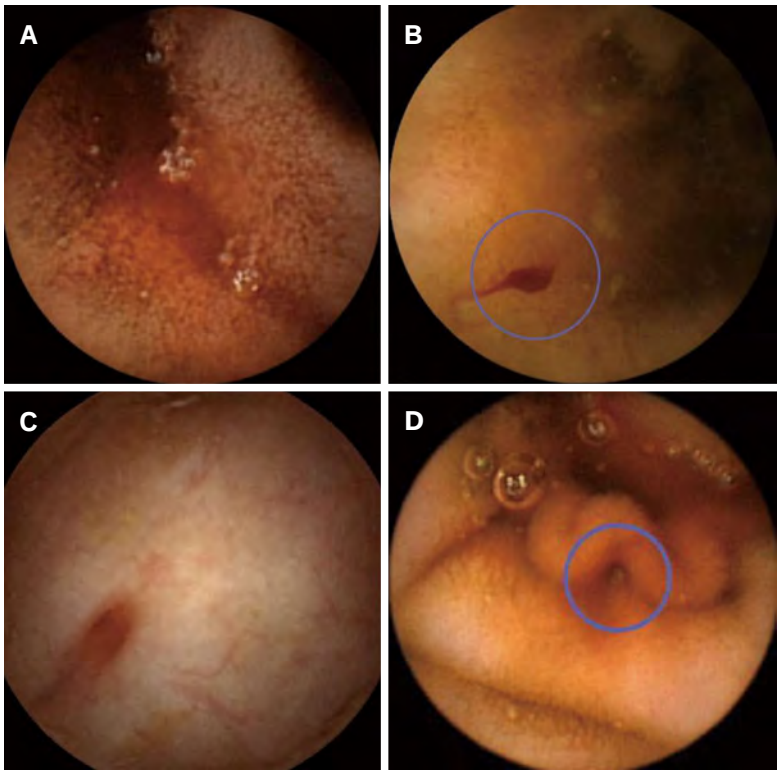

Figure 1 Capsule endoscopy. A: Significant amounts of fresh blood in the first jejunal loop; B: A very small mucosal break suggestive of Dieulafoy's lesion in the jejunum (circle); C: A bleeding mucosal break on the colonic mucosa; D: A small mucosal break in the proximal jejunum (circle).

be a very efficient diagnostic and therapeutic procedure for these lesions. Patients with small bowel DLs-like frequently require more than one enteroscopy until the source is identified and treated; a mean of 1.5 enteroscopy per case being reported[2].

Previous case reports document a correct diagnosis of DLs-like achieved by VCE, with subsequently successful guided therapeutic procedures[9-11]. All our cases also argue for the good performance of VCE in identifying and guiding therapy, even in hemodynamically unstable patients (cases 1 and 4). An important issue to consider in these patients is the timing of both VCE and enteroscopy. In active bleeding patients, with hemodynamically instability, VCE is not routinely used, as it may be considered time consuming; however, recent studies report a good diagnostic yield in identifying the site of acute bleeding in 75% of patients and the source of bleeding in 67% of cases[6-8]. Based on these findings, successful guided therapy was performed in 73%-76% of patients[7,8]. In these studies, no specific results or discussion are presented for DLs-like. Our patients presented clinical signs of active bleeding at the time of VCE, which substantially increased the chance for detecting blood or lesions. Capsule endoscopy images were interpreted immediately after the procedure and were followed by emergency endoscopy in the next few hours.

To the best of our knowledge, no previous report describes colon DL-like diagnosed with colon VCE. Previous studies reported missing bleeding lesions localized in the stomach or cecum, by conventional endoscopies and identified on capsule endoscopy[14,15]. In case 3, previous colonoscopy did not find fresh blood in the colon, meaning that DL was inactive. A second colonoscopy guided by findings on VCE enabled the correct identification of the lesion and treatment.

In our patients, after correct localization of the bleeding active site by VCE, only one interventional endoscopy per patient was needed for treatment (3 push enteroscopies and 1 colonoscopy), hemostasis being achieved by hemoclipping. VCE findings provided a very good selection for subsequent therapeutic procedures, reducing the number of interventional diagnostic maneuvers. Hemoclipping is a highly effective therapy, very well documented for gastric DLs-like[16]. Angiography can identify and treat DLs by embolization, being used in difficult to treat cases[3]. Surgery could be regarded as an alternative treatment only if the bleeding is not stopped by endoscopic or angiographic procedures[3].

No pathogenetic association between NSAIDs and DLs-like has been identified in the literature[17,18]. However, as DLs-like seem to be more frequent in older patients with significant comorbidities[4] that may require NSAIDs and/or anticoagulants, it is assumed that concomitant administration of these agents prolongs and increases the severity of bleeding, as was observed in cases 1 and 4. In addition, differential diagnosis may be challenging, as NSAIDs enteropathy can display a wide spectrum of features from mucosal breaks to large bleeding ulcers[19]. In actively bleeding NSAIDs enteropathy, multiple erosions or ulcers could be documented as necro-inflammation, representing the background pathology induced by these drugs[20].

The limitations of this report are related to the retrospective analyses of cases and inclusions of patients with active OGIB, highly suspicious of DLs-like. Despite these limitations, this case series emphasizes the role of

capsule endoscopy in reducing the number of diagnostic enteroscopies in DLs-like.

In conclusion, VCE should be considered in active OGIB, even in hemodynamically unstable patients, because it is able to identify the site of bleeding and to guide therapeutic procedures.

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CASE REPORT

Endoscopic appearance of AIDS-related gastrointestinal lymphoma with *c-MYC* rearrangements: Case report and literature review

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Abstract

Acquired immune deficiency syndrome (AIDS)-related lymphoma (ARL) remains the main cause of AIDS-related deaths in the highly active anti-retroviral therapy (HAART) era. Recently, rearrangement of *MYC* is associated with poor prognosis in patients with diffuse large B-cell lymphoma. Here, we report a rare case of gastrointestinal (GI)-ARL with *MYC* rearrangements and coinfected with Epstein-Barr virus (EBV) infection presenting with various endoscopic findings. A 38-year-old homosexual man who presented with anemia and was diagnosed with an human immunodeficiency virus infection for the first time. GI endoscopy revealed multiple dish-like lesions, ulcerations, bloody spots, nodular masses with active bleeding in the stomach, erythematous flat lesions in the duodenum, and multiple nodular masses in the colon and rectum. Magnified endoscopy with narrow band imaging showed a honeycomb-like pattern without irregular microvessels in the dish-like lesions of the stomach. Biopsy specimens from the stomach, duodenum, colon, and rectum revealed diffuse large B-cell lymphoma concomitant with EBV infection that was detected by high tissue EBV-polymerase chain reaction levels and Epstein-Barr virus small RNAs *in situ* hybridization. Fluorescence *in situ* hybridization analysis revealed a fusion between the immunoglobulin heavy chain (IgH) and *c-MYC* genes, but not between the IgH and BCL2 loci. After 1-mo of treatment with HAART and R-CHOP, endoscopic appearance improved remarkably, and the histological features of the biopsy specimens revealed no evidence of lymphoma. However, he died from multiple organ failure on the 139th day after diagnosis. The cause of his poor outcome may be related to *MYC* rearrangement. The GI tract involvement in ARL is rarely reported, and its endoscopic findings are various and may be different from those in non-AIDS GI lymphoma; thus, we also conducted a literature review of GI-ARL cases.

Key words: Acquired immune deficiency syndrome-related lymphoma; Non-Hodgkin-lymphoma; Epstein-Barr virus infection; *c-MYC* rearrangement; Endoscopic appearance

Core tip: Endoscopic findings in gastrointestinal-ac-

quired immune deficiency syndrome (GI-AIDS) related lymphoma (ARL) are miscellaneous and may be different from non-AIDS GI lymphoma. We report a rare case of GI-ARL with *MYC* rearrangements and coinfected with Epstein-Barr virus infection, and there are multiple findings involving stomach, duodenum, and colon and rectum. Magnified endoscopy with narrow band imaging showed a honeycomb-like pattern without irregular microvessels in the dish-like lesions of the stomach. Moreover we conducted literature review of GI-ARL. To our knowledge, this is the first report of GI-ARL with *MYC* arrangements and presenting an atypical endoscopic appearances.

Tanaka S, Nagata N, Mine S, Igari T, Kobayashi T, Sugihara J, Honda H, Teruya K, Kikuchi Y, Oka S, Uemura N. Endoscopic appearance of AIDS-related gastrointestinal lymphoma with *c-MYC* rearrangements: Case report and literature review. *World J Gastroenterol* 2013; 19(29): 4827-4831 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i29/4827.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i29.4827

INTRODUCTION

Non-Hodgkin-lymphoma (NHL) occurs in 5%-10% of individuals with an human immunodeficiency virus (HIV) infection. The early diagnosis of acquired immunodeficiency syndrome (AIDS)-related lymphoma (ARL) is highly important because patients with ARL tend to exhibit an aggressive clinical course, short survival, and poor treatment response. Chromosomal translocations of 8q24, encoding the c-myc oncogene, are considered to be associated with NHL oncogenesis, and are normally seen in patients with Burkitt lymphoma[1]. Recently, *MYC* rearrangements have been seen occasionally in diffuse large B-cell lymphoma (DLBCL) and are associated with a poor prognosis[2]. Here, we report a rare case of gastrointestinal (GI)-ARL with MYC rearrangements and an Epstein-Barr virus (EBV) infection presenting with various endoscopic findings. As the endoscopic findings in ARL are variable and may be different from those of non-AIDS GI lymphoma, we conducted a literature review of GI-ARL cases.

CASE REPORT

A 38-year-old homosexual man was admitted to our hospital with shortness of breath and multiple lymphadenopathy. He was diagnosed with an HIV infection for the first time. Physical examination showed slight upper abdominal tenderness, hepatomegaly, and splenomegaly without watery or bloody stools. Blood sample tests showed a low CD4 lymphocyte count (240 cells/μL), high quantity of HIV RNA (2.9×10^7 copies/mL), anemia (hemoglobin, 93 g/L), high lactate dehydrogenase (4.882 U/L), low serum albumin (24 g/L), and high EBV-PCR levels (9.0×10^5 copies/μg DNA). The patient was *Helicobacter pylori* (*H. pylori*) negative with the titer of the *H. pylori*-antibody under 3.0 U/mL. To diagnose anemia, we performed upper and lower GI endoscopy and revealed multiple dish-like elevated lesions (Figure 1A and B), bloody spots (Figure 1C), and ulcers in the body of the stomach (Figure 1D). Magnification endoscopy with narrow band imaging (NBI) showed a honeycomb-like pattern at the edge of the elevated lesions (Figure 1E), but there was no irregular microvascular pattern in the ulcers (Figure 1F). We also found erythematous flat lesions in the duodenum (Figure 1G). Lower GI endoscopy showed multiple nodular masses in the colon and rectum (Figure 1H). Biopsy from these lesions revealed pleomorphic, atypical lymphoid cells with eosinophilic cytoplasm, marked nucleoli, and vesicular nuclei with hematoxylin and eosin staining (Figure 2). Immunohistochemistry showed positive staining for CD20, CD79a, CD38, MUM-1, BCL2, CD30, EMA, and latent membrane protein 1 and no staining for CD138, BCL6, and CD10 (Figure 2). Biopsy specimens from the upper and lower GI tract also revealed positive EBER *in situ* hybridization and high EBV-PCR levels (100000 copies/μg DNA). We also conducted a biopsy from the right inguinal lymph node. Fluorescence *in situ* hybridization analysis revealed fusion between the immunoglobulin heavy chain (IgH) and *c-MYC* genes, but not between the IgH and BCL2 loci. Computed tomography showed splenomegaly, slight hepatomegaly, and lymphadenopathy. Positron emission tomography detected radioisotope uptake within the bone marrow, lymph nodes, spleen, and gallbladder. The final diagnosis was DLBCL clinical stage 4B, according to the Ann Arbor Staging Classification for Lymphomas, and concomitant with an EBV infection. The patient was administered oral highly active anti-retroviral therapy (HAART) and R-CHOP chemotherapy. After 1 mo of treatment, the endoscopic appearance of the elevated lesions, blood spots, and ulcers had improved. The histological features of the biopsy specimens revealed no evidence of NHL. However, after 7 cycles of R-CHOP chemotherapy, blood sample tests showed high levels of lactate dehydrogenase (2568 U/L), hyperferritinemia (31810 ng/mL), and cytomegalovirus (CMV)-PCR (200 copies/μg DNA). Bone marrow aspiration revealed infiltration by activated histiocytes and hemosiderin-filled macrophages. The patient showed CMV viremia, tumor lysis syndrome, and hemophagocytic syndrome. He died of multiple organ failure on the 139th day after diagnosis.

DISCUSSION

The incidence of ARL has not decreased over time despite the widespread use of chemotherapy and the improved management of long-term HAART[3]. Furthermore, ARL remains the most frequently observed AIDS-defining event leading to death[3]. Therefore, the early diagnosis of ARL is very important. In 5%-10% of cases with DLBCL with typical morphology, a *MYC* rearrangement (MYC+) is also observed, and these cases are considered a sub-category of DLBCL[2]. The presence of

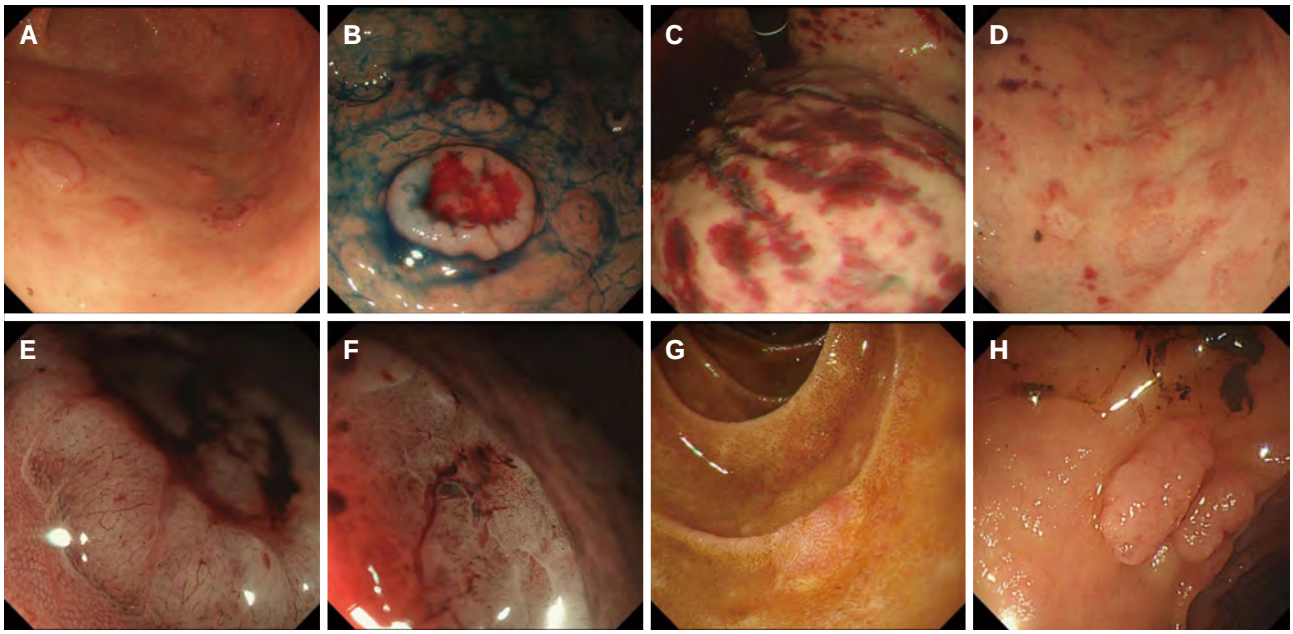

Figure 1 Upper and lower gastrointestinal endoscopic findings. A: Multiple elevated lesions in the body of the stomach; B: Multiple dish-like lesions with bleeding dyed with indigo carmine; C: Bloody spots in the body of the stomach; D: Ulceration with bleeding in the upper body of the stomach; E: Narrow band imaging (NBI) with magnification showing a honeycomb-like pattern at the edge of the elevated lesion; F: Irregular microsurface pattern in ulceration with NBI; G: Erythematous flat lesions in the duodenum; H: Multiple nodular masses in the colon and rectum.

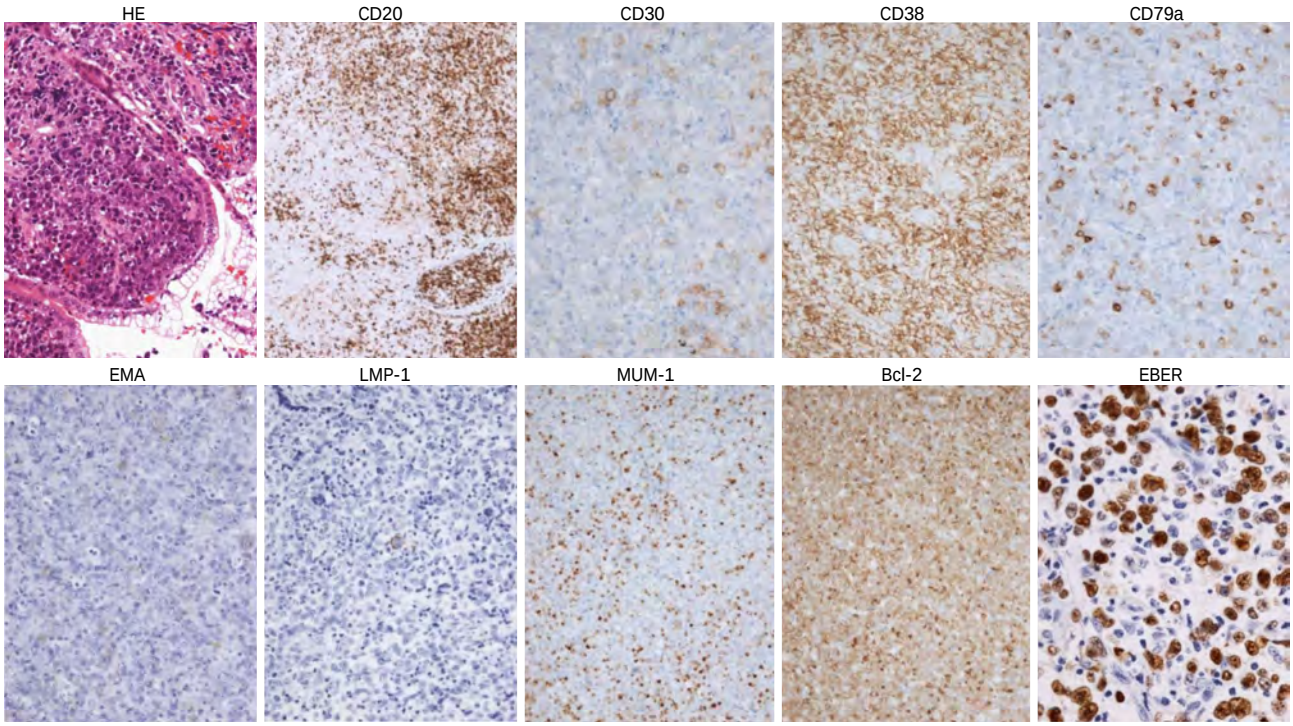

Figure 2 Histological findings and immunostaining of the biopsy specimen. Pleomorphic, atypical lymphoid cells with eosinophilic cytoplasm, marked nucleoli, and vesicular nuclei with hematoxylin and eosin staining (× 10). Immunohistochemistry shows positive staining for CD20 (× 4), CD30 (× 20), CD38 (× 10), CD79a (× 20), EMA (× 10), LMP-1 (× 10), MUM-1 (× 10), and BCL2 (× 10). Biopsy specimens revealed positive EBER-*in situ* hybridization (× 40). LMP-1: Latent membrane protein 1; MUM-1: Multiple myeloma oncogene 1; EMA: Epithelial membrane antigen.

a *MYC* rearrangement is associated with inferior overall survival and there is a trend for a reduction in event-free survival[4]. Our case had a poor prognosis in spite of their temporary clinical and endoscopic improvement after chemotherapy. We suggest that the aggressive course of his illness was related to *MYC* rearrangement.

Endoscopy with biopsy is essential to make a definite diagnosis of GI-ARL; however, it is usually indicated for patients with GI symptoms. In our case, he had only slight tenderness, but we performed endoscopy to inves-

Table 1 Literature review of the endoscopic findings in acquired immune deficiency syndrome-related lymphoma patients

Ref.	Patients	Involved GI tract	Endoscopic findings
Friedman[9]	NHL (n = 12)	Esophagus, stomach, duodenum, small bowel, colon, rectum	Multiple bulky tumor masses, small polypoid lesions
Heise *et al*[10]	NHL (n = 48)	Esophagus, stomach, duodenum, small bowel, colon, rectum	Bulky tumor mass, polypoid lesions, deep well-defined ulceration, necrotic abscesses
Corti *et al*[11]	Burkitt lymphoma (45-yr man)	Duodenum	Pseudo-polypoid masses
Andrews *et al*[12]	NHL (n = 30)	Stomach, small bowel, colon	Tumor mass, ulceration, nodular lesions
Mani *et al*[13]	Plasmablastic lymphoma (40-yr man)	Esophagus, stomach	Tumor mass, ulceration
Mahmoudi *et al*[14]	Lymphoma (38-yr woman)	Stomach	Ulceration, non-specific inflammation
Chow *et al*[15]	41-yr woman	Esophagus	A flat and solitary ulcer
Cappell *et al*[16]	GI lymphoma in AIDS patients (n = 6)	Esophagus, stomach, duodenum	Multiple volcano-like masses, ulceration, stricture with ulceration, nodular lesions, tumor mass, large sessile polypoid lesions
Yehya *et al*[17]	44-yr man	Colon	Mass with ulceration
Rezende *et al*[18]	NHL in AIDS patients (n = 6)	Stomach	Polypoid lesions, ulceration
Fujita *et al*[19]	41-yr man	Stomach	Multiple submucosal tumors with ulceration
Nakazuru *et al*[20]	68-yr man	Duodenum	Well-defined ulceration, auricle-like shaped mass with scattered tiny white spots

NHL: Non-Hodgkin-lymphoma; GI: Gastrointestinal.

tigate the cause of his anemia, which enabled us to make a diagnosis. The knowledge of the distinctive endoscopic appearance of GI-ARL also leads to an early diagnosis, but its detailed characteristics remain unknown. Therefore, we reviewed the English literature in the MEDLINE database by searching with the key words "HIV", "lymphoma", and "endoscopy" (Table 1). We identified 65 reports, but there was no description of endoscopic findings in 53; finally, we selected 12 reports.

The most frequently involved GI sites are the stomach (24%), small bowel (10%), and colon/rectum (7%). The most common site of involvement in the colon is the cecum (45%-75%), followed by the rectum[5]. However, in immunocompetent patients, GI lymphomas are found in the stomach in 50%-80% of all cases and rarely in the colon, small bowel, or perianal region[5]. There are a wide range of endoscopic findings in ARL. Findings of multiple, polypoid lesions, nodular masses, bulky tumor mass, or ulcerations are commonly found. Our search of the literature revealed no findings of multiple bloody spots with exudates or flat erythematous lesions in GI lymphoma. Previous study has shown that *H. pylori*-induced T-cell plays an important role in gastric mucosa-associated lymphoid tissue lymphoma development[6]. However, *H. pylori* was negative in this case, thus other mechanisms have been suggested. We believed that EBV activation caused by the immunosuppression in AIDS patients plays a role in the pathogenesis of the various and unique endoscopic findings, such as multiple nodular masses, bloody spots, and ulcers, because high EBV DNA blood levels are considered to be associated with the development of NHL[7].

In this case, we also perform magnified endoscopy using NBI. NBI endoscopy for diagnosing superficial gastric cancer is accurate for the diagnosis of cancer when the diagnostic triad of the disappearance of fine mucosal structures, microvascular dilation, and heterogeneity is used[8]. Our case did not satisfy this triad, suggesting that NBI endoscopy is a useful technique for the differential diagnosis of epithelial tumors and non-epithelial tumors.

Endoscopic biopsy is highly valuable to diagnose GI-ARL. We need to re-realize that GI-ARL can present with various endoscopic findings and involve the entire GI tract, which may be different from non-AIDS GI lymphoma due to differences in the immune status of patients with these conditions. NBI endoscopy may be used to differentiate GI-ARL from gastric cancer.

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CASE REPORT

Laparoscopic cholecystectomy in patients with anesthetic problems

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Abstract

Laparoscopic cholecystectomy is a standard operation for benign gallbladder disease. As experience with laparoscopic cholecystectomy has increased, the procedure has become possible in patients with anesthetic problems. Patients with ankylosing spondylitis or severe kyphosis represent a challenging group to anesthesiologists and laparoscopic surgeons since these diseases are associated with difficult intubation, restrictive ventilatory defects, and cardiac problems. The relatively new approach of awake fiberoptic intubation is considered to be the safest option for patients with anticipated airway difficulties. Laparoscopic cholecystectomy is usually performed under general anesthesia but considerable difficulties in anesthetic management are encountered during laparoscopic surgery; for example, hemodynamic instability may develop in patients with cardiopulmonary dysfunction due to pneumoperitoneum and position changes during the operation. Nonetheless, regional anesthesia can be considered as a valid option for patients with gallbladder disease who are poor candidates for general anesthesia due to cardiopulmonary problems. We report three cases of laparoscopic cholecystectomy successfully performed in patients with anesthetic problems that included cardiopulmonary disease, severe kyphosis, and ankylosing spondylitis.

Key words: Laparoscopic cholecystectomy; Kyphosis; Ankylosing spondylitis; Anesthetic problems

Core tip: This paper reported three cases of laparoscopic cholecystectomy successfully performed in patients with anesthetic problems that included cardiopulmonary disease, severe kyphosis, and ankylosing spondylitis. This study demonstrates that regional anesthesia can be considered as a valid option for patients with gallbladder disease who are poor candidates for general anesthesia due to cardiopulmonary problems.

Kim BS, Joo SH, Joh JH, Yi JW. Laparoscopic cholecystectomy in patients with anesthetic problems. *World J Gastroenterol* 2013; 19(29): 4832-4835 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i29/4832.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i29.4832

INTRODUCTION

Laparoscopic cholecystectomy is a standard procedure for the management of gallbladder disease. As experience with laparoscopic cholecystectomy has increased, laparoscopic cholecystectomy in patients with anesthetic problems has become possible. Ankylosing spondylitis is a challenge to the anesthesiologist and to the laparoscopic surgeon because it is associated with difficult intuba-

tion, restrictive ventilatory defects, and cardiac problems. Kyphotic patients may also suffer considerable perioperative morbidity such as cardiac problems and respiratory insufficiency. While laparoscopic cholecystectomy is classically performed under general anesthesia, regional anesthesia must be considered as a valid option for patients with gallbladder disease who are poor candidates for general anesthesia. We report three cases of laparoscopic cholecystectomy successfully performed in patients with anesthetic problems that included cardiopulmonary disease, severe kyphosis, and ankylosing spondylitis. All patients were informed about the operative and anesthetic procedures and provided consent.

CASE REPORT

Case 1

A 71-year-old, American Society of Anesthesiology (ASA) physical status Ⅲ male patient (168 cm, 56 kg) underwent laparoscopic cholecystectomy due to acute cholecystitis with cholelithiasis. The patient had undergone a right pneumonectomy nine years previous to this procedure (Figure 1). A standard pulmonary function test revealed a moderate obstructive and restrictive pattern. Hypokinesia of the apical anterior and septum segments was observed on echocardiography. Pulmonology consultation reported a moderate postoperative risk that could be fatal if postoperative pneumonia developed. Cardiology consultation reported a postoperative risk of 1%-5% based on modified Goldman cardiac risk criteria[1]. Because severe postoperative complications were anticipated, segmental spinal anesthesia was performed and CO_2 inflation to an intra-abdominal pressure of 8 mmHg. Laparoscopic cholecystectomy was performed; the procedure was smooth and uneventful. The patient was discharged 3 d after surgery.

Case 2

A 42-year-old male patient (173.6 cm, 68.9 kg) suffering from ankylosing spondylitis was admitted to our hospital presenting with right upper quadrant pain. Abdominal ultrasound revealed a 2 cm sized cholelithiasis for which he was scheduled to undergo laparoscopic cholecystectomy. The patient was ASA Ⅲ and Mallampati grade Ⅳ on airway examination. The patient had fixed rigidity of the cervical spine and could not lie supine (Figure 2A) and his mouth opening was restricted. Accordingly, he was placed in a supine position with three pillows under his head. The anesthesiology team decided to perform fiberoptic intubation through the nasotracheal tree while the subject was conscious (Figure 2B). The patient was informed of an anticipated difficult orotracheal intubation. Laparoscopic cholecystectomy was performed without any complication. There were no perioperative complications related to airway management, and the patient was discharged 3 d after surgery.

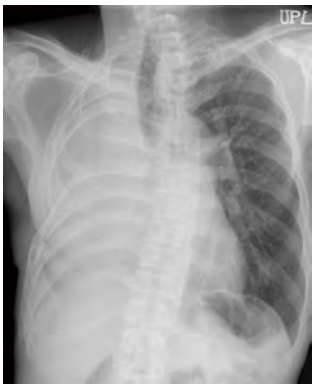

Figure 1 Chest posterior to anterior of case 1. The patient had undergone a right pneumonectomy.

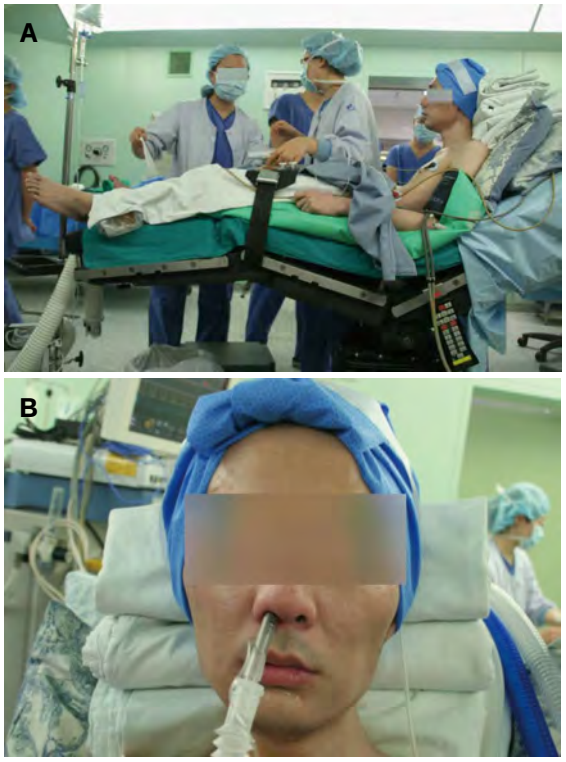

Figure 2 A 42-year-old male patient suffering from ankylosing spondylitis was admitted to our hospital presenting with right upper quadrant pain. A: Operating position used for case 2; B: Awake fiberoptic intubation through the nasotracheal tree used for case 2.

Case 3

A 54-year-old female patient (140 cm, 45.6 kg) with ASA physical status Ⅲ suffering from severe thoracolumbar kyphosis (Figure 3) presented with right upper quadrant pain. The patient was diagnosed with acute cholecystitis on abdominal ultrasound and was scheduled to undergo laparoscopic cholecystectomy. Moderate restrictive pattern was found following a pulmonary function test. The patient could not lie supine and needed thick pillows beneath her head for support. Her thoracic cage was tilted anteriorly resulting in a low-ly-

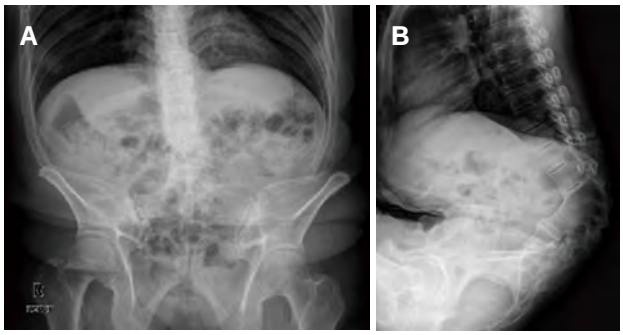

Figure 3 Thoracolumbar spine image for case 3. The anteroposterior (A) and lateral (B) views are shown.

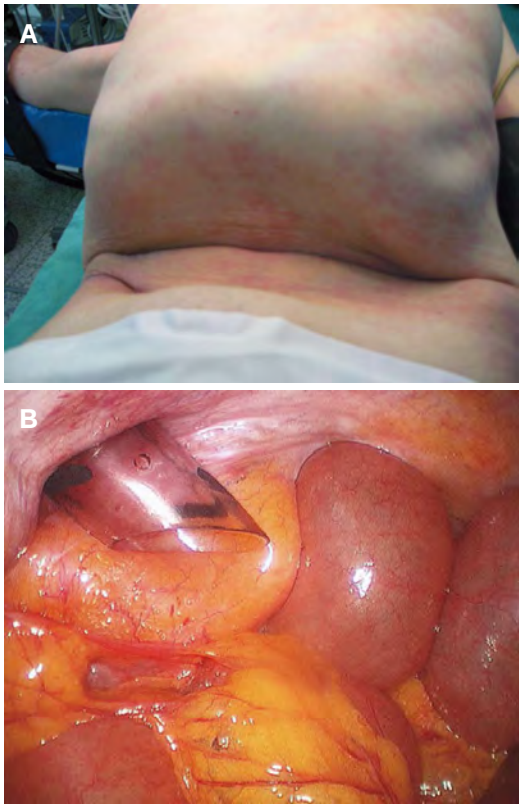

Figure 4 Insertion of the trocar in case 3. A: The narrow abdomen provided a challenge for trocar insertion; B: Internal view of trocar placement in the narrow abdomen.

ing costal margin and narrow abdomen. It was difficult to insert trocars because of narrow abdomen (Figure 4). CO_2 was used to inflate her intra-abdominal pressure to 8 mmHg. The patient underwent laparoscopic cholecystectomy successfully and discharged from hospital at 5 d after surgery.

DISCUSSION

Considerable difficulties in anesthetic management are encountered during laparoscopic surgery. Hemodynamic instability in patients with cardiopulmonary dysfunction may develop due to pneumoperitoneum and position changes during the operation. While laparoscopic cholecystectomy is usually performed under general anesthesia, regional anesthesia has been used in special high-risk cases[2]. However, laparoscopic cholecystectomy under general anesthesia has been reported as safe and feasible even in high-risk patients[3]. Here, we show that carbon dioxide pneumoperitoneum with an intra-abdominal pressure of 8-12 mmHg for high-risk patients can be safe if the patients are well monitored and hemodynamic instability is actively treated. However, the patient in case 1 was at risk of post-operative death if pneumonia developed in the remnant lung, and was scheduled for the regional anesthesia. Due to the harmful effects of high intra-abdominal pressure, it is recommended that the lowest pressure possible that allows adequate exposure of the operative field be used[4]. A laparoscopic cholecystectomy was successfully carried out by maintaining CO_2 pressure < 8 mmHg during the operation with the active participation of the attending anesthesiologists.

Ankylosing spondylitis is a chronic and progressive inflammatory disease involving articulations of the spine and adjacent tissue. Oral intubation is difficult in ankylosing spondylitis patients with cervical spine rigidity. Recently, awake fiberoptic intubation has been increasingly used, and considered to be the safest option for patients with anticipated airway difficulties[5,6]. To secure the airway, we used awake fiberoptic intubation in case 2 with written informed consent of the patient. In addition to airway management, anesthesiologists need to be concerned with restrictive ventilator defects and airway problems associated with ankylosing spondylitis. Therefore, preoperative examination for cardiovascular and pulmonary function is required[7].

Kyphosis is a deformity of the spine. Anesthesia for kyphosis in common with ankylosing spondylitis is often challenging. There is usually secondary involvement of the respiratory, cardiovascular, and neurologic systems. A detailed pre-anesthetic assessment and optimization of the respiratory and cardiovascular systems should be done[8]. It is desirable to maintain the lowest intra-abdominal pressure to prevent the adverse hemodynamic effect of pneumoperitoneum in restrictive lung disease. Another concern for the laparoscopic surgeon for a patient with kyphosis is positioning of the patient and surgical approach to the abdomen. Increasing numbers of older patients are presenting with benign gallbladder disease with senile kyphosis caused by aging or osteoporosis. Laparoscopic surgery is minimally invasive, and in severely kyphotic patients the procedure may prove difficult in approaching the narrow abdomen. In the third case, we performed this difficult surgery because the chest of the patient was tilted anteriorly and the abdominal cavity was narrowed by low-lying costal margin. Abnormalities of the spine can alter the contour of the pelvic vessels. Kyphosis, scoliosis, and the other skeletal abnormalities can affect vascular anatomy and must be considered during trocar insertion[9]. Severe senile kyphosis is not a contraindication for laparoscopic surgery[10].

Patients with spinal deformity such as ankylosing spondylitis and kyphosis form a challenging group to anesthesiologists and laparoscopic surgeons because of difficulties in intubation, positioning, and abdominal approaches. Careful preoperative anesthetic assessment for respiratory dysfunction, cardiac impairment, difficult airway, and associated specific problems in patients with ankylosing spondylitis or kyphosis is imperative. In patients with severely compromised respiratory function the final surgical plan should be a joint decision between surgeon and anesthesiologist. The patient and family should be warned about the possibility of postoperative ventilation and prolonged respiratory weaning and support.

As newer and more extensive laparoscopic procedures have been implemented, close cooperation between the laparoscopic surgeon and the anesthesiologist is required. We have performed laparoscopic cholecystectomy in seven patients with kyphosis. No perioperative complications occurred. While several studies[11] about obstetrics and gynecology and spine surgery in patients with ankylosing spondylitis and kyphosis have been reported, only one report has addressed laparoscopic cholecystectomy[12,13].

In conclusion, we successfully performed three cases of laparoscopic cholecystectomy in patients with anesthetic problems including cardiopulmonary disease, severe kyphosis, and ankylosing spondylitis.

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CASE REPORT

Idiopathic chronic ulcerative enteritis with perforation and recurrent bleeding: A case report

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Abstract

Idiopathic chronic ulcerative enteritis (ICUE) is a distinct entity without a defined etiology and is rarely seen in the clinic. Patients with ICUE mainly present with insidious abdominal symptoms such as chronic abdominal pain and intermittent gastrointestinal hemorrhage and symptoms of malnourishment in the early stages of the disease. ICUE is always difficult to diagnose. However, as the disease progresses, patients have a variety of acute abdominal complications such as hemorrhage, perforation, or ileus. Surgical intervention is always needed, and the condition can recur and require repeat laparotomy. When diffuse ulceration of the small bowel is present in the absence of recognizable causes, it is classified as nonspecific or idiopathic. The histological examination always demonstrates an acute, chronic inflammatory infiltration without giant cells, granulomas, or villous atrophy. The etiology of ICUE has not been identified, and its pathogenesis is poorly understood; therefore, radical surgical resection is considered the best available treatment. Here, we report a case of ICUE characterized by nonspecific, multiple, small intestinal ulcers resulting in perforation and recurrent bleeding. The differential diagnosis and the treatment are also discussed.

Key words: Idiopathic ulcerative enteritis; Small intestinal ulcer; Perforation; Gastrointestinal hemorrhage; Bleeding.

Core tip: This is a case report of idiopathic chronic ulcerative enteritis (ICUE) with perforation and recurrent bleeding. Complete clinical material of the patient is presented in this case report. We also provide several images, including colonoscopy, radiography, computed tomography, and pathology. All of this material reflected the clinical characteristics of ICUE. When patients present with multiple, nonspecific, small intestinal ulcers without a defined etiology, a diagnosis of ICUE should be considered. This case report will help diagnose and treat patients with similar clinical symptoms.

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INTRODUCTION

Idiopathic chronic ulcerative enteritis (ICUE) is uncommon. It was first described by Nyman in 1949 as a kind of ulcerous jejunoileitis[1]. Since then, numerous descriptive terms have been used, including ulcerative jejunitis[2], chronic ulcerative jejunoileitis[3], nongranulomatous ulcerative jejunoileitis[4], and chronic nonspecific ulcerative duodenojejunoileitis[5]. These terms all attempt to describe a type of small bowel ulcerative disorder in the absence of a recognizable cause. Patients with ICUE present with hemorrhage, perforation, or obstruction and often need

emergency surgical intervention. The pathogenesis of ICUE is not clear, and it is associated with a high mortality rate[6], therefore, surgical treatment remains a major challenge in clinical practice. Thus far, radical surgical resection is considered the best available treatment[7].

Here, we report a rare case of ICUE characterized by nonspecific, multiple, small intestinal ulcers resulting in perforation and recurrent bleeding, and discuss the differential diagnostic considerations and treatment.

CASE REPORT

A 78-year-old man was admitted to the hospital with intermittent dull pain in the upper quadrant of his abdomen of > 2 mo duration. The pain occurred irregularly and had no obvious relationship with meals. He reported experiencing nausea, vomiting, heartburn, diarrhea, melena, anorexia, fatigue, and weight loss of 12.5 kg. No hematemesis, hematochezia, tenesmus, or stools with mucus or pus were reported. The patient had a history of intestinal resection after trauma 10 years before. His mother had died of bladder cancer. Gastroscopy showed reflux esophagitis, duodenitis, and superficial gastritis throughout the stomach. No abnormality was noted on colonoscopy. On admission, his temperature (37 ℃) and pulse (78 beats/min and regular) were normal. However, his blood pressure was a little lower than average (90/45 mmHg). Physical examination revealed tenderness in the right upper quadrant of the abdomen, without rebound tenderness or guarding. The bowel sounds were normal. Several enlarged lymph nodes could be palpated bilaterally in the neck and axilla. The lymph nodes were hard, stationary, and painless.

After admission, the patient had intermittent diarrhea, recurrent subxiphoid pain, abdominal distension, and anorexia. Screening for markers of viral hepatitis (A-E), cytomegalovirus, Epstein-Barr virus, herpes simplex virus, and varicella zoster virus was negative. Stool cultures for the pathogens *Shigella, Salmonella, Vibrio cholerae, Mycobacterium tuberculosis* and *Clostridium difficile* (*C. difficile*), and *C. difficile* toxin A were negative. Stool culture was positive for intestinal *Escherichia coli* and enterococci, which accounted for 90% and 10% of normal intestinal flora, respectively. Multiple ulcers with a segmented pattern were noted in the terminal ileum on review of the colonoscopy (Figure 1). From the transverse to the sigmoid colon, the mucosa was rough and had erosive patches. The histopathology of biopsy specimens demonstrated acute and chronic nonspecific inflammation in the mucosa and submucosa, with a large quantity of plasma cell infiltration in the wall of the ileum, forming focal erosion and ulcers. The vasculature of the lamina propria was dilated and edematous. Radiographs of the whole digestive tract showed thickening of the terminal ileum mucosa, and peristalsis was poor (Figure 2), but the emptying function was normal. Abdominal computed tomography (CT) showed mild intestinal dilatation and wall thickening in the terminal ileum, and moderate ascites in the abdominal cavity (Figure 3). The mesenteric vasculature was normal, and the mesenteric lymph nodes were considered to have reactive hyperplasia. The proton pump inhibitor omeprazole was given to suppress acid production and protect the gastric mucosa, alleviating symptoms caused by the gastrointestinal tract ulceration. Meanwhile, the patient was given nutritional support to improve his nutritional status, probiotics to regulate intestinal flora, and an infusion of albumin combined with diuretic therapy to relieve edema. One month later, hemafecia occurred and worsened. Moreover, the patient's general condition worsened, with anemia and hypoalbuminemia. On day 36 of hospitalization, his intermittent transient abdominal pain suddenly worsened. Symptoms of peritoneal irritation and severe distention of the entire abdomen emerged, with a rapid pulse and low blood pressure. The patient was diagnosed with diffuse peritonitis and septic shock. Emergency laparotomy and intraoperative endoscopy revealed multiple bleeding ulcers scattered in the ileum and perforation at the side of mesenteric margin. About 120 cm of the ileum had multiple bleeding ulcers, and the ulcerative perforation was resected. Although it had some superficial ulcers, the preserved proximal small intestine was not bleeding. Management was aimed at avoiding postoperative short bowel syndrome. The edematous small intestinal wall and poor nutritional sta-

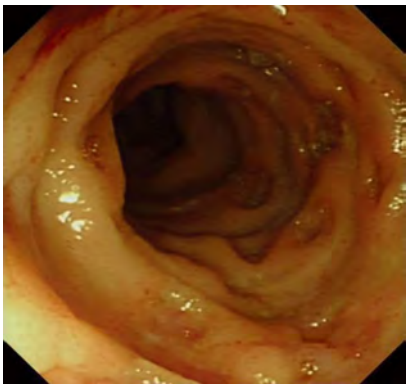

Figure 1 Colonoscopy demonstrated multiple ulcers with a segmented pattern in the terminal ileum.

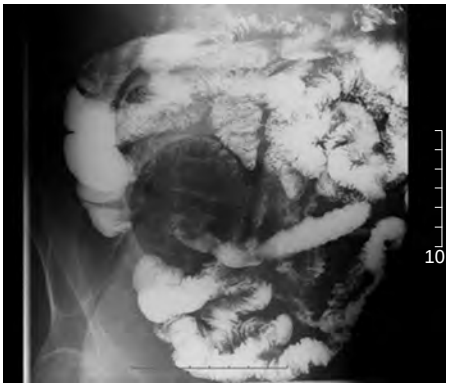

Figure 2 Radiography of the whole digestive tract showed that the terminal ileal mucosa was thickened and peristalsis was poor.

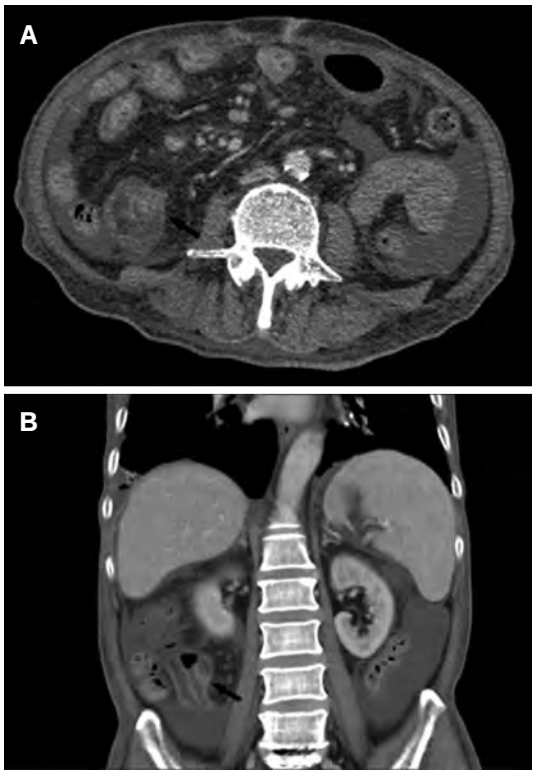

Figure 3 Abdominal computed tomography showed mild intestinal dilatation and wall thickening in the terminal ileum (marked with black arrow), and moderate ascites in the abdominal cavity. A: Horizontal slice; B: Coronal slice.

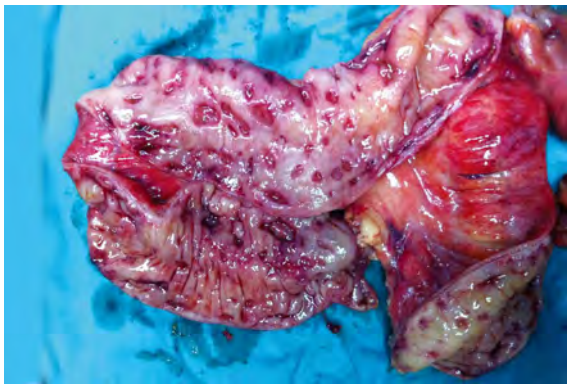

Figure 4 Multiple intestinal ulcers in the longitudinal section of the resected terminal ileum.

tus could have led to anastomotic leakage, therefore, a proximal and distal ileum double-lumen enterostomy was performed in the right lower quadrant of the abdomen.

Gross pathological examination was performed after the intestinal specimen was cut longitudinally. The intestinal ulcers were round or oval with a diameter of 0.3-1.5 cm. In some ulcers, the long axis was parallel to the intestinal long axis (Figure 4). A perforated ulcer could be seen at the side of the mesenteric margin. The mesentery was thick and edematous, with enlarged mesenteric lymph nodes. Postoperative histological examination demonstrated partial intestinal villous atrophy and multiple nonspecific ulcers in the terminal ileum (Figure 5A). The examination also showed chronic inflammatory infiltration, with extensive small lymphocytes and plasma cells in the lamina propria, submucosa, and serosa (Figure 5B). The mesenteric lymph nodes had histiocytes, which contained lymphocytes and plasma cells in the dilated sinuses. Phagocytosis similar to that of Rosai-Dorfman disease was noted. After surgery, the patient's vital signs were normal. However, on day 4, a large amount of bloody fluid began to leak from the ileal stoma. A blood transfusion and intravenous infusion did not alleviate symptoms of hemorrhagic shock. Bedside enteroscopy through the stoma showed multiple intestinal hemorrhages. Old and new ulcers were bleeding along 30 cm of the proximal intestine. Iced saline with norepinephrine and thrombin were used alternatively to irrigate the surface of the ulcers under enteroscopic guidance. The hemorrhage of the intestinal ulcers was controlled, but it recurred a few days later. Conservative treatment was continued. The patient's general condition gradually deteriorated due to recurrent bleeding. Ultimately, the patient died of pneumonia and respiratory functional failure.

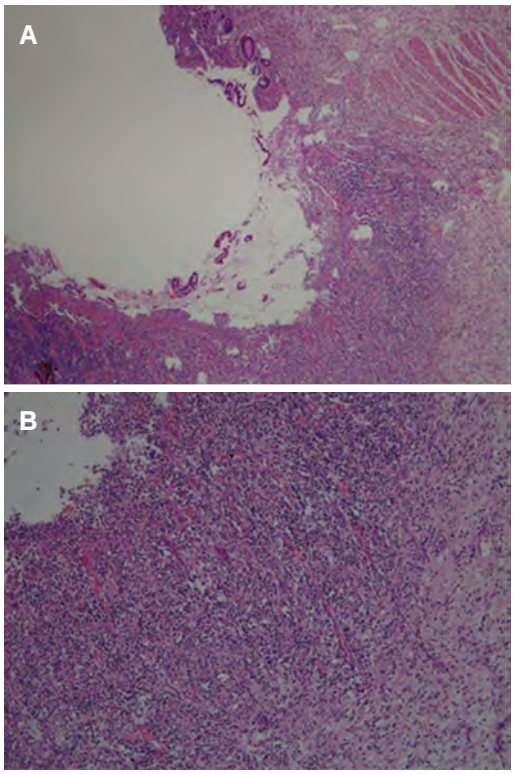

Figure 5 Histological examination. A: Glands of the small intestinal mucosa were necrotic and exfoliated, with intestinal villous atrophy and ulcer formation [hematoxylin and eosin (HE) stain, × 40]; B: Extensive small lymphocytes and plasma cell infiltration in the lamina propria, submucosa, and serosa of the terminal ileum (HE stain, × 100).

DISCUSSION

A variety of clinical conditions can lead to small intestinal ulcerations. Common causes include ischemia, trauma, nutritional disturbance, immune disorders, infection, drugs, and hormones. However, these causes can be eas-

ily excluded through medical history and laboratory test results. Therefore, most patients with small bowel ulcers have recognizable pathology. After a wide differential diagnosis, only those without an underlying disorder can be diagnosed as having ICUE.

The symptoms of small intestinal ulcers are nonspecific and insidious in the early stages. Patients with small intestinal ulcers usually present with chronic abdominal pain, diarrhea, a positive fecal occult blood test, intermittent hemafecia or melena, and a variety of types of malnutrition, including iron deficiency anemia, hypoalbuminemia, and weight loss. The diagnosis is often delayed or overlooked because the small intestine is the part of the alimentary canal most remote from both the mouth and the anus. Consequently, it is the most difficult to investigate. Patients typically experience exacerbations and complications such as intestinal hemorrhage, perforation, or obstruction, which always require emergency surgical intervention. With development of medical techniques such as gastrointestinal radiography, double-balloon enteroscopy[8], and capsule endoscopy[9], small intestinal ulcers can be diagnosed more accurately before surgery.

The patient complained chiefly of chronic abdominal pain for 2 mo, with many nonspecific gastrointestinal symptoms and increasing malnutrition. Later, symptoms of small intestinal ulcer bleeding and perforation emerged. We ruled out infective, ischemic, and tumor-related diseases. According to the laboratory results, we first excluded bacterial infection. With no history of atherosclerosis, hyperlipidemia, congestive heart failure, or arrhythmia, combined with normal findings on enhanced CT and ultrasonography of mesenteric vessels, we also ruled out mesenteric ischemia. Although the superficial and abdominal lymph nodes were enlarged, we obtained biopsy specimens from multiple sites during endoscopy and bone marrow biopsy and conducted a marrow chromosome examination to rule out lymphoma.

We needed to differentiate ICUE from diseases such as Crohn's disease, Behcet's disease, nonsteroidal anti-inflammatory drug (NSAID)-induced enteropathy, and cryptogenic multifocal ulcerous stenosing enteritis (CMUSE) because they also may cause multiple small intestinal ulcers.

During surgery, we found that the multiple intestinal ulcers mainly diffused in the terminal ileum. The ulcers were sharply demarcated with a round or oval shape, and the intervening mucosa was normal. The diameter of the ulcers ranged from 0.3 to 1.5 cm, with varying depths. The superficial ulcers were limited to mucosa or submucosa, while the deep ones can reach the serosa and even form a transmural perforation. The lesions were different from those seen in Crohn's disease, which are generally longitudinal, with a cobblestone appearance and fistulas.

ICUE can also be differentiated to the intestinal involvement of Behcet's disease. In Behcet's disease, ulcers are usually found from terminal ileum to the cecum, with transmural inflammation extending to the serous membrane and crater-shaped ulcer margins. In addition, Behcet's disease usually has a triad of symptoms consisting of aphthous stomatitis, genital ulcers, and ocular symptoms, which this patient did not have.

Since 1960, it has been recognized that NSAIDs can cause small intestinal ulcers. The macroscopic lesions of NSAID-induced enteropathy are characterized by multiple circumferential ulcers with severe concentric stenosis, referred to as "diaphragm disease"[10]. The different pathological characteristics in the patient and no history of NSAID use excluded NSAID-induced enteropathy.

CMUSE also can cause nonspecific multiple intestinal ulcers. Perlemuter *et al*[11] summarized the clinicopathological features of CMUSE as unexplained small intestinal strictures, superficial ulceration of the mucosa and submucosa, no biological signs of systemic inflammatory reaction, a chronic or recurrent clinical course even after surgery, and a positive response to use of corticosteroids. For our patient, the syndrome was characterized by recurrent intestinal bleeding and perforation, ulcers that were not limited to the submucosa, and no ulcerative stenosis or obstruction. Therefore, we excluded the diagnosis of CMUSE.

The etiology of ICUE is unknown, and symptoms of the disease are nonspecific and insidious. In the early stages, patients usually present with chronic abdominal pain and symptoms of malabsorption that do not respond to conservative treatments such as a gluten-free diet or adrenocorticosteroids. The clinical course could be severe and rapidly fatal due to complications such as hemorrhage, perforation, sepsis, and cachexia[12]. Surgical treatment is always required, after which the condition may recur and require repeat laparotomy. In many reports, ICUE was diagnosed after several operations and exclusion of other underlying disorders. The diagnosis was confirmed by histological features characterized by the diffuse nongranulomatous ulcerations called multiple nonspecific small intestinal ulcers. Some patients survive after radical, aggressive surgical resection[7]. In the present case, considering the advanced age and poor nutritional status of the patient and the risk of short bowel syndrome, part of the affected segment was preserved. Nevertheless, the conserved superficial ulcers deteriorated, and multiple new ulcers emerged; both were bleeding after surgery. Corticosteroid therapy is not effective in ICUE[13], and it is never used in patients with gastrointestinal hemorrhage, therefore, we avoided corticosteroid therapy. The ulcers were deep and transmural, thus, we believed that multiple intestinal perforations would emerge as the disease progressed. Postoperative aggravation of the ulcerative injury in the small intestine precluded reoperation and led to patient's debility and death.

In conclusion, early diagnosis and treatment are important in ICUE. Radical surgical resection is considered the best available treatment for patients presenting with abdominal ulcerative complications such as hemorrhage, perforation, and obstruction, although the true etiology of ICUE is unknown. When patients present with multiple, nonspecific, small intestinal ulcers in the absence

of well-documented causes, a diagnosis of ICUE should be considered. However, patients of advanced age with compromised nutritional status and severe complications cannot undergo aggressive surgery. In these patients, the prognosis is usually guarded.

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EDITORIAL

Acoustic radiation force impulse of the liver

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Abstract

Acoustic radiation force impulse (ARFI) imaging is a new and promising ultrasound-based diagnostic technique that, evaluating the wave propagation speed, allows the assessment of the tissue stiffness. ARFI is implemented in the ultrasound scanner. By short-duration acoustic radiation forces (less than 1 ms), localized displacements are generated in a selected region of interest not requiring any external compression so reducing the operator dependency. The generated wave scan provides qualitative or quantitative (wave velocity values) responses. Several non-invasive methods for assessing the staging of fibrosis are used, in order to avoid liver biopsy. Liver function tests and transient elastography are non-invasive, sensitive and accurate tools for the assessment of liver fibrosis and for the discrimination between cirrhotic and non-cirrhotic liver. Many published studies analyse ARFI performance and feasibility in studying diffuse liver diseases and compare them to other diagnostic imaging modalities such as conventional ultrasonography and transient elastography. Solid focal liver lesions, both benign and malignant, are common findings during abdominal examinations. The accurate characterization and differential diagnosis are important aims of all the imaging modalities available today. Only few papers describe the application of ARFI technology in the study of solid focal liver lesions, with different results. In the present study, the existing literature, to the best of our knowledge, about ARFI application on diffuse and focal liver pathology has been evaluated and results and statistical analyses have been compared, bringing to the conclusion that ARFI can be used in the study of the liver with similar accuracy as transient elastography in diagnosing significant fibrosis or cirrhosis and has got some advantages in respect to transient elastography since it does not require separate equipment, better displays anatomical structures and measurements can be successfully carried out almost in every patient.

Key words: Acoustic radiation force impulse imaging; Sonoelastography; Diffuse liver pathology; Focal liver lesion

Core tip: In the present study, the existing literature, to the best of our knowledge, about acoustic radiation force impulse (ARFI) application on diffuse and focal liver pathology has been evaluated and results and statistical analyses have been compared, bringing to the conclusion that ARFI can be used in the study of the liver with similar accuracy than transient elastography in diagnosing significant fibrosis or cirrhosis and has got some advantages in respect to transient elastography since it does not require separate equipment, better displays anatomical structures and measurements can be successfully carried out almost in every patient.

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INTRODUCTION

Acoustic radiation force impulse (ARFI) imaging is a new and promising ultrasound-based diagnostic technique that, evaluating the wave propagation speed, allows the assessment of the tissue stiffness[1-3]. ARFI is implemented in the ultrasound scanner and by using a conventional probe, without any need for external compression so reducing the operator dependency, it evaluates deep tissues stiffness providing complementary informations potentially useful for the diagnosis[1-6]. By short-duration acoustic radiation forces (less than 1 ms), it generates localized displacements in a selected region of interest (ROI; a box with dimension of 1 cm × 0.5 cm), identified on a conventional B-mode (Figure 1) image[7,8]. Depending on the interactions with the transducer[8,9], the generated wave scan provides qualitative (imaging) or quantitative (wave velocity values, measured in m/s) responses, by Virtual Touch Tissue Imaging and Virtual Touch Tissue Quantification, respectively (Siemens, Erlangen, Germany).

DIFFUSE LIVER DISEASES

Biopsy provides an extremely valuable contribution to the assessment of liver status in the case of chronic disease, offering information both on fibrosis and necro-inflammatory activity. However, not only the risk of complications, which have been reported with a frequency of 5%-20% for minor complications and 0.3%-0.5% for major complications[10] including also exceptional cases of death, but also contraindications, such as coagulopathy, poor patients cooperation or lack of consent, tend to limit its use, especially for repeated use over time. Furthermore, insufficient sampling and inter-observer variability may occur[11].

Considerable efforts have been made to develop non-invasive methods for assessing the staging of fibrosis, in order to avoid liver biopsy. In this setting the ideal method should be simple, inexpensive, easily available, repeatable and accurate.

Liver function tests [alanine aminotransferase (ALT), aspartate aminotransferase (AST), total proteins, serum albumin, gamma-globulins, gamma glutamyl transpeptidase (GGT), total bilirubin, alkaline phosphatase, prothrombin time/international normalized ratio] can be performed prior to the liver biopsy. The 2 main scoring systems used to predict and evaluate liver fibrosis are AST-platelet ratio index (AST level and platelet count) and FIBROMAX (Biopredictive, France) that combines the measurement of 10 indirect parameters adjusted for age, sex, weight and height: α2-macroglobulin, haptoglobin, apolipoprotein A1, total bilirubin, GGT, ALT, AST, fasting glucose, triglycerides and total cholesterol.

Transient elastography (TE) (Fibroscan, Echosense, Paris, France) has proved to be a non-invasive, sensitive and accurate tool for the assessment of liver fibrosis and particularly for the discrimination between cirrhotic and non-cirrhotic liver and its use is rapidly spreading. However, since it requires separate equipment, it means that at least one other examination is necessary in addition to conventional ultrasonography (US) of the liver, requiring additional time and costs after B-mode ultrasonography. Moreover, during TE examination, only A-mode imaging is displayed on the screen in order to select the area of scanning and, consequently, ligaments, vascular structures or even lesions, may inadvertently be included in the ROI, possibly affecting the final results.

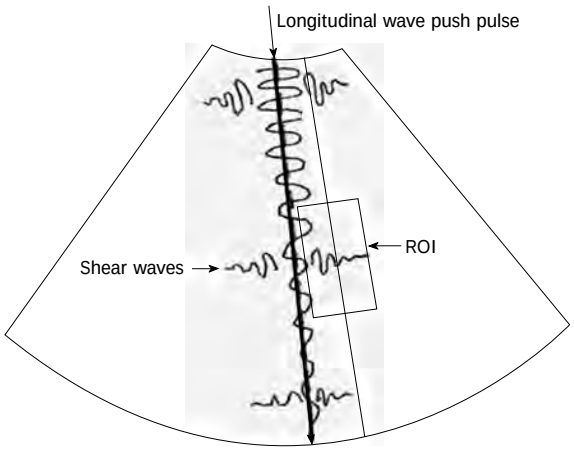

Figure 1 Acoustic radiation force impulse virtual touch tissue quantification technical scheme. ROI: Region of interest.

ARFI imaging offers the possibility of performing a quantitative measurement of the elasticity of the hepatic parenchyma during conventional US evaluations, without requiring additional transducers or other equipment[7].

Many studies analyse ARFI performance in studying diffuse liver diseases. Piscaglia *et al*[12] show that Virtual Touch Tissue Quantification is able to identify the presence of cirrhosis with good accuracy and produces results correlated with those obtained by transient elastography with Fibroscan. They performed measurement in the right lobe, by means of an intercostal scan, a condition which offers high inter-observer reproducibility (r = 0.874 in their series[12]).

The great advantage of fibrosis assessment using Virtual Touch Tissue Quantification is the fact that it can be performed at the same time as conventional US investigation. US is routinely used worldwide in the management of patients with chronic liver disease and is the first imaging technique employed when liver disease is suspected. With conventional US, certain features are highly specific for predicting severe fibrosis or cirrhosis (surface nodularity: specificity 95%; caudate lobe hypertrophy: 91%), but are not very sensitive (sensitivity of 54% and 41% respectively)[13]. Piscaglia *et al*[12] affirm that results in performing ARFI imaging have found to be similar to those of other works, all of which showed an area under the receiver operating characteristic curve (AUROC) above 0.9 for the diagnosis of cirrhosis with a cut-off value of 1.77 m/s (sensitivity 93%, specificity 85.1%). This cut-off value is very similar to those reported by Friedrich-Rust *et al*[7] (1.75 m/s), Sporea *et al*[14] and Takahashi *et al*[15], but differs from the ones obtained

Table 1 Mean wave propagation velocity values

Ref.	Value (m/s)
The right lobe of healthy liver	
Eiler *et al*[33]	1.16
Sporea *et al*[48]	1.19
Karlas *et al*[49]	1.19
Jaffer *et al*[50]	1.12
Marginean *et al*[51]	1.18 ± 0.27
Crespo *et al*[52]	1.06
Kircheis *et al*[53]	1.09 ± 0.13
Yoon *et al*[54]	1.06
Colombo *et al*[18]	1.40
Sporea *et al*[55]	1.28 ± 0.43
Noruegas *et al*[56]	1.11
Rizzo *et al*[57]	0.99
Karlas *et al*[58]	1.15 ± 0.17
Sporea *et al*[59]	0.97 ± 0.19
Son *et al*[60]	1.07 ± 0.11
Rifai *et al*[61]	1.10 ± 0.17
Kuroda *et al*[62]	0.99 ± 0.21
Popescu *et al*[63]	1.15 ± 0.21
Piscaglia *et al*[12]	1.13
Toshima *et al*[64]	1.15
Horster *et al*[65]	1.19
Goertz *et al*[66]	1.16 ± 0.11
Goertz *et al*[35]	1.09
D'Onofrio *et al*[32]	1.56
Fierbinteanu-Braticevici *et al*[17]	< 1.185 (cut-off)
Friedrich-Rust *et al*[7]	1.10
A liver with severe fibrosis (> F3)	
Ye *et al*[67]	1.69
Sporea *et al*[48]	1.43
Karlas *et al*[49]	1.43
Chen *et al*[68]	2.43 ± 0.13
Sporea *et al*[69]	1.60 ± 0.49 HBV; 1.55 ± 0.63 HCV
Crespo *et al*[52]	1.77
Kircheis *et al*[53]	1.44 + 0.26
Yoon *et al*[54]	1.89
Friedrich-Rust *et al*[70]	1.55
Colombo *et al*[18]	1.44
Sporea *et al*[55]	1.64 ± 0.51
Noruegas *et al*[56]	1.48
Rizzo *et al*[57]	1.70
Karlas *et al*[58]	1.70 if non-viral
Sporea *et al*[59]	1.71 ± 0.52
Kuroda *et al*[62]	1.61 ± 0.79 F3; 2.35 ± 1.11 F4
Toshima *et al*[64]	1.88
Sporea *et al*[21]	1.78 ± 0.77
Fierbinteanu-Braticevici *et al*[17]	> 1.54 (cut-off)
Takahashi *et al*[15]	2.57 ± 0.52 mean value for F4 (cut-off > F3 = 1.44)
Lupsor *et al*[16]	1.520 ± 0.575
Friedrich-Rust *et al*[7]	1.64

HBV: Hepatitis B virus; HCV: Hepatitis C virus.

in other studies[16,17] that reported slightly higher thresholds. In their series the good performance of the ARFI technique with the previously reported cut-off value was also confirmed by the testing in a population with cirrhosis proven by biopsy as the reference standard.

Other works compare ARFI imaging to TE by means of Fibroscan, such as the one from Colombo *et al*[18]. They similarly found that TE and ARFI are both highly effective in diagnosing cirrhosis, but they came to the conclusion that TE is probably more accurate in predicting significant fibrosis (AUROC of TE 0.897, AUROC of ARFI 0.815), although they could not demonstrate a statistically significant difference between the two curves. Their results were consistent with Boursier *et al*[19] and Lupsor *et al*[16] who found the same diagnostic accuracy for cirrhosis, but better performance of TE in predicting significant fibrosis (F2 or higher), but were at variance with three studies which instead found similar accuracies of TE and ARFI in diagnosing significant fibrosis[7,20,21].

Another interesting finding was that Virtual Touch Tissue Quantification measurements could be successfully carried out in all patients enrolled[12], while TE was unsuccessful in 7% of cases (*e.g.*, in patients with narrow intercostal spaces and in those with morbid obesity), as reported also in literature[22-26]. A possible explanation for this is that Virtual Touch Tissue Quantification may not be limited by narrow intercostal spaces or even by moderate excess weight, as it only requires the visible liver is not deeper than a fixed distance from the skin surface (in order to put the ROI in the parenchyma), while with TE the liver must not be more than 25 mm from the skin.

Regarding steatosis and inflammatory changes in diffuse liver disease, there is no agreement in the possible use of ARFI in diagnosing these parenchymal changes and in the effects of these changes themselves on ARFI measurements[20,27,28]. It seems unlikely that changes that minimally affect the parenchymal stiffness will be at this moment accurately depicted and diagnosed by using this non invasive technique.

NORMAL AND PATHOLOGICAL VALUES

Mean normal values and mean values indicating severe fibrosis (Table 1) range about 0.8-1.7 m/s (Figure 2A) and about 1-3.4 m/s (Figure 2B) respectively.

Fierbinteanu-Braticevici *et al*[17] demonstrated that ARFI elastography could predict fibrosis of F2 METAVIR stage or higher with a validity of 90.2%. They calculated the optimal cut-off point between stages F1 and F2 to be 1.21 m/s. At this value, ARFI elastography had a sensitivity of 89.4% and a specificity of 100%. In their work, they also demonstrated that ARFI can predict even better F3 and F4 stage fibrosis. The optimal cut-off value to identify fibrosis stage F3 or higher was 1.54 m/s, with sensitivity and specificity of 97% and 100% respectively. The optimal cut-off value in predicting cirrhosis (stage F4) was 1.94 m/s with a sensitivity of 100% and a specificity of 98.1%.

In chronic viral hepatitis, the knowledge of the stage of liver fibrosis is important for prognosis and for decisions about antiviral treatment[29]. Fibrosis staged higher than F2 is an indicator for antiviral treatment, hence the great therapeutic value of a highly accurate diagnostic test. Moreover, early detection of significant fibrosis (F3 or higher) is essential since patients with significant fibrosis are at high risk of developing complications, such as portal hypertension or hepatocellular carcinoma, and consequently need specific follow-up[17].

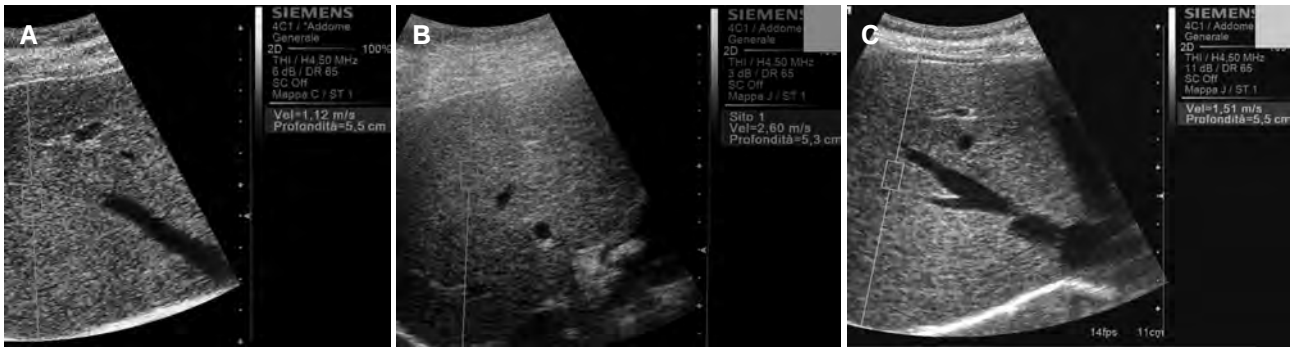

Figure 2 Acoustic radiation force impulse imaging of the liver. A: Normal value in healthy liver. B: Cirrhosis. C: Outlier value in healthy liver.

At present, it's difficult to determine the real impact of ARFI in the early diagnosis of hepatic fibrosis[17,27]. According to Fierbinteanu-Braticevici *et al*[17], in fact, there is a values overlap between F0-F1 and F2 fibrosis stages. The increase in liver propagation velocity has been demonstrated to be more important between stages F2 and F3 than between F1 and F2. This is consistent with the fact that the increase in fibrous tissue is more important between stages F2 and F3 than between F1 and F2. This limit of ARFI was overcome by the fact that fibrosis staged F2 or higher is considered a hallmark of progressive liver disease, therefore these are the patients in which there is a stronger indication for treatment as compared with patients with no or mild fibrosis[30,31].

There is in fact a range of variability of normal and pathological values in the Literature (Table 1). So what is important is to give the correct task to this new technique at present. The correct use of this technique has to be based on the true possibility of this system to detect changes in liver stiffness related to the development of different amount of fibrosis. The risk that absolutely should be avoided is to overestimate pathology and to look for inconsistent diseases. Therefore, in conclusion, the normal cut-off values must not be too strict but perhaps they also have to be adapted from time to time in relation to clinical and technical setting and from measurement to measurement.

In example, variability in the normal value is reported in literature[32] with a mean value of about 1.5 m/s in healthy subjects. This result can be considered an outlier[33] but however possible (Figure 2C). Moreover higher values can be obtained measuring in the left lobe[12,32] and in the superficial part of the right lobe[32]. This last aspect can be contrarily absent in child[33] due to a lower age-related fibrosis in the superficial liver parenchyma. Also in other published series Virtual Touch Tissue Quantification results in the right and left liver lobes did not appear to be strictly similar and, on average, the stiffness values were found to be higher in the left lobe than in the right lobe, at least in patients with chronic hepatitis (68% of patients had higher values in the left lobe than in the right lobe). Furthermore the diagnostic capacity to establish the histological degree of liver fibrosis (with a reference biopsy taken in the right lobe) was lower in Virtual Touch Tissue Quantification measurements from the left lobe than from the right lobe. These data, however, are not to be considered as a limitation of Virtual Touch Tissue Quantification to date, since they may perhaps more correctly reflect real differences and heterogeneity in the disease progression rates between the two lobes. It was in fact demonstrated that when two biopsies were taken in the two lobes during laparoscopy, a difference in one fibrosis stage between the two lobes occurred in up to 33% of cases[34]. However since our reference standard for the assessment of fibrosis in chronic liver disease is biopsy of the right lobe of the liver, it is recommended to measure liver stiffness by Virtual Touch Tissue Quantification in this lobe. Moreover, an approach with multiple measurements in various liver sites is worthy of further investigation as it may lead to interesting and original diagnostic results. In addition, Goertz *et al*[35] suggest to perform Virtual Touch Tissue Quantification *via* intercostal access in order to minimize invalid measurements and standard deviation. In their series, in fact, values taken subcostally were slightly higher than those measured through an intercostal approach.

Also some technical aspects need to be taken into account because they may explain some variability among published data. In example, the new release of the system is based on two acoustic pulses laterally to the ROI one by one at both sides and the maximum depth of the system nowadays achievable is 8 cm. Based on these considerations, the data published in the more recent papers should be more indicative of what can be obtainable with the new systems.

A recent study by Han *et al*[36], compares ARFI performance to Doppler parameters and describes a weak but significant relationship between liver stiffness, measured by ARFI, and the parameters related to the portal pressure, as measured by Doppler US in patients with liver cirrhosis at different Child-Pugh stages, but having no oesophageal varices. The study demonstrates a positive correlation between the median ARFI sonoelastographic velocity, which reveals liver stiffness, and the flow parameters of Doppler US, which reflects portal hypertension. All these features, however, appear in advanced stage of disease.

Regarding the possible role of ARFI, it can be for sure

Table 2 Diagnostic accuracy of non invasive methods for identifying severe liver fibrosis (> F3)

	AUROC	Ref.
Laboratory test (APRI score)	0.80	Lin *et al*[71]
	0.76	Friedrich-Rust *et al*[7]
	0.84	Leroy *et al*[72]
Transient elastography	0.87	Boursier *et al*[73]
	0.96	Ferraioli *et al*[74]
	0.90	Friedrich-Rust *et al*[7]
Acoustic radiation force impulse	0.91	Friedrich-Rust *et al*[7]
	0.90	Lupsor *et al*[16]
	0.99	Fierbinteanu-Braticevici *et al*[17]

AUROC: Area under the receiver operating characteristic curve; APRI: Aspartate aminotransferase platelet ratio index.

employed in the follow up of cirrhotic patients in order to avoid multiple biopsies comparing the result before and after treatment. Liver biopsy is not suitable for repeated evaluations because it is invasive and can cause major complications (0.3%-0.5%)[37]. Moreover liver fibrosis is a sequential and continuous process, and the staging of liver fibrosis should be evaluated frequently (Table 2). In contrast to liver biopsy, ARFI imaging is not invasive and can be repeated many times in the same patient[27].

FOCAL LIVER LESIONS

Solid focal liver lesions are common findings during abdominal examinations. The accurate characterization and differential diagnosis are important aims of all the imaging modalities available today[38-43]. Only three papers describe the application of ARFI technology in the study of solid focal liver lesions, with different results[44-46].

The first human images of hepatic malignancies acquired *in vivo* using the ARFI technique or any other elasticity imaging technique appeared in the work of Fahey *et al*[44]. His group compared B-mode and ARFI images both qualitatively (assessing the lesion margins definition by B-mode ultrasonography and ARFI imaging) and quantitatively (comparing the images contrast for both the techniques). They came to the conclusion that lesions margins definition at ARFI imaging was superior to that seen at B-mode US imaging (qualitative analysis). They also calculated that ARFI imaging can provide improvements in defining the contrast of tissue masses demonstrating, in fact, that the mean contrast for suspected hepatocellular carcinoma (HCC) in B-mode imaging was 2.9 dB (range 1.5-4.2 dB) *vs* 7.5 dB (range 3.1-11.9 dB) in ARFI images, with all HCCs appearing less stiff (brighter) than regional cirrhotic non-tumorous liver parenchyma. Moreover, the mean contrast for hepatic metastases in B-mode images was 3.1 dB (range 1.2-5.2 dB) *vs* 9.3 dB (range 5.7-13.9 dB) in ARFI images. Metastatic lesions in fact are stiffer (darker) than regional non-cirrhotic, non-neoplastic liver parenchyma. Fahey *et al*[44] also stated that combined US/ARFI could find application in tumor screening, lesion characterization and early detection of disease. Since HCC screening is not considered cost-effective in regions with low prevalence, due to the low sensitivity of both sonography and serum AFP sampling[47], ARFI imaging can improve sensitivity and cost-efficiency given its low cost, its capability of improving tumor contrast in comparison to US alone. If ARFI imaging had been proven to be a feasible alternative to contrast-enhanced ultrasound for liver applications, it could hold potential advantages related to the cost and complexity of the imaging protocol used for HCC screening. The authors, however, did not take into account the mechanical response of benign abdominal masses to applied radiation forces, thus they couldn't evaluate the ability of ARFI in differentiating benign from malignant liver masses.

More recent works about ARFI imaging applied to solid focal liver lesions are the ones from Cho *et al*[45] and from Gallotti *et al*[46]. The first one evaluates ARFI values calculated on HCCs, metastases, cholangiocarcinomas and hemangiomas, the second one evaluated in addition adenomas and focal nodular hyperplasia (FNHs), but did not consider cholangiocarcinomas.

Benign lesions

In regard to hemangiomas, Gallotti *et al*[46] agree with Cho *et al*[45] about the great variability of this type of lesions (mean wave velocity value of the lesion 2.30 m/s; mean wave velocity value of the surrounding parenchyma 1.45 m/s), since its stiffness depends on the amount of fibrotic septa which divide the dilated vascular space.

For the first time in Gallotti *et al*[46] paper also FNHs and adenomas were studied. FNH resulted the stiffest lesions after metastases and cholangiocarcinomas, indipendently from their dimensions and from the presence or absence of central scar. In fact, even if present, the ROI has to be located out of the fibrotic central scar. The high stiffness (mean wave velocity value of the lesion 2.75 m/s; mean wave velocity value of the surrounding parenchyma 1.57 m/s[46]) is explained with the well known high fibrotic content of this type of lesion. Thus, if the result will be confirmed by further studies, the cut-off of 2 m/s, suggested by Cho *et al*[45] to distinguish benign from malignant lesions, can no longer be used.

On the other hand, adenomas showed wave velocity values similar to those observed in the surrounding liver[46]: this is a solid focal liver lesion, the softest analysed (mean wave velocity value of the lesion 1.25 m/s; mean wave velocity value of the surrounding parenchyma 1.40 m/s). The presence of cells similar to normal hepatocytes and few stroma explain the low mean wave velocity value calculated in adenomas compared to other focal liver lesions.

Malignant lesions

According to Fahey *et al*[44], but inconsistent with results of Cho *et al*[45] despite the similar diameter of the lesions, in Gallotti *et al*[46] almost all the HCCs evaluated resulted in softer lesions compared to the surrounding cirrhotic liver (mean wave velocity value of the lesion 2.17 m/s;

mean wave velocity value of the surrounding parenchyma 2.99 m/s), and the main elastographic value was significantly lower than that of the surrounding parenchyma. This discrepancy might be explained by the difference in the severity of the cirrhosis of the background liver in each study population. In Cho *et al*[45] the degree of liver cirrhosis of patients with HCCs was likely to be less severe (15 out of 20 patients with HCCs had chronic liver disease of Child-Pugh classification A) compared with that seen in the other studies, assuming that the liver is stiffer with more severe liver cirrhosis.

There is concordance[44-46] about the fact that all metastatic lesions (mean wave velocity value of the lesion 2.87 m/s; mean wave velocity value of the surrounding parenchyma 1.63 m/s[46]) and, when considered, cholangiocarcinomas, are stiffer than the surrounding liver. This is probably due to the presence of fibrous content potentially found in many of these lesions. The presence of necrotic degeneration, mainly in the biggest masses, does not influence the results since the ROI for the stiffness calculation has to be accurately positioned out of the necrotic portion. Summarizing, based on the preliminary results of the study of solid focal liver lesions[44-46], it can be also concluded that ARFI seems to be an useful in the following scenarios: (1) for differential diagnosis between adenomas and FNHs; (2) for the study of metastases; and (3) for the study of HCCs in cirrhotic liver. Future perspective could be the application of ARFI in liver lesion detection by using volumetric automated acquisition.

CONCLUSION

Virtual Touch Tissue Quantification is a new non invasive imaging based technique able to estimate liver stiffness diagnosing cirrhosis with a good accuracy. The first assessment of patients with a suspicion of liver disease can be therefore easily performed with both conventional ultrasonography and Virtual Touch Tissue Quantification for liver stiffness assessment in a single step.

In conclusion, several studies about ARFI application in diffuse liver pathology have been made, and most of them state that ARFI itself can be used in the study of the liver with similar accuracy than transient elastography in diagnosing significant fibrosis[7,20,21] or cirrhosis[12,16,19]. However, ARFI has got some advantages in respect to TE since it does not require separate equipment and consequently it is not necessary an additional examination in addition to conventional US, saving time and costs. Moreover, during TE examination, only A-mode imaging is displayed on the screen in order to select the area of scanning and, consequently, ligaments, vascular structures or even lesions, may inadvertently be included in the ROI, possibly affecting the final results.

Another interesting finding is that Virtual Touch Tissue Quantification measurements can be successfully carried out almost in every patient while TE is unsuccessful in 7% of cases (*e.g.*, in patients with narrow intercostal spaces and in those with morbid obesity), as reported also in literature[22-26]. On the contrary, there are just few indications about ARFI and focal liver lesions, so further studies are needed in order to find ARFI the correct place in the everyday clinical practice.

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REVIEW

New ultrasound techniques for lymph node evaluation

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Abstract

Conventional ultrasound (US) is the recommended imaging method for lymph node (LN) diseases with the advantages of high resolution, real time evaluation and relative low costs. Current indications of transcutaneous ultrasound and endoscopic ultrasound include the detection and characterization of lymph nodes and the guidance for LN biopsy. Recent advances in US technology, such as contrast enhanced ultrasound (CEUS), contrast enhanced endoscopic ultrasound (CE-EUS), and real time elastography show potential to improve the accuracy of US for the differential diagnosis of benign and malignant lymph nodes. In addition, CEUS and CE-EUS have been also used for the guidance of fine needle aspiration and assessment of treatment response. Complementary to size criteria, CEUS could also be used to evaluate response of tumor angiogenesis to anti-angiogenic therapies. In this paper we review current literature regarding evaluation of lymphadenopathy by new and innovative US techniques.

Key words: Lymph nodes; Ultrasound; Endoscopic ultrasound; Lymph node metastasis; Lymphoma

Core tip: The differentiation of malignant from benign lymph nodes by ultrasound, computed tomography and magnetic resonance imaging traditionally relies mainly on size measurements and topographic distribution. However, sensitivity and specificity in the differentiation of benign and malignant lymph nodes are disappointing using only size parameters. The presented paper is intended to discuss, comment and illustrate the clinical important work-up of lymphadenopathy with respect of recently introduced imaging techniques including contrast enhanced ultrasound and elastography.

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INTRODUCTORY CONSIDERATIONS

The differentiation of malignant from benign lymph nodes by ultrasound (US), computed tomography (CT) and magnetic resonance imaging (MRI) traditionally relies mainly on size measurements and topographic distribution[1-3]. However, sensitivity and specificity in the differentiation of benign and malignant lymph nodes are disappointing using only size parameters. Reasons for the low accuracy include that malignant lymph node infiltration occurs in up to 30% in lymph nodes of less than 5 mm which has been shown for lung, esophageal, gastric, pancreatic and rectal carcinoma[4-10]. The evaluation of shape and border often adds no or only little more information

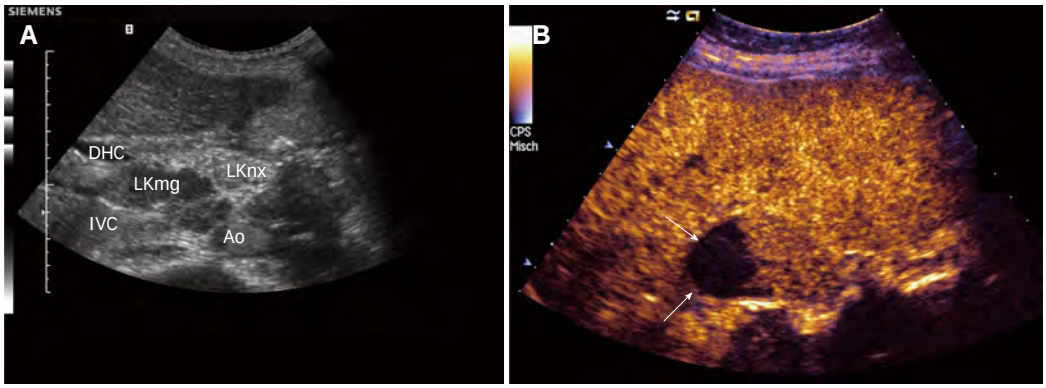

Figure 1 Lymph node infiltration, carcinoma. A: With lymph node (LN) specific contrast agents malignant infiltration can be delineated (LKmg) as focal hypoenhancement in the upper part of this perihepatic LN. The lower part (LKnx) shows normal (physiological) enhancement; B: With SonoVue®. Necrotic (non-enhancing, arrows) areas can be detected within this perihepatic lymph node. Necrotic areas are typically for carcinoma infiltration and tuberculosis. IVC: Inferior vena cava; Ao: Aorta; DHC: Common bile duct.

to exclude malignancy[11,12]. New imaging methods should be able to delineate the early and circumscribed malignant infiltration and to improve ultrasound guided biopsy.

Colour Doppler ultrasound (CDI) adds value for the differentiation of malignant from normal or reactive nodes by displaying the macrovessel architecture. Normal LNs generally show hilar predominant normal vascularity. Inflammatory lymph nodes are typically more vascularised without changes of the predominant hilar vessel architecture. In contrast metastatic lymph nodes present peripheral or mixed vascularity and loss of hilar type of vascularisation[13].

Contrast enhanced CDI has improved the viusalisation of macrovessels (angioarchitecture) but does not allow evaluation of microvessels[14]. Demonstration of malignant neovascularisation, *e.g.*, vessels penetrating the LN capsule, has been used as the characteristic feature of lymph node metastases.

Spectral Doppler ultrasound contributes to differentiation of malignant and benign solid neoplasia[15]. Likewise, normal and inflammatory lymph nodes show lower vascular resistance [resistive index (RI)] as compared to malignant lymph nodes[16] but overall results are disappointing.

Although Doppler ultrasound techniques have extended the opportunities for the differentiation of malignant from benign lymph nodes by displaying changes of macrovascularity and the vascular resistance[13,17,18], they do not improve lymph node detection rate and vascularity is often not detected in small lymph nodes[19]. Therefore, Doppler techniques and contrast enhanced Doppler techniques in general have not significantly improved the diagnostic work up of lymphadenopathy. There is a need for new imaging techniques for better characterisation of lymph nodes with the opportunity to assess also the internal microvessel architecture of lymph nodes and tissue elasticity for detection of early circumscribed malignant infiltration.

In the presented paper we discuss current knowledge about recent advances in ultrasound technology for improved lymph node evaluation.

CONTRAST ENHANCED ULTRASOUND

Contrast enhanced ultrasound (CEUS) is the application of ultrasound contrast agents (UCA) to traditional sonography. The currently used UCA are microbubbles stabilized by a shell which has a high degree echogenicity. Since their physical size is just 1-4 micrometres in diameter (equal to or smaller than red blood cells), UCA allow depiction of both the macrovasculature and the microvasculature[20]. CEUS has been introduced more than ten years ago and guidelines have been published for the liver[20,21] and non-liver indications[22]. Currently 4.8 mL SonoVue® is recommended for imaging superficial LNs with a high frequency probe and for imaging the mediastinal and abdominal LNs with a high frequency endoscopic probe in CE-EUS.

CEUS techniques provide information on vascularisation and perfusion patterns, and exploit the differences in blood flow characteristics between normal and pathological tissue but knowledge about lymph node evaluation is limited[22]. CEUS could be helpful by identifying changes in vascular architecture of macro- and micro-vessels and avascular areas as signs of malignant infiltration.

Carcinoma

Carcinoma infiltration causes the development of pathological vessels (neoangiogenesis) and, therefore, a change of the perfusion pattern with heterogeneous enhancement due to the presence of caliber changes of the neoplastic vessels and arteriovenous shunts[23-27]. Focal hypoenhancement may result from the partial insufficiency of blood-supply due to overpressure in the LN caused by the neoplastic infiltration. Malignant lymph nodes not only have a greater number of peripheral vessels, but also longer contrast enhancement duration than benign lymph nodes[28]. Destructive avascular necroses are an important imaging sign for malignant infiltration (Figures 1-3). Avascular areas are detected by the lack of contrast agent uptake in the necrotic zones and the peripherally located pronounced hyperenhancement (rim enhancement)[29,30]. The contrast enhancement pattern of focal cortical

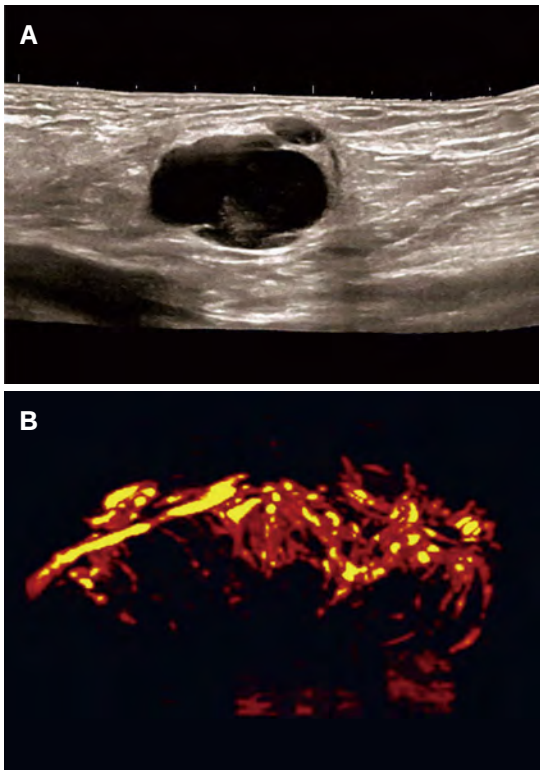

Figure 2 Carcinoma infiltration. Typically vessel destruction with chaotic vessels in the lymph node can be observed. B-mode (A) and 3D angiographic mode (B).

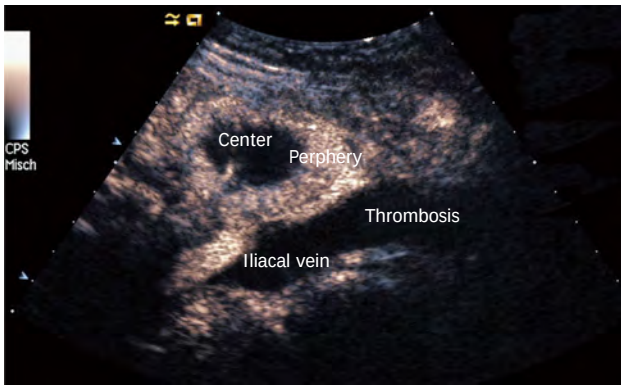

Figure 3 Prostate carcinoma infiltration of the pelvis. Typically vessel destruction with interruption of vessel architecture can be observed in patients with carcinoma. The center of the lymph node is almost non-enhancing except one visible vessel whereas the periphery shows hyperenhancement. Thrombosis of the iliac vein is indicated as well.

thickening has been also identified as an important sign to differentiate benign and malignant lymphadenopathy. In benign lymph nodes contrast enhancement within the cortex is homogeneous, whereas in malignant lymph the cortical thickening is less well vascularized than the adjacent normal lymph node parenchyma[24].

In conclusion, criteria for carcinomatous lymph node infiltration on CEUS are centripetal inhomogeneous enhancement and perfusion defects.

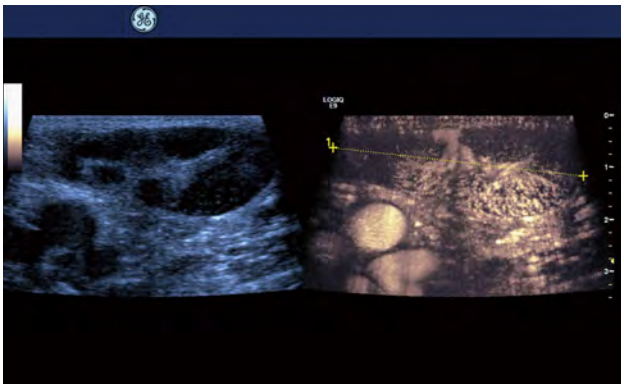

Figure 4 Lymph node infiltration (50 mm), non-Hodgkin's lymphoma. Typically the hilum predominant vessel architecture is preserved (between markers).

Lymphoma

It is essential to consider lymphoma separately because some of its features are different from other LN disease[13,31]. The very few studies published so far showed that in lymphoma contrast enhancement patterns are highly variable. The most often observed pattern is intense homogeneous enhancement, which is not different from reactive inflammatory lymph nodes[25,31] (Figure 4).

In conclusion, there is evidence that the vascular pattern of lymphomatous lymph node infiltration resembles that of non-malignant nodes.

Inflammation

Most inflammatory processes do not change the hilum predominant vessel architecture of lymph nodes. According to the majority of published papers, normal and inflammatory LNs are characterized by a centrifugal and homogeneous enhancement pattern[23-26] (Figure 5). Therefore, inflammation changes the enhancement pattern only by the amount (peak) enhancement but not by changes of distribution. It is worth mentioning that non-destructive necrosis, which is reflected in avascular areas on CEUS, can be also found in granulomatous lymphadenitis, *e.g.*, cat-scratch disease (bartonellosis), tuberculosis and sarcoidosis.

Treatment response

Changes and reduction of intranodal vascularity may be the first sign of response to antineoplastic treatment as shown for gastrointestinal stromal tumors and renal cell carcinoma[22,32]. Since tumour growth depends on neovascularization, CEUS can also help to detect focal nodular tumour recurrence in scars and to guide biopsy[33]. In Hodgkin's disease well demarcated avascular areas have been described as a typical sign of treatment response[34,35].

Dynamic contrast enhanced ultrasound

Quantification software (time intensity curve analysis) has been used for the differential diagnosis of benign and malignant LNs but results so far are conflicting[36]. There

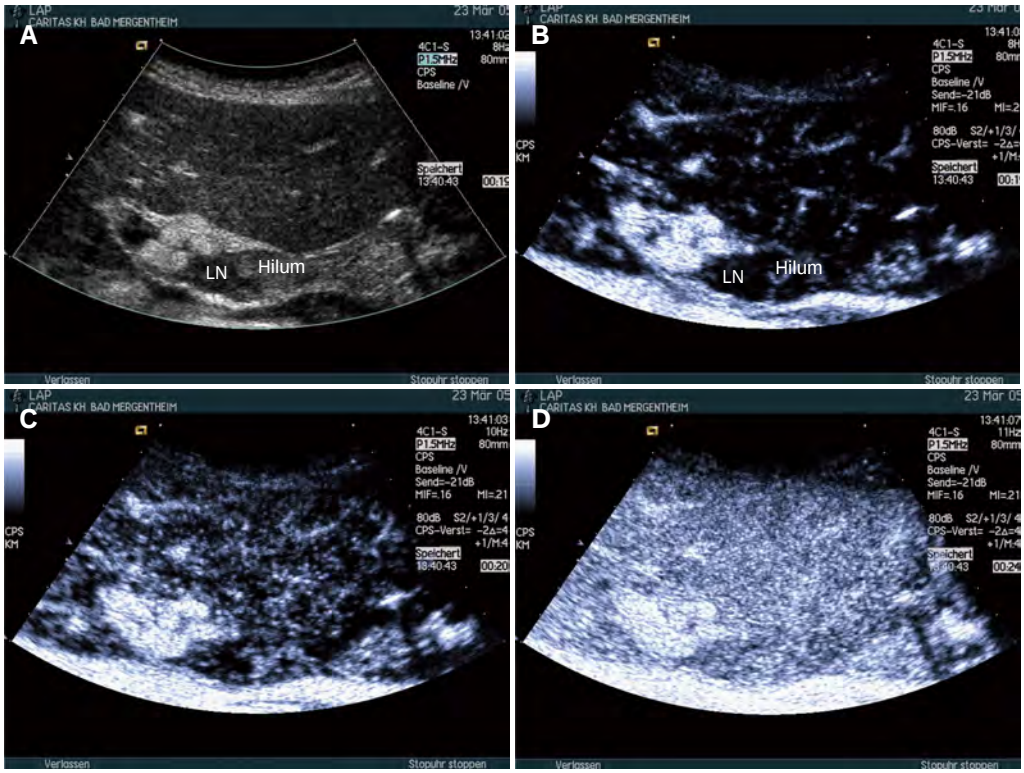

Figure 5 Inflammatory perihepatic lymph node dorsal in the hepatoduodenal ligament. Inflammation most often shows no changes of the symmetric lymph node vascularity and homogenous contrast enhancement (A-D). The hilum is indicated. LN: Lymph node.

are two studies showing that the difference of intensity between the hypervascular and hypovascular regions was significantly higher in metastatic than in non-metastatic LNs[26,37]. Steppan *et al*[38] reported that malignant compared to benign lymph nodes showed higher maximum intensity and duration of enhancement while Yu *et al*[25] reported no significant differences on maximum intensity. Time to peak intensity and area under the curve of malignant lymph nodes and lymphomas were less than that of benign LNs. Ahuja could demonstrate a reduction of vessel density (vascularity) and delay in the time to peak enhancement after treatment. It has to be mentioned that the changes in peak enhancement were operator dependent[33]. Since evidence is inconsistent quantification techniques cannot be generally recommended for clinical use. European Federation for Ultrasound in Medicine and Biology (EFSUMB) has published recommendations on the use of dynamic contrast enhanced ultrasound (DCE-US) discussing the current use and limitations in detail[39].

In conclusion, contrast enhanced techniques compared to conventional ultrasound may improve the differential diagnosis of benign LNs from malignant LNs and provide a more accurate selection of nodes to be submitted to fine-needle aspiration biopsy[25,28].

ELASTOGRAPHY

Elastography is a non-invasive method in which the stiffness of the tissue can be imaged as colour map or shear wave velocity. Two main forms of elastography have been studied for the evaluation of lymph nodes. One form is strain elastography (SE). The ultrasound probe is used to palpate the tissue[40] usually transcutaneously but optionally also intra-operatively or *via* an endoscope[41-45]. The tissue deformation produced (*i.e.*, strain) is assessed by following the way the speckle in the image moves, usually with a tracking algorithm working on the radio-frequency data. The data can then be used to form an image that is coded in colour or grey-scale to show the pattern of strain, which is inversely related to tissue stiffness. Therefore, SE allows assessment and visualization of relative elasticity differences. The area to be evaluated is defined by a ROI in a similar way to CDI[44,46]. New technical developments allow for averaging over several frames to calculate the mean histogram value which corresponds to overall elasticity within a selected area[47]. Comparing two different areas within the ROI allows calculation of the strain ratio. SE is the most commonly used method for the evaluation of lymph nodes. The other forms of elastography are shear wave elastography techniques (SWE) which include transient elastography (TE) (*e.g.*, Fibroscan™, Echosens, France), Acoustic Radiation Force Impulse imaging (*e.g.*, ARFI, Siemens, Germany) and Supersonic Shear Wave Imaging (SSI) (Supersonic, France). In shear wave elastography the "pushing" ultrasound beam causes minute displacements in soft tissue, which depend on the magnitude of tissue stiffness. Using tracking algorithms, the resulting shear waves can be detected sonographically. So far only SSI has been studied for the evaluation of LN. In elastographic images

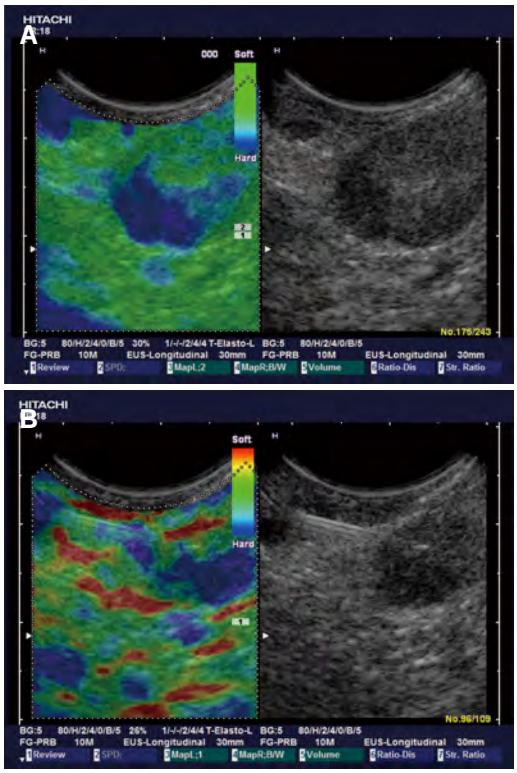

Figure 6 Colorectal carcinoma with presacral circumscribed lymph node metastasis proven by colonic endoscopic ultrasound using Fine Needle Aspiration Cytology. Sonoelastography reliability test evaluation reveals typically harder (blue) area in the lymph node.

of normal lymph nodes the nodal cortex is significantly harder than the medulla and the hilum[48,49].

EFSUMB has prepared recommendations on the use of elastography. In two sets of papers the techniques are explained in more detail[50,51].

Carcinoma

Typically the well differentiated carcinoma at least initially infiltrates lymph nodes in a circumscribed manner (focally stiffer, harder) (Figure 6), whereas the undifferentiated carcinoma leads to a diffuse (stiffer, harder) infiltration.

Suspected cervical LN metastases from hypopharyngeal and thyroid carcinomas have been recently investigated using SE (real time elastography)[39,52]. An elasticity index has been created by comparing the elasticity of the LN with the surrounding head and neck muscle tissue (muscle to LN strain ratio). Using a ratio of > 1.5 as an indicator of malignant infiltration, sensitivity was 82% and specificity 98% which is superior to the best B-mode criteria[52]. These data have been reproduced by Tan *et al*[53]. Moreover, interobserver agreement with SE was very high (kappa 0.828-0.946)[53].

Applying a higher cut-off value for strain ratio (1.78) Teng *et al*[54] at the cost of an only moderate specificity (65%) reported a very high sensitivity (98%) for discriminating malignant from benign suspicious cervical lymph nodes.

Other authors used a scoring system (percentage of blue-coding lymph node area) to differentiate malignant from benign lymph nodes in head and neck cancer patients. A blue coded (hard) area of > 50% of total lymph node area (score 3 and 4) or observation of a central necrosis (score 5) predicted malignant infiltration with high accuracy and added value to traditional ultrasound criteria[55-57].

So far two papers are published for the differential diagnosis of lymph nodes using shear wave elastography on 55 cervical enlarged lymph nodes using SSI. Malignant nodes were homogeneously stiffer than benign lymph nodes. The sensitivity (42%), specificity (100%) and accuracy (62%) were promising defining a cut-off level of 30.2 kPa[58]. The intra- and inter-observer reliability of shear wave elastography using SSI was shown to be fair to excellent for 176 neck lesions according to the intraclass correlation coefficients (0.78-0.85)[59].

In conclusion, elastography seems to be a very promising diagnostic tool for the differentiation between benign and malignant lymph nodes. This is reflected by a recent meta-analysis which reported a pooled sensitivity and specificity for the diagnosis of malignant lymph nodes of 74% and 90% for elasticity scores, and 88% and 81% for strain ratio, respectively[60]. However, to date studies comparing the two techniques of elastography (SE and SWE) are lacking.

Lymphoma

Knowledge of strain imaging in lymphoma is very limited. So far different lymphoma cannot be differentiated. Initial experience suggests that focal lymph node infiltration (Figure 7A) is indicative for low grading of follicular lymphoma whereas diffuse and homogenous lymph node infiltration is typically found in high grade lymphoma (Figure 7B).

Inflammation

Most inflammatory processes do not change the elastographic architecture of lymph nodes. The hilum in normal lymph nodes remains softer than the stiffer cortex also in inflammatory lymph nodes. Circumscribed softer (and stage dependent also stiffer) lymph node areas are found in tuberculosis but this has only been shown in few cases.

ENDOSCOPIC ULTRASOUND

Diagnostic and therapeutic endoscopic ultrasound has been established in the last thirty years[61,62]. The technique can be also applied with colour Doppler imaging as discussed above. Recently CE-EUS and real time endoscopic elastography (RTE-EUS) have been introduced.

Contrast enhanced endoscopic ultrasound

Contrast enhanced endoscopic ultrasound (CE-EUS) is CEUS performed with an endoscopic probe, which can be performed on both Doppler mode with high MI and contrast specific mode with low MI[63] to also guide therapeutic procedures. The dose of the ultrasound contrast agent (UCA) should be 4.8 mL for SonoVue®. CE-EUS

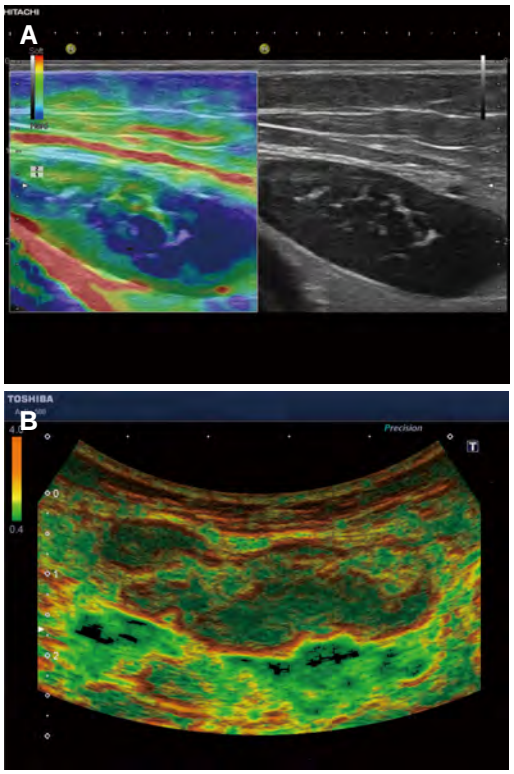

Figure 7 Non-Hodgkin's lymphoma involving the inguinal region. A: Sonoelastography reliability test evaluation reveals typically asymmetric and circumscribed infiltrated harder (blue) lymph node tissue in low grade follicular cell lymphoma; B: Elastography (acoustic structured quantification) reveals mainly homogenous diffuse infiltration in high grade follicular cell lymphoma.

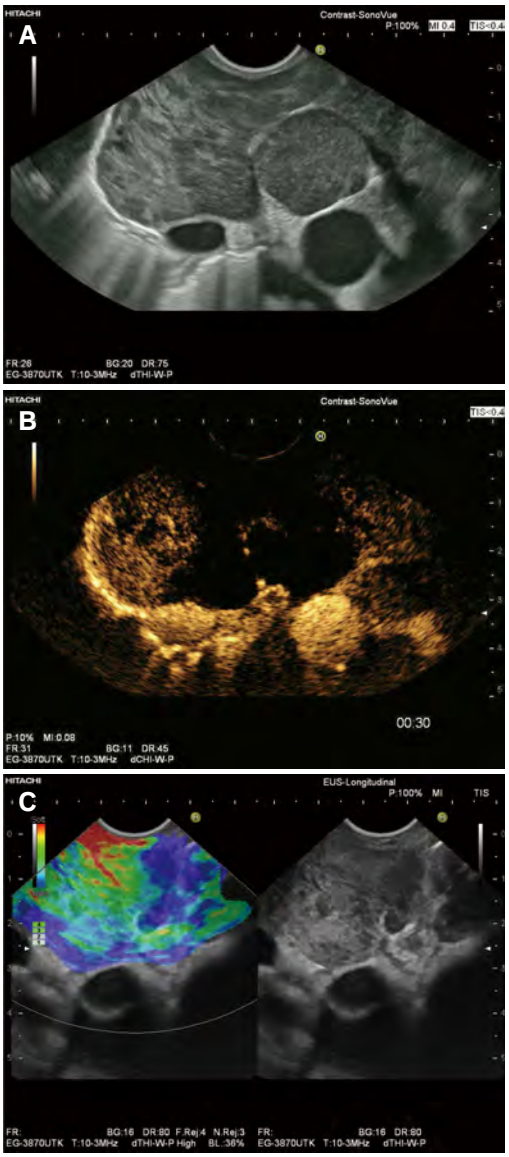

Figure 8 Endosonography of enlarged subcarinal lymph nodes in Non-Hodgkin Lymphoma. A: B-mode reveals two enlarged lymph nodes; B: contrast enhanced endoscopic ultrasound demonstrates extensive avascular (necrotic) areas in the lymph nodes; C: real time endoscopic elastography indicates hard, infiltrated areas (blue color), thus targeting endoscopic ultrasound-guided biopsy.

can improve the detection of small intranodal vessels and thus could be useful in characterization of LNs[3,64] (Figure 8). CE-EUS has improved our understanding of gastrointestinal (subepithelial) tumors[65-67], differential diagnosis of pancreatic neoplasia[15,68-75] and other organ infiltration[74,75] through analysis of perfusion patterns.

There are only a few reports about the usefulness of contrast enhanced endoscopic Doppler ultrasound in the differentiation between malignant and benign lymphadenopathy. Kanamori *et al*[64] performed CE-EUS with high MI on 46 patients in whom EUS revealed LN in the mediastinum or abdominal cavity and suggested that CE-EUS is useful for differentiating benign from malignant LNs by detecting defects of enhancement in malignant nodes. The sensitivity, specificity, and accuracy rate of CE-EUS were 100%, 86.4% and 92.3%, respectively. In another study by Hocke *et al*[3], high MI CE-EUS was performed in 122 patients, and it was found that CE-EUS improved the specificity in diagnosing benign LNs as compared to B-mode EUS by analysing arteries and veins. However, it did not improve the accurate identification of malignant LNs and therefore could not replace EUS-guided fine-needle aspiration[3].

To the best of our knowledge, there is only one report on the application of low MI CE-EUS for the discrimination of benign and malignant abdominal lymph nodes. A Japanese group investigated 43 patients with intra-abdominal lesions of undetermined origin, which were suspected to be malignant lymph nodes, and evaluated the enhancement pattern after injection of the UCA Sonazoid®. Final pathological examination revealed that 35 lesions in fact were lymph nodes. All but one of the malignant lesions showed a heterogeneous enhancement pattern, whereas none of the benign lesion displayed heterogeneous enhancement. Most interestingly the interobserver agreement was very high (kappa 0.953)[78].

Endosonographic elastographic lymph node evaluation (strain imaging)

Endoscopic elastography is real time elastography performed with an endoscopic probe, which has led to further improvement in B mode imaging results for classification of benign and malignant LNs (Figure 8), particularly by

targeting LNs for needle sampling. Janssen *et al*[79] reported on 50 patients, 66 LNs were described elastographically (dominant colour/tissue hardness and guidance for tissue samples) and the elastogram data later compared with the histological findings obtained in the same session from fine needle biopsy. This study revealed that benign LNs exhibited predominantly intermediate homogeneous deformation (yellow/green), while malignant LNs were characterized by a quantitative dominance of hard (blue) units. The accuracy, which could be consistently reproduced by two more reviewers (kappa 0.84), for benign *vs* malignant LNs was about 85%. Intra- and interobserver agreement was also high in one recent study using visual assessment of the elastography image to differentiate between malignant and benign lymph nodes[80]. However, the same group found that EUS elastography did not perform better than EUS morphology in differentiating between malignant and benign lymph nodes in patients with resectable upper gastrointestinal cancer[45]. These findings conflict with the results of two other groups, which showed superior accuracy of EUS elastography strain ratio and histogram analysis, respectively, in comparison with conventional EUS criteria in differentiating malignant and benign lymph nodes in the nodal staging of esophageal cancer[81,82].

Săftoiu *et al*[41] used similar criteria for qualitative analysis in their study. In computer analysis, accuracy for differential diagnosis of malignant *vs* benign LNs increased slightly from 93% to 95%. In a follow-up study[47], they reached an accuracy for differentiation between benign and malignant LNs of 89%, using the computer based histogram analysis of video sequences, while this was significantly superior to the B-Mode image analysis (accuracy 53%). Another recent study with pathological confirmation yielded however lower values for sensitivity, specificity and accuracy, based on strain ratio calculations[45].

A recent meta-analysis calculated a sensitivity of 88% and a specificity of 85%, respectively, of EUS elastography for differentiating between benign and malignant lymph nodes[83].

In conclusion, the sensitivity of an imaging procedure critically depends on spatial resolution, which in elastography is as good as in conventional ultrasound since both depend on the same physical rules. The smallest LN metastases may escape both B-mode diagnosis and endosonographic fine needle biopsy. Elastography can detect the smallest metastasis-related changes in tissue hardness and it is considered to be potentially useful for target selection prior to endosonographic guided tissue sampling[10].

RTE can be recommended for discrimination of benign and malignant lymph nodes by identifying malignant regions that should be targeted for EUS-FNA (Figure 8).

SENTINEL LYMPH NODE EVALUATION

The detection or exclusion of sentinel lymph node (SLN) micrometastases is critical in staging cancer, especially breast cancer and melanoma, because it directly affects patient's prognosis and surgical management. It is well known that conventional US is not able to detect SLN in most cases. However, studies showed that low MI CEUS can be used for detecting SLN, which may become a potential application in clinical routine, like lymphoscintigraphy[32,84-89]. The application of CEUS for the investigation of SLN has shown promising results in animal models but the technique has not been sufficiently evaluated in humans. About 1 mL of contrast agent (*e.g.*, SonoVue®) is injected subcutaneously (intralymphatic) near the tumour site and the enhanced lymphatics are traced to the sentinel lymph node. Initial experience indicates that the method is not toxic and performs as well as blue dye or radioisotope methods. The current literature has been recently reviewed[90] and the topic is not the subject of this paper.

PANORAMIC IMAGING, 3D AND 3D-CEUS

Panoramic imaging, 3D[91] and 3D-CEUS[92,93] have been used for improved anatomic and topographic description of lymphadenopathy but have not gained additional information except improved presentation of results to clinicians.

CONCLUSION

The currently possible lymph node detection rate is limited by a minimal required lymph node size which is between 5-10 mm. Since about one third of malignant infiltrations occur in lymph nodes which are not detectable by all imaging methods, reliable exclusion of malignant lymph node infiltration is almost impossible. Therefore, current imaging methods mainly focus on the improved detection of early malignant infiltration in detectable lymph nodes, *e.g.*, to guide neoadjuvant treatment strategies.

Ultrasound techniques (CEUS, CE-EUS and elastography) demonstrate high spatial resolution which is important for early detection of malignant lymph node infiltration (Table 1). CEUS compared with conventional CDI could improve the visualization of vessels in LNs which is essential for the evaluation of vessel distribution. The visualization of avascular necrotic deposits of neoplastic cells is helpful for the differentiation of benign and malignant lymphadenopathy. The identification of hypoenhancing areas in malignant lymph nodes may guide biopsy for improved early detection of malignant infiltration.

In addition, the strictly intravascular distribution of intravenously injected contrast agents (*e.g.*, SonoVue®) allows the assessment of neoangiogenesis which is of importance for treatment evaluation under antiangiogenetic treatment.

CEUS cannot be recommended for the diagnosis of lymphoma so far. However, CEUS may be a tool to assess the treatment response by indentifying the reduction of vascularisation, *e.g.*, in Hodgkin's disease.

Table 1 Criteria on lymph node characterization using different ultrasound modes

Lymphadenopathy more (most) likely	B-mode	(Contrast enhanced) Colour Doppler	Vascular resistance	CEUS (contrast special imaging mode)	Elastography
Inflammatory	Preserved architectur, homogeneous, thin cortex	Preserved vessel architecture, hilar vascularity with or without tree like branching.	Lower, RI < 0.8, PI < 1.6	Homogeneous enhancement from the hilum, centrifugal enhancement	No data, most often normal architecture (except tuberculosis)
Malignant infiltration (metastasis)	Destroyed architecture (capsule), eccentric hypoechoic cortical thickening, inhomogeneity of the internal structure, loss of echogenic hilum, surrounding edema	Peripheral or mixed vascularity, inhomogeneous vessel density, split arteries, torturous course of vessels	Higher, RI > 0.8, PI > 1.6, often variable at different sites	Centripetal enhancement, different intra-nodal enhancement levels, inhomogeneous wash-out, perfusion defects	Initially circumscribed. SR in diffuse infiltration > 1.5 (1.78)
Lymphoma	Focal or global hypoechoic cortical thickening, usually without echogenic hilum, peri-nodular edema, pseudocystic appearance	Often but not always preserved vessel architecture, rich vascularity	Intermediate RI and PI	Intense homogeneous enhancement, starts with diffuse bright spots, peripheral hypo- or non-enhancement	No data; wide range of appearance applying qualitative criteria

CEUS: Contrast enhanced ultrasound; RI: Resistive index; PI: Pulsatility index.

Elastography is mainly helpful in delineating the very early circumscribed malignant infiltration for improved US- and EUS-guided fine needle aspiration (biopsy). Additionally, normal elastographic architecture of enlarged inflammatory lymph nodes can be helpful to prove a benign inflammatory disease, *e.g.*, sarcoidosis.

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REVIEW

Is diabetes mellitus a risk factor for pancreatic cancer?

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Abstract

The relationship between diabetes mellitus and the risk of pancreatic cancer has been a matter of study for a long period of time. The importance of this topic is due to two main causes: the possible use of recent onset diabetes as a marker of the disease and, in particular, as a specific marker of pancreatic cancer, and the selection of a population at risk for pancreatic cancer. Thus, we decided to make an in-depth study of this topic; thus, we carried out an extensive literature search in order to re-assess the current knowledge on this topic. Even if diabetes is found a decade before the appearance of pancreatic cancer as reported in meta-analytic studies, we cannot select those patients already having non detectable pancreatic cancer, at least with the imaging and biological techniques available today. We believe that more studies are necessary in order to definitively identify diabetes mellitus as a risk factor for pancreatic cancer taking into consideration that approximately 10 years are needed to diagnose symptomatic pancreatic cancer. At present, the answer to the as to whether diabetes and pancreatic cancer comes first similar to the adage of the chicken and the egg is that diabetes is the egg.

Key words: Diabetes mellitus; Obesity; Pancreatic neoplasms; Risk factors; Clinical studies

Core tip: Even if diabetes is found a decade before the appearance of pancreatic cancer we cannot select those patients already having non detectable pancreatic cancer, at least with the imaging and biological techniques available today. We believe that more studies are necessary in order to definitively identify diabetes mellitus as a risk factor for pancreatic cancer taking into consideration that approximately 10 years are needed to diagnose symptomatic pancreatic cancer. At present, the answer to the as to whether diabetes and pancreatic cancer comes first similar to the adage of the chicken and the egg is that diabetes is the egg.

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INTRODUCTION

The relationship between diabetes mellitus and the risk of pancreatic cancer has been a matter of study for a long period of time. The importance of this topic is due to two main causes: the possible use of recent onset diabetes as a marker of the disease and, in particular, as a specific marker of pancreatic cancer, and the selection of a population at risk for pancreatic cancer[1].

SEARCH STRATEGY

Taking into consideration diabetes mellitus irrespective of type, there is a lack of agreement regarding the data; thus, we decided to make an in-depth study of this topic. On July 24, 2012, we carried out a PubMed/Medline search using the following strategy: ("Diabetes Mellitus" [Mesh]

Table 1 Diabetes as a risk factor for pancreatic cancer according to diabetes duration

Meta-analysis	Studies evaluated (*n*)		Diabetes duration (yr)	Risk	95%CI
Everhart *et al*[76]	20	All studies evaluated	> 1	RR = 2.1	1.6-2.8
	11	All case-control studies	> 1	RR = 1.8	1.1-2.7
	9	All cohort studies	> 1	RR = 2.6	1.6-4.1
	11	All studies	> 5	RR = 2.0	1.2-3.2
	6	All case-control studies	> 5	RR = 1.8	0.86-3.8
	6	All cohort studies	> 5	RR = 2.4	0.85-7.0
Li *et al*[64]	3		≤ 2	OR = 2.9	2.1-3.9
	3		3-5	OR = 1.9	1.3-2.6
	3		6-10	OR = 1.6	1.2-2.3
			11-15	OR = 1.3	0.9.2.0
			> 15	OR = 1.4	1.0-2.0
Huxley *et al*[80]	9		1-4	RR = 2.05	1.87-2.25
	9		5-9	RR = 1.54	1.31-1.81
	7		≥ 10	RR = 1.51	1.16-1.96
Ben *et al*[84]	3		< 1	RR = 5.38	3.49-8.30
	5		1-4	RR = 1.95	1.65-2.31
	4		5-9	RR = 1.49	1.05-2.12
	4		≥ 10	RR = 1.47	0.94-2.31

or "Diabetes Mellitus, Type 2" [Mesh] or "Diabetes Mellitus, Type 1" [Mesh]) and "Pancreatic Neoplasms" [Mesh] and ("humans" [MeSH Terms] and English [lang]); other papers were manually extracted from the references of the papers selected. From 1966, a total of 787 papers were found and, of these, we selected 74 papers[2-75] and nine meta-analyses[76-84].

ANALYSIS OF LITERATURE AND CLINICAL CONSIDERATIONS

One of the first studies on the relationship between pancreatic cancer and diabetes is that of Maruchi *et al*[2] who found that there was an association between pancreatic carcinoma and diabetes from 1935 through 1974 only in cases of confirmed pancreatic carcinoma in residents of Olmsted County, Minnesota. In their series, 17% of the patients were diabetic (19/113) and nine cases (8%) of diabetes had appeared at least 2 years before the diagnosis of pancreatic cancer. Twenty years later, our group carried out a case-control study matching a large number of patients with and without pancreatic cancer[24]. The main findings were as follows: in the majority of cases, the diabetes was diagnosed at the same time as the cancer or within a few years prior to its identification, suggesting that it was the cancer which caused the diabetes. In fact, diabetes mellitus of long duration (> 7 years) had essentially no association with pancreatic cancer whereas, in a small group of patients who had had diabetes of a 5-7 years duration when the cancer was diagnosed, the association was statistically significant. Finally, all the patients in whom the diagnosis of diabetes had been made prior to that of the tumor had non-insulin-dependent diabetes, and no association was found with the insulin-dependent form.

Taking into account all findings in the literature, all the studies and the meta-analyses found an association between diabetes mellitus and pancreatic cancer at the time of diagnosis. However, little is known about glucose tolerance and insulin secretion in patients with this tumor. Gapstur *et al*[85] prospectively studied the postload plasma glucose concentration in 84 patients with pancreatic cancer in order to determine the presence of an independent association between postload plasma glucose concentration and the risk of pancreatic cancer mortality among people without self-reported diabetes. Compared to a postload plasma glucose level of 119 mg/dL or less and, after adjusting for age, race, cigarette smoking and body mass index, the relative risks (95%CI) of pancreatic cancer mortality were 1.65 (1.05-2.60) for postload plasma glucose levels between 120 mg/dL and 159 mg/dL, 1.60 (0.95-2.70) for levels between 160 mg/dL and 199 mg/dL and 2.15 (1.22-3.80) for levels of 200 mg/dL or more. Such an association appeared to be stronger in men than in women. Estimates were only slightly lower after excluding 11 men and 2 women who died from pancreatic cancer during the first 5 years of follow-up. Elevated body mass index and serum uric acid concentration were also independently associated with an elevated risk of pancreatic cancer mortality in men only. This study provides evidence for a positive, dose-response relationship between postload glycemia and pancreatic cancer mortality. The possible mechanisms underlying the increased pancreatic cancer risk among patients with diabetes mellitus is the involvement of insulin resistance and hyperinsulinemia. In addition, whereas postoperative diabetes was seen in all long-standing diabetic patients, and in some patients with intolerance fasting glucose and normal fasting glucose, the diabetes was resolved in more than 50% of patients with new-onset diabetes despite removal of half of the beta-cell mass[52]. Thus, it seems that diabetes is caused by pancreatic cancer. The answer to whether the diabetes is a specific marker of the disease comes from the study of Aggarwal *et al*[86]; these authors retrospectively reviewed the medical records of 500 consecutive patients with cancer (lung, breast, prostate, colorectal cancers and pancreatic cancer) and 100 non-cancer controls, and found that whereas the prevalence of diabetes mellitus in pancreatic cancer is high, diabetes mellitus prevalence in other common cancers is no different from that in non-cancer controls. Thus, diabetes mellitus is not useful as an early or specific marker of pancreatic cancer.

Controversies also exist between the association of long-standing diabetes mellitus and pancreatic cancer; some, epidemiological studies have excluded the possibility that long-standing diabetes mellitus is a risk factor for pancreatic cancer[2,6,24,32,36,41,44,45,51,52,54,59,60,64,70] whereas others (Table 1) have found a relation-

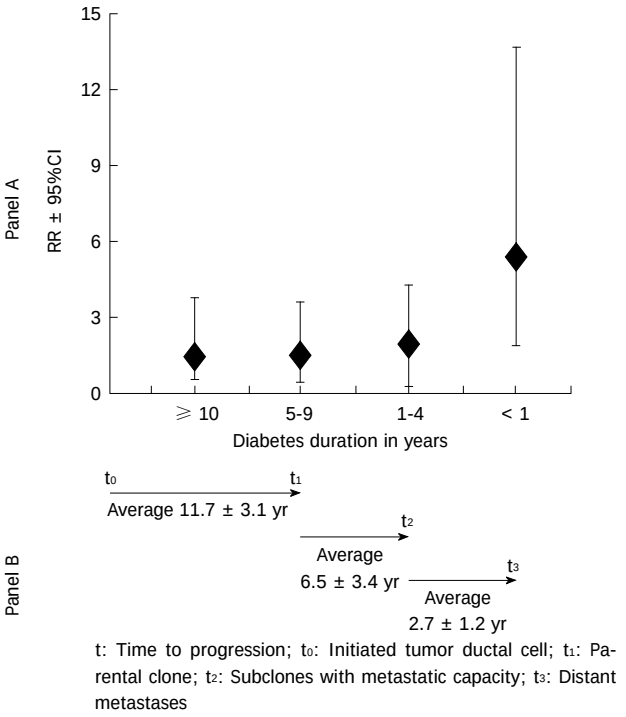

Figure 1 Relative risks and quantitative analysis. Panel A: Relative risks (RR) for the association between diabetes and pancreatic cancer according to the duration of the diabetes (originated from[63]). The risk disappears after 10 years; Panel B: Quantitative analysis of the timing of the genetic evolution of pancreatic cancer indicates that at least a decade is necessary between the occurrence of the initiating mutation and the birth of the parental, non-metastatic founder cell, that at least five more years are required for the acquisition of metastatic ability and patients usually die on an average of 2 years thereafter (originated from[87]).

ship[3-5,7-31,33,35,37-40,42,43,46-49,53,55-58,61,63,65-69,71-75]. It should be pointed out that, in papers showing an association between long standing diabetes and pancreatic cancer there are some biases due to self-reported diabetes which could result in misclassification, heterogeneity among individuals with diabetes in terms of physiologic status, sequelae and treatment which could also confuse this relationship. In addition, Yachida *et al*[87], sequencing the genomes of seven pancreatic cancer metastases to evaluate the clonal relationships among primary and metastatic cancers, found that clonal populations which give rise to distant metastases are represented within the primary carcinoma (but these clones are genetically evolved from the original parental, non-metastatic clone) and they performed a quantitative analysis of the timing of the genetic evolution of pancreatic cancer found at least a decade between the occurrence of the initiating mutation and the birth of the parental, non-metastatic founder cell. At least five more years are required for the acquisition of metastatic ability and patients die an average of 2 years thereafter[87]. Thus, even if diabetes is found a decade before the appearance of pancreatic cancer as reported in meta-analytic studies, we cannot select those patients already having non detectable pancreatic cancer, at least with the imaging and biological techniques available today (Figure 1).

CONCLUSION

We believe that more studies are necessary in order to definitively identify diabetes mellitus as a risk factor for pancreatic cancer taking into consideration that approximately 10 years are needed to diagnose symptomatic pancreatic cancer. At present, the answer to the question posed by Magruder *et al*[83] as to whether diabetes and pancreatic cancer comes first similar to the adage of the chicken and the egg is that diabetes is the egg.

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REVIEW

MicroRNAs may solve the mystery of chronic hepatitis B virus infection

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Abstract

Hepatitis B virus (HBV) infection is a global public health problem that causes persistent liver diseases such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma. A large amount of people die annually from HBV infection. However, the pathogenesises of the HBV-related diseases are ill defined and the therapeutic strategies for the diseases are less than optimum. The recently discovered microRNAs (miRNAs) are tiny noncoding RNAs that regulate gene expression primarily at the post-transcriptional level by binding to mRNAs. miRNAs contribute to a variety of physiological and pathological processes. A number of miRNAs have been found to play a pivotal role in the host-virus interaction including host-HBV interaction. Numerous studies have indicated that HBV infection could change the cellular miRNA expression patterns and different stages of HBV associated disease have displayed distinctive miRNA profiles. Furthermore, the differential expressed miRNAs have been found involved in the progression of HBV-related diseases, for instance some miRNAs are involved in liver tumorigenesis and tumor metastasis. Studies have also shown that the circulating miRNA in serum or plasma might be a very useful biomarker for the diagnosis and prognosis of HBV-related diseases. In addition, miRNA-based therapy strategies have attracted increasing attention, indicating a promising future in the treatment of HBV-related diseases.

Key words: MicroRNA; Hepatitis B virus; Hepatitis B; Host-virus interaction; Biomarker; Therapy

Core tip: The cellular microRNAs (miRNAs) involved in host-hepatitis B virus (HBV) interaction and each stage of HBV-related disease show distinctive miRNA expression profiles at the tissue or serum level indicating that miRNAs have marked potential in detecting or treating of HBV infection.

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INTRODUCTION

The hepatitis B virus (HBV) is a hepadnavirus that causes persistent liver diseases and have a major effect on global public health[1,2]. HBV, discovered in 1966[3], is transmitted among humans by contact with the blood, semen or vaginal fluid of an infected person. Approximately, one third of the world's population have infected HBV, and more than 350 million people have developed chronic HBV infection[4-6]. The severity of HBV-related disease

varies widely, from a self-limited infection to acute hepatitis and from asymptomatic chronic infection to cirrhosis and hepatocellular carcinoma[7,8]. The factors affecting the prognosis of HBV infection have not been determined. miRNAs was discovered recently and researchers have determined that it plays a pivotal role in host-virus interactions[9-11]. By using the functions of miRNA, we may explain the mechanism of chronic HBV infection and discover novel biomarkers as well as new therapies for HBV associated diseases.

Since numerous researches discovered that RNA does more than simply serves an intermediary function in "central dogma"[12], the door to a brand new world of RNA had been opened. The genomes of organisms produce two types of RNA, and mRNAs belong to the first type which can be used as translation templates. Besides, genomes manufacture a variety of noncoding RNAs, including the components of the machinery of gene expression and regulatory RNAs[13]. MicroRNAs (miRNAs) are noncoding RNAs, and their mature forms are approximately 22 nucleotides (nt) in length. When these RNAs were initially described in *Caenorhabditis elegans* (*C. elegans*)[14], they were hypothesized to be peculiar to nematodes[12,13]. Subsequent work revealed that miRNAs are common tiny nucleic acid molecules that can be found in plants[15], animals[16] and other organisms[17]. To date, the record of miRNAs has increased significantly. MiRbase 19, released in August of 2012, increased the numbers of recognized hairpin and mature miRNAs to 21264 and 25141, respectively[18-23]. In human, while the expression profiles of some miRNAs in different cells or tissues are similar, other miRNAs may exhibit temporal or tissue-specific patterns[24,25], suggesting that miRNA may be involved in numerous physiological or pathological processes[26].

The biogenesis and action mechanism of these tiny but potential molecules had been detailed described[24,25,27]. Briefly, they are not born so small, in other words, they have some larger progenitors. The processing of the mature miRNA ancestors (primary and precursor miRNAs) is closely related to RNA polymerase Ⅱ (pol Ⅱ), Drosha, the GTP-dependent Ran/Exportin 5 complex, and the Dicer enzyme[24-32]. Generally, by binding to the 3' untraslated regions of their target mRNAs, miRNAs can serve as gene expression regulators, fine-tune the expression primarily at the post-transcriptional level and play critical roles in a variety of physiological and pathological processes, including antiviral defense, developmental timing, cell apoptosis, cell proliferation, tumor generation and so on[24,30,33-39]. One computational prediction indicated that more than 30% of animal genes may be subject to regulation by miRNAs, which emphasizes the importance of miRNA-mediated gene regulation[40,41].

HOST-VIRUS INTERACTION AT THE MIRNA LEVEL

Viruses are generally harmful to human, in order to protect our health, the battle between virus and host break out shortly after infection initiated. In this war, a large amount of reports have indicated that cellular miRNAs serve a key role in protecting the host. However, we may be disappointed at the truth that viruses can use miRNAs as their weapons to fight the host. Remarkably, some features of miRNAs ensure their effectiveness as virally encoded regulators of host and viral gene expression: they are small, lack of immunogenicity and functional flexibility[42]. To facilitate an understanding of the intricacies of host-virus cross-talk mediated by miRNAs, we designed an illustration (Figure 1) base on the review of Scaria *et al*[9] In the interaction between virus and host, miRNAs can be divided into cellular miRNAs and viral miRNAs. To cellular miRNAs, their expression profiles changed at the infected state and the abnormal miRNAs often closely relate to the viral life cycle as well as host disorder. To viral miRNAs, they can evolved to regulate both viral and cellular gene expression[42].

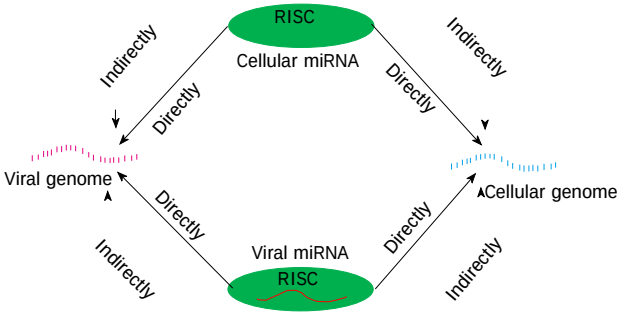

Figure 1 Logical model of host-virus cross talk mediated by microRNAs. Viral microRNAs (miRNAs) and cellular miRNA take part in the host-virus interactions. Moreover, viral and cellular miRNA can influence the expression of viral and cellular genome. RISC: RNA-induced silencing complex.

Cellular miRNAs in host-virus interaction

Studies have noted that miRNA-mediated gene regulation involve in diverse biological processes in the mammalian system, including cellular miRNAs influence viral reproduction and pathogenesis[42,43]. Sometimes, viruses may exploit celluar miRNAs to facilitate certain steps of their life cycle, a living example is hepatitis C virus (HCV) use miR-122, a liver-specific cellular miRNA, to enhance its replication of itself by targeting the viral 5' non-coding region[34,44]. Another study showed that miR-122 knockdown reduced the HCV load in infected chimpanzees[45] and the interferon-mediated down-regulation of miR-122 that contributes to antiviral effects[46]. In contrast, miR-122 serve as an antiviral role in HBV life cycle. For instance, Qiu *et al*[47] found that the miR-122 over-expression inhibited HBV expression, whereas the depletion of endogenous miR-122 resulted in increased production of HBV in transfected cells. Their subsequent study suggested that the miR-122 inhibitor also caused an increase in cellular heme oxygenase-1, which can decrease HBV covalently closed circular DNA (cccDNA) levels both *in vitro* and *in vivo* by reducing the stability of the HBV core protein[48]. A recent study by Wang *et al*[1], indicated that miR-122 expression in the liver was significantly down-

regulated in patients with HBV infection compared with healthy controls. Depletion of endogenous miR-122 and over-expression of miR-122 led to enhanced HBV replication and inhibited viral production, respectively. Cyclin G1 was identified as an miR-122 target that specifically interacted with p53, resulting in the specific binding of p53 to the HBV enhancer elements and simultaneous abrogation of the p53-mediated inhibition of HBV transcription. Ji *et al*[49] found that miR-122 was significantly up-regulated in HBV-infected patients and could inhibit HBV replication in Huh7 and HepG2 cells. Overall, to HCV and HBV, miR-122 can promote and inhibit viral replication respectively. In other words, cellular miRNAs can influence viral lifecycles by accelerative or suppressive mechanisms.

Studies have reported the involvement of cellular miRNAs in numerous host-virus interactions. HIV-1 can use cellular miRNAs to repress the expression of viral proteins and evade the host immune system respose[11,50]. The replication of primate foamy virus can be inhibited by cellular miR-32[43]. miR-24 and miR-93 were responsible for the increased vesicular stomatitis virus replication in variant Dicer1d/d allele mice[51]. The above instances indicate the diversity of miRNA activity and indicate that host-derived miRNAs are essential for the host-virus interactions.

Viral miRNAs in host-virus interaction

A number of the miRNAs that participate in the interaction between host and virus are viral. Pfeffer *et al*[52] initially discovered the existence viral miRNAs in the Epstein-Barr virus (EBV). Analogous to cellular miRNAs, viral miRNAs have multifaceted functions[42], that generally benefit the virus in maintaining its replication, latency and evasion of the host immune system[11]. Barth *et al*[53] showed that miR-BART2 down-regulates the viral DNA polymerase BALF5, inhibiting the transition from latent to lytic viral replication in EBV. Analogously, miR-BART-1p, miR-BART16 and miR-BART17-5p have been found to repress the translation of latency-associated membrane protein LMP-1 mRNA[11,54]. Additional examples of viral miRNAs that regulate viral gene expression are found in HCMV, SV40, MDV, HIV-1 and other viruses[11].

Although numerous miRNA-produced viruses have been identified, the HBV-encoded miRNAs have not been confirmed experimentally but have been suggested by computation[55,56]. This discrepancy may be the result of the limitations of current technology and HBV-derived miRNAs could be found in the future.

EMPHASIZING THE ROLE OF MIRNAS IN HBV INFECTION

A number of cases of host-virus interaction at the miRNA level have been mentioned above. To emphasize the role of miRNAs in HBV infection, we intend to report additional details about the interaction between miRNAs and HBV (Figure 2).

Understanding the mechanisms of miRNAs influence HBV infection requires the knowledge that HBV is a noncytopathic virus that replicates preferentially in the hepatocytes. cccDNA which serves as a template for transcription of all viral RNA is synthetized And after HBV DNA enters the hepatocyte nucleus. The HBV genome is 3.2 kb in length and contains four overlapping open reading frames. It can transcribe viral pregenomic RNA that reverses transcription to synthesize the viral DNA genome and encode the hepatitis B virus surface antigen (HBsAg), hepatitis B virus core protein, viral reverse DNA polymerase (Pol) and X protein. Two enhancers, Ⅰ and Ⅱ, have been shown to function as two master regulators of the four viral promoters[57-59]. Although the viral miRNAs encoded by HBV have not been verified[56], there are cellular miRNAs capable of inhibiting or stimulating HBV viral replication and gene expression. In addition, the products of HBV can alter the miRNA expression profiles.

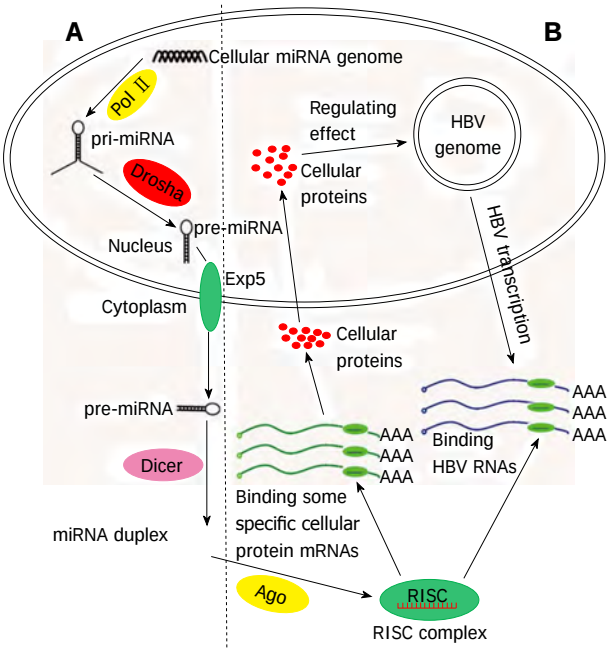

Figure 2 The biogenesis of human cellular microRNAs and the mechanism of the alteration hepatitis B virus gene transcription and replication. For simplicity, not all participators are shown. A: The biogenesis of microRNAs (miRNAs); B: The mechanism of cellular miRNAs regulates hepatitis B virus (*HBV*) gene transcription and replication can be direct and indirect. Cellular miRNAs can target to HBV transcripts (HBV surface antigen mRNA, HBV x mRNA, DNA polymerase mRNA, *etc*.), causing the alteration of HBV expression. Cellular miRNAs can also target to the mRNAs of a number of key regulatory proteins (liver-enriched transcription factors, nuclear receptors, heme-oxygenase-1, DNA methyltransferases, *etc*.) in the process of HBV transcription and replication. Consequently, the amount of these proteins was changed, and the *HBV* gene transcription and replication were altered. RISC: RNA-induced silencing complex. Pol: Polymerase.

Cellular miRNAs targeting to HBV transcripts

A study of Zhang *et al*[60], in attempt to determine whether host-encoded miRNAs affect HBV replication, antisense oligonucleotides of 328 identified human miRNAs were orderly transfected into HepG22.2.15 cells. The expression level of HBsAg, hepatitis B e antigen and

cell proliferation were detected by enzyme-linked immunosorbent assay and methyltestosterone assay. Compared to the experimental controls, miR-199a-3p and miR-210 efficiently reduced the HBsAg expression without affecting HepG2 2.2.15 cell proliferation. Furthermore, they used the bioinformatics method to analyze six miRNAs, and the outcome suggested a putative binding site for miR-199a-3p in the HBsAg coding region and a binding site for miR-210 in the HBV pre-S1 region, respectively. Potenza *et al*[61] used MiRanda to analyze the HBV genome and found seven sites that were potential targets for human liver miRNAs. Their subsequent validation test found that hsa-miR-125a-5p interferes with the HBV translation and down-regulation of the expression of the surface antigen. These findings indicate that cellular miRNAs can alter HBV gene expression by targeting to HBV transcripts.

Cellular miRNA affects HBV replication

Cellular miRNAs can affect viral translation and change viral replication. In addition to the instance of the miR-122 inhibition of HBV replication, there are other cases about host miRNAs altering HBV replication. A study by Hu *et al*[33] suggested that miR-141 suppressed HBV replication by reducing HBV promoter activities through the down-regulation of peroxisome proliferator-activated receptor alpha. DNA hypermethylation might be closely related to the suppression of HBV cccDNA transcription[56], and miR-152 might be a factor involved in the regulation of the methylation of HBV cccDNA[62,63]. Zhang *et al*[64] revealed that cellular miRNAs do not consistently inhibit HBV replication. Collectively, miRNAs can directly or indirectly alter HBV replication.

HBV infection can change the host miRNA expression pattern

A recently study by Wei *et al*[65] showed that the hepatitis B virus x (HBx) protein expression was found to have a significant inverse correlation with miR-101 expression in HBx-expressing HepG2 cells compared to control HepG2 cells. Ren *et al*[66] found that Drosha (a regulator of the biogenesis of miRNAs) mRNA and protein expression were down-regulated in cells expressing the HBV genome, and that the mechanism was related to a reduction in the activity of the Drosha gene promoter. By using RNA interference to knockdown the HBX gene, the expression of Drosha was significantly restored. Their date showed that HBV could inhibit Drosha expression by inhibiting the promoter activity and in turn, leading to an alteration of the host miRNA profiles[66]. These studies suggested that HBV infection can alter the miRNA expression profiles.

MIRNA PROFILES OF HBV-ASSOCIATED DISEASE

The consequences of HBV infection are diverse and can be ranged from asymptomatic chronic infection to cirrhosis and hepatocellular carcinoma. Numerous studies have detected that cellular miRNAs could influence the lifecycle of HBV and HBV could change the miRNA expression profiles, reversely. Taking these factors into consideration, the miRNA profiles may change along with the severity of HBV associated disease. So, we concentrated on the miRNAs expression patterns and their potential role in HBV associated chronic hepatitis, cirrhosis and HCC in the following contents.

miRNA profiles of chronic hepatitis B

The miRNA profiles of chronic hepatitis B (CHB) from numerous studies are controversial and complicated. On the one hand, a series of study indicated that the miRNA expression patterns of CHB are particular at the tissue or serum level[1,67-69]. For instance, a study of Ura *et al*[68] suggested that the miRNA expression profiles in chronic hepatitis B were different from those in the healthy controls and those in HBV-associated HCC, and hepatitis C. To the contrary, appliying massively parallel signature sequencing to conduct an in-depth analysis of the miRNomes in normal human, hepatitis and HCC liver tissues, Hou *et al*[70] found that, except for in HCC, the known miRNAs exhibited a similar distribution in each library based on classification of the transcripts permillion degrees.

miRNA profiles of liver cirrhosis

A well-known trilogy of hepatitis B is that chronic hepatitis B progresses into liver cirrhosis and HCC. An increasing number of studies have focused on the expression patterns of miRNAs during the cirrhotic stage to uncover their function in the progression of hepatitis B and to seek novel therapies for cirrhosis. Roderburg *et al*[71] investigated the role of miRNAs in liver fibrosis by carbon tetrachloride and bile duct ligation models of liver fibrosis. Fibrosis-inducing injuries cause the abnormal expression of many miRNAs. All three members of the miR-29 family were significantly down-regulated under the disposes of these models. To correlate these findings with HBV in human, they measured the miRNA profiles of human liver samples, and found miR-29 family members were down-regulated in the fibrotic/cirrhotic tissues compared with the non-fibrotic tissues. In conclusion, miR-29 family members were down-regulation both in mouse models and in human fibrotic livers. Hepatic stellate cells (HSCs) play a key role in liver fibrosis[72,73]. Roderburg group's further study revealed that miR-29b was down-regulated in HSCs, upon exposure to fibrotic stress. On a cellular level, miR-29 down-regulation in murine HSC cells was mediated by transforming growth factor (TGF)-β as well as inflammatory signaling and nuclear factor κB (NF-κB). Forced expression of miR-29b in murine HSCs can result in the repression of collagen expression[71].

Additional studies report on miRNA regulation in the progression of liver fibrosis. Compared with quiescent HSCs, Lakner *et al*[73] verified that miR-19b was a regu-

lator of TGF-β signaling in activated HSCs, it play an inhibitory effect in HSC-mediated fibrogenesis. Another study suggested that liver fibrosis could cause the down-regulation of miR-150 and miR-194 in HSC, and that their over expressions could repress HSC activation.

miRNA profiling in HBV-related HCC

Chronic hepatitis B is closely relate to HCC. In recent years, numerous studies that focused on the miRNA profiling in HBV-related HCC identified a number of deregulated miRNAs which are critical for the generation of HCC. Gao *et al*[74] isolated miRNAs from formalin fixed paraffin embedded dysplastic nodules (DNs), small HCCs, and their corresponding nontumorous livers. They investigated the expression changes of seven cancerrelated miRNAs, which have been reported to be frequently deregulated in human cancers and might play a role in liver carcinogenesis. They frequently observed the down-regulation of miR-145 and miR-199b as well as the up-regulation of miR-244 in premalignant DNs, moreover these alterations persisted throughout the HCC development. By restoring miR-145 in both HepG2 and Hep3B HCC cells, they found that cell proliferation, cell migration and cell invasion were significantly inhibited. What's more, an anti-miR-145 inhibitor could impair these inhibitory functions of miR-145. This study suggested that miRNA deregulation was an early event and may accumulate throughout the generation of HBV-associated HCC[74]. A study from Hou *et al*[70] identified miR-199a/b-3p which consistently decreased in HCC, and its decrement significantly correlates with poor survival of HCC patients. Huang *et al*[63] suggested that miR-152 was aberrantly expressed and involved in the regulation of the abnormal DNA methylation status in HBV-related HCC.

Interestingly, one miRNA was found to be up-regulated and contribute to enhancing HBV-related HCC metastasis by repressing the expression of fibronectin[75]. Zhang *et al*[75] reported that the levels of miR-143 were significantly increased in p21-HBx transgenic mice and HCC patients with metastatic HBV-HCC. Furthermore, they found that the over-expression of miR-143 was transcribed by NF-κB and facilitates the invasive and metastatic behavior of liver tumor cell. In an athymic nude mouse model, they found that high levels of miR-143 administered by intratumoral administration could remarkably promote HCC metastasis. And they used p21-HBx transgenic mice to show *in vivo* that local liver metastasis and distant lung metastasis were significantly inhibited by blocking miR-143. What's more, fibronectin type Ⅲ domain containing 3B was identified *in vivo* and *in vitro* as the target of miR-143[75].

A NOVEL DIAGNOSTIC BIOMARKER OR PROGNOSTIC PREDICTOR

The expression profiles of miRNAs in different stages of HBV-associated diseases are always inconsistent. Moreover, a portion of miRNAs are closely related to the stage

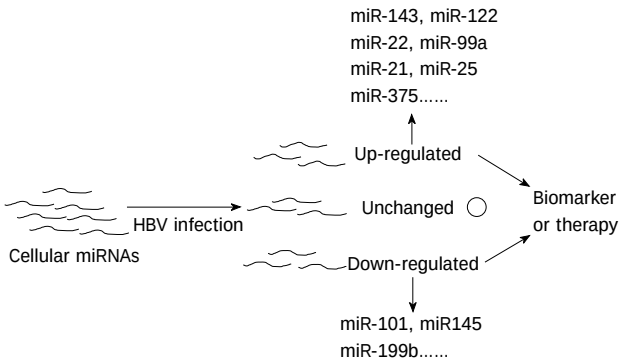

Figure 3 The aberrant expressed microRNAs and their potential use in hepatitis B virus infection. Hepatitis B virus (HBV) infection can alter the expression profiles of cellular microRNAs (miRNAs). Except the unchanged miRNAs, up-regulated (such as miR-143, miR-122, miR-22 *etc.*) and down-regulated miRNAs (miR-101, miR145, miR-199b *etc.*) are promising for detecting and treating HBV-related diseases.

of this liver disease and often play a crucial role in their progression[68,69,71,73-75]. Therefore miRNAs can serve as the role of biomarker in the diagnosis of HBV-related disease (Figure 3). Studies have reported that miRNAs could be stably detected in plasma and serum[76-78]. Chen *et al*[77] demonstrated that miRNAs could be found in the plasma and serum of humans and that their levels in serum were stable, reproducible, and consistent among individuals of the same species. In their study, Solexa was employed to sequence all of the serum miRNAs of healthy Chinese subjects and to identify specific expression patterns of serum miRNAs for lung cancer, colorectal cancer, and diabetes. They validated two non-small cell lung cancer-specific serum miRNAs in an independent trail using quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays. These results showed the existence of human serum miRNAs and suggested that these miRNAs contain fingerprints for diverse diseases[77]. Hence, assaying miRNA profiles could become a novel approach for detecting HBV-related diseases.

More powerful biomarkers are needed to compensate for the defects of the existing diagnostic means for detecting HBV-related liver injury and HCC[69,79-83]. In blood samples, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are the most widely used enzymatic indicators for liver damage[84]. But enhanced ALT and AST activities have been detected in some other clinical disorders[81,82]. In clinical practice, these two markers are not always consistent with histomorphological alterations[80]. One of the reasons for the high mortality in HBV-related HCC is that the tumors are frequently identified after metastasis at a stage in which curative resection is no longer feasible[69]. We rely on radiology imaging methods such as ultrasonography, computed tomography, and magnetic resonance imaging to find a liver mass to diagnose HCC. These methods can not diagnose small lesions accurately[69]. The most commonly used serum HCC markers, α-fetoprotein (AFP), has insufficient sensitivity and specificity[69,85,86].

Collectively, there is an urgent need for novel strate-

gies in the detection of HBV-related disease, and miRNAs could become a novel and powerful biomarker.

miRNAs can be used to detect liver injury and HBV infection

Several recent reports suggested that miRNAs could be used as an indicator of liver injury and HBV infection[49,67,84,87]. Zhang *et al*[84] selected and validated miRNA biomarkers using an extensive set of plasma samples from patients with HBV infection, patients with skeletal, and healthy controls. Combining these experimental results with their further investigation in liver injury mouse models, these authors reported that the plasma miR-122 concentration presented a disease severity-dependent change in the patients and mouse models that earlier than the alteration in aminotransferase activity. Their findings suggested that miR-122 had potential as a blood marker for liver injury including HBV associated injury[84]. Waidmann *et al*[67] investigated the relationship between miR-122 and HBV infection, suggested that the serum levels of miR-122 can discriminate between HBV infected patients and healthy controls. Ji *et al*[49] found that the numbers of circulating miRNAs increased with the symptom severity of HBV infected patients and that the expression of miR-122 was significantly up-regulated in these patients.

miRNAs other than miR-122 had been reported to get the ability of indicating HBV infection. Hayes *et al*[87] found a number of disease-specific serum miRNAs of HBV infection, including miR-122, miR-22, and miR-99a which were up-regulated at least 1.5-fold in the serum of HBV-infected patients.

miRNAs may become the diagnostic and prognostic marker of HBV-positive HCC or HCC

Early diagnosis of HCC plays a vital role in reducing mortality, but the existing strategies are not effective. A number of miRNAs had been found to have the potential to become the diagnostic and prognostic markers of HCC[69,88-94].

Tomimaru *et al*[69] measured the plasma miR-21 levels of different subjects including HCC patients and chronic hepatitis patients. In their study, plasma miR-21 was significantly reduced after a curative resection in HCC, and the level in the HCC subjects was significantly higher than the levels in the patients with chronic hepatitis and healthy controls. These authors found that miR-21 could differentiate HCC from healthy controls with high sensitivity and specificity. In theory, miR-21 was superior to AFP in the diagnosis of HCC. In a study with several phases, Qi *et al*[93] found that the serum miR-122 level was significantly higher in HCC patients compared to healthy controls and post-operative subjects. Their findings indicate that miR-122 might serve as a novel biomarker for the detection of HCC in healthy subjects but is not useful for the detection of HCC in patients with chronic HBV infections[93].

Li *et al*[92], employed Solexa sequencing to screen and qRT-PCR to validate miRNAs in serum samples. Thirteen miRNAs were found that could accurately distinguish not only HBV cases from healthy and HCV individuals, but also HBV-postive HCC subjects from healthy and HBV subjects. Additionally, in a comparison of miRNA expression in the serum of HCC subjects and healthy controls, six miRNAs were found to be significantly elevated in the samples from HCC. Three miRNAs (miR-25, miR-375, and let-7f) can be used to separate HCC cases from healthy controls. In the prediction of HCC, miR-375 had an ROC of 0.96 (specificity: 96%; sensitivity: 100%).

Although the outcomes of these studies are not uniform, the data have shown that miRNAs are promising for detecting HCC or HBV-positive HCC. A number of reports have indicated that the expression of miRNAs could anticipate the prognosis of HCC[89,91]. Using Kaplan-Meier estimates and the log-rank test, Li *et al*[91] showed that high expression of has-miR-125b was related to good survival and a subsequent transfection assay showed that forced expression of miR-125b in the HCC cell line could perceptibly repress the cell growth and phosphorylation of Akt. Budhu *et al*[89] created a unique 20-miRNA metastasis signature that could significantly predict HCC tissues with venous metastases from metastasis-free solitary tumors with a 10-fold cross-validation. In the corresponding noncancerous liver tissues they could not identify significant miRNAs. A survival risk prediction analysis revealed that the majority of metastasis-related miRNAs were related to survival. Their additional validation experiments revealed that the 20-miRNA tumor signature could serve as a survival and relapse predictor of HCC[89].

Although miRNAs have significant potential, a number of problems remain. Too many miRNAs have been identified to be practically applied for routine clinical use, and the accuracy of the miRNA signatures has not been adequately evaluated[95]. These factors may result in inaccuracy or incorrect diagnosis and prediction outcomes.

EMPLOYING MIRNAS OR ANTAGOMIR IN HBV THERAPEUTIC

The closely relationship between miRNAs and HBV-related diseases offers an opportunity to use miRNAs or antagomir in the treatment of these diseases (Figure 3). The feasibility of this method has been demonstrated[96-99]. Grimm *et al*[100] showed that anti-HBV shRNAs might cause serious toxicity *in vivo*. Although a miRNA-based strategy is promising, its therapeutic application must be dependent on rigorously demonstrated safety, efficient delivery to target tissues and optimization shRNAs dosing and sequencing[100,101]. To obtain an optimal solution for a miRNA-based strategy, Keck *et al*[102] produced improved HBV RNAi triggers, Ely *et al*[103] designed pri-miRNA expression cassettes and linear DNA sequences that expressed antiviral micro-RNA shuttles[104], and Xiangji *et al*[105] developed a lentiviral miRNA-based sys-

tem. Improved miRNA-based therapeutic methods could successfully inhibit HBV replication or expression. A promising miRNA-based HBV therapy method has not been well established but could be designed successfully in the future.

CONCLUSION

In this review, we limited our focus to the role of miRNAs in host-virus interactions, especially in host-HBV interactions. HBV infection is a global issue, but the pathogenesises and therapies of HBV-related diseases are not well defined. In the years since miRNA was discovered in *C. elegans* and subsequent studies revealed that miRNA are involved in many physiological and pathological processes in humans, scientists have observed that miRNAs played a key role in viral diseases and could serve a guardian or aggressor role. Regarding to HBV infection, cellular miRNAs were found to influence HBV translation and replication and HBV was found to change expression profiles of cellular miRNA. This finding led to the possibilities of miRNAs serving as biomarkers and of miRNAs or antagomirs serving as therapeutic tools in HBV-related diseases (Figure 3). Studies have indicated that the blood or tissue samples from the different stages of HBV-related disease presented distinctive miRNA expression patterns and that miRNA-based therapy is feasible.

Although many experimental studies have confirmed the capacity of miRNAs or antagomirs to detect or treat HBV-related diseases, adequate evaluation of their accuracy, efficacy, and cost-effectiveness is required. Further research into the relationship between miRNAs and chronic HBV infection may increase the understanding of hepatitis B virus infection and miRNAs could become accurate biomarkers and powerful therapy tools.

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ORIGINAL ARTICLE

Thiopurines related malignancies in inflammatory bowel disease: Local experience in Granada, Spain

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Abstract

AIM: To investigate the incidence of neoplasms in inflammatory bowel disease (IBD) patients and the potential causative role of thiopurines.

METHODS: We performed an observational descriptive study comparing the incidence of malignancies in IBD patients treated with thiopurines and patients not treated with these drugs. We included 812 patients which were divided in two groups depending on whether they have received thiopurines or not. We have studied basal characteristics of both groups (age when the disease was diagnosed, sex, type of IBD, *etc.*) and treatments received (Azathioprine, mercaptopurine, infliximab, adalimumab or other immunomodulators), as well as neoplasms incidence. Univariate analysis was performed with the student *t* test, χ^2 test or Wilcoxon exact test as appropriate. A logistic regression analysis was performed as multivariate analysis. Statistical significance was establish at *P* values of less than 0.05, and 95%CI were used for the odds ratios.

RESULTS: Among 812 patients included, 429 (52.83%) have received thiopurines: 79.5% azathioprine, 14% mercaptopurine and 6.5% both drugs. 44.76% of patients treated with thiopurines and 46, 48% of patients who did not receive this treatment were women ($P > 0.05$). The proportion of ulcerative colitis patients treated with thiopurines was 30.3% compare to 66. 67% of patients not treated ($P < 0.001$). Mean azathioprine dose was 123.79 ± 36.5 mg/d (range: 50-250 mg/d), mean usage time was 72.16 ± 55.7 mo (range: 1-300 mo) and the accumulated dose along this time was 274.32 ± 233.5 g (1.5-1350 g). With respect to mercaptopurine, mean dose was 74.7 ± 23.9 mg/d (range: 25-150 mg/d), mean usage time of 23.37 ± 27.6 mo (range: 1-118 mo), and the accumulated dose along this time was 52.2 ± 63.5 g (range: 1.5-243 g). Thiopurine *S*-methyltransferase activity was tested in 66% of patients treated with thiopurines, among which 98.2% had an intermediate or high activity. Among the patients treated with thiopurines, 27.27% (112 patients) and 11.66% (50 patients) received treatment with Infliximab and Adalimumab respectively, but only 1.83% (7 patients) and 0.78% (3 patients) received these drugs in the group of patients who did not received thiopurines ($P < 0.001$ and $P < 0.001$ respectively). Finally, 6.8% (29 patients) among those treated with thiopurines have received other immunesupresants (Methotrexate, Tacrolimus, Cyclosporin), compare to 1% (4 patients) of patients not treated with thiopurines ($P < 0.001$). Among patients treated with thiopurines, 3.97% developed a malignancy, and among those not treated neoplasms presented in 8.1% ($P = 0.013$). The most frequent neoplasms were colorectal ones (12

cases in patients not treated with thiopurines but none in treated, $P < 0.001$) followed by non-melanoma skin cancer (8 patients in treated with thiopurines and 6 in not treated, $P > 0.05$).

CONCLUSION: In our experience, thiopurine therapy did not increase malignancies development in IBD patients, and was an efective and safe treatment for these diseases.

Key words: Malignancy; Neoplasm; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Thiopurines; Azathioprine; Mercaptopurine

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic disorder which is characterised by episodes of inflammatory activity alternated with episodes of remission of this inflammation. Ethiology is mostly unknown, and there is no curative treatment, being the goal of current therapies to maintain patients in remission.

Among the drugs used for the treatment of this disease there are corticoids, immunomodulators (azathioprine, mercaptopurine, cyclosporine or tacrolimus) and biological therapies (infliximab and adalimumab) which interfere in the altered inflammatory and immunological process of these patients in order to induce and maintain clinical remission. Given the usefulness of these drugs for treating this disease, their relative safety with regards to side effects and the tendency to be more strict in symptoms control (top-down *vs* set-up tendency), immunomodulators and biological therapies have become drugs extensively used in this field. However, these treatments imply new challenges, as their hypothetical ability to induce neoplastic diseases, determined by their interference with the immune response which could limit its ability to control dysplastic cells, favouring the development of tumours. Indeed, Azathioprine has been classified as carcinogenic by the International Agency for Research on Cancer. Regarding this relationship between thiopurines and neoplastic diseases, several studies dealing with this adverse effect[1] have been published.

Thiopurines are drugs known to be antiproliferative given that their main effect is that they prevent the proliferation of T lymphocytes, promoting the incorporation of thiopurine analogues into DNA, which prevents the synthesis of purine nucleotides[2], as well as blocking several enzymes involved in the synthesis of DNA, RNA and proteins. All of those mechanisms block the proliferation and functions of the lymphocytes, inhibiting the synthesis of antibodies, and reducing the number of circulating monocytes and granulocytes, which is more evident in tissues and situations with a high cellular turnover.

The presence of thiopurine analogues in the DNA not only alters its structure by preventing cellular proliferation, but also increases the risk of mutagenesis[3-6]. This theoretic possibility becomes more evident in recent studies which describe an increased number of somatic mutations in circulating leukocytes in patients treated with thiopurines in comparison to patients who are not treated[7], more so, the number of mutations is proportional to the dose and duration of the treatment with thiopurines.

Also, this structurally altered DNA becomes more sensitive to radiation, especially ultraviolet radiation (UVA), which creates reactive oxygen species with the potential to modify genetic material and nearby proteins[2,8-11]. Hypothetically, those events determine an increased susceptibility neoplasms. For instance, we find the highest number of non-melanoma skin cancer cases in patients with thiopurines prescribed as immunosuppressants in organ transplant[12]. Other studies have suggested a relation between the development of malignancies and the total thiopurine doses, its metabolite levels and thiopurine *S*-methyltransferase (TPMT) mutations[13-17].

Therefore, with the aim of review the incidence of thiopurine induced neoplasms in our IBD patients and treated with thiopurines at the University Hospital Virgen de las Nieves (Granada, Spain), we designed a retrospective observational study, comparing our results with previously published papers.

MATERIALS AND METHODS

An observational, retrospective study, analysing data from patients with a confirmed IBD diagnosis from 1996 to the present was designed. Data were collected from the electronic clinical charts of each patient, as well as from our local database. We included demographical variables and specific data such as TPMT activity, use of thiopurines (azathioprine and mercaptopurine), as well as other immunosuppressants and biological agents. We also studied the appearance of neoplastic diseases, age of diagnosis and type of disease.

Statistical analysis

Resulting variables were analysed by means of SPSS 18 software (Chicago, IL, United States). Univariate analysis was performed with the χ^2 test, Fisher exact test, Student *t* test or Wilcoxon as appropriate, we used 95%CI for de odds ratios and statistical signification was considered when $P < 0.05$.

Multivariable logistic-regression analyses involving treatment regimen and prespecified baseline characteristics, which were the ones with statistical significance in

Table 1 Patients' characteristics *n* (%)

	Thiopurines (*n* = 429)	No thiopurines (*n* = 383)	*P* value
Age of diagnosis of IBD (yr)	30.85	40.13	< 0.001
Sex			
Female	192	178	> 0.050
Male	237	205	> 0.050
Ulcerative colitis[1]	*n* = 130	*n* = 263	< 0.001
Extension			
E1	5 (3.85)	51 (19.4)	
E2	42 (32.3)	103 (39.2)	
E3	83 (63.85)	109 (41.4)	
Severity			
S1	51 (39.2)	150 (57)	
S2	58 (44.6)	102 (38.8)	
S3	21 (16.2)	11 (4.2)	
Crohn's disease[2]	*n* = 299	*n* = 120	< 0.001
Age of diagnosis (yr)			
A1	33 (11)	6 (5)	
A2	214 (71.6)	68 (56.7)	
A3	52 (17.4)	46 (38.3)	
Location			
L1	137 (45.8)	46 (38.3)	
L2	54 (18)	34 (28.3)	
L3	96 (32.1)	39 (32.5)	
L4	12 (4.1)	1 (0.8)	
Behaviour			
B1	155 (51.8)	105 (87.5)	
B2	55 (18.4)	10 (8.3)	
B3	89 (29.8)	5 (4.2)	
P	73 (24.4)	15 (12.5)	
Neoplasms	*n* = 17	*n* = 31	0.013

[1]Patients with ulcerative colitis treated with thiopurines had more severity and disease extension; [2]Patients with Chron's disease treated with thiopurines more usually had ileal or ileocholic location and an inflammatory or penetrating behaviour; Patients under thiopurine treatment had a lower age at diagnosis. IBD: Inflammatory bowel disease.

Table 2 Montreal classification of inflammatory bowel disease

IBD	Montreal classification
Crohn disease	
Age of diagnosis	A1 below 16 yr
	A2 between 17 and 40 yr
	A3 above 40 yr
Location	L1 ileal
	L2 colonic
	L3 ileocolonic
	L4 isolated upper disease
Behaviour	B1 non-stricturing, non-penetrating
	B2 stricturing
	B3 penetrating
	P perianal disease
Ulcerative colitis	
Extent	E1
	Ulcerative proctitis (distal to the rectosigmoid junction)
	E2
	Left sided UC (distal to the splenic flexure)
	E3
	Extensive UC (proximal to the splenic flexure)
Severity	S0
	Clinical remission (asymptomatic)
	S1
	Mild UC: Passage of four or fewer stools/d with or without blood, absence of any systemic illness, and normal inflammatory markers
	S2
	Moderate UC: Passage of more than four stools per day but with minimal signs of systemic toxicity
	S3
	Severe UC: Passage of at least six bloody stools daily, pulse rate of at least 90 beats/min, temperature of at least 37.5 ℃, haemoglobin of less than 10.5 g/100 mL, and ESR of at least 30 mm/h

IBD: Inflammatory bowel disease; ESR: Erythrocyte sedimentation rate; UC: Ulcerative colitis.

univariate analyses and also the factors with a potential or already recognized influence on neoplasm development, were performed to evaluate the risk of malignancies. A stepwise procedure was used to identify independent risk factors for neoplasm development (with $P = 0.05$ as the threshold level for variables to be entered into the model and retained in the final model).

An online pubmed search was performed with the terms "thiopurines", "azathiopurine", "mercaptopurine", "neoplasm", "malignancy", "inflammatory bowel disease", "ulcerous colitis (UC)" and "Crohn's disease (CD)", in order to review published data on thiopurines, neoplasms and IBD.

RESULTS

Eight hundred and twelve patients with confirmed IBD diagnosis were included; 48.4% had UC and 45.6% were women (Table 1). The average age of diagnosis was 35.23 ± 16.5 years (range: 5-85 years), 34.99 ± 16.7 years for females (range: 5-85 years) and 35.43 ± 16.4 years (range: 5-82 years) for males.

Among CD patients (Table 1), 67.3% had been diagnosed between 16 to 40 years (A2 in Montreal classification), the most frequent location (43.7%) was ileal (L3 of Montreal classification) (Table 2)[18] and the most usual pattern (62.1%) was inflammatory (B1 of Montreal). In UC (Table 1), 48.9% had pancolitis (E3 in Montreal classification) and 51.1% presented as a mild inflammatory bout (S1 of Montreal).

With regards to the treatments used (Table 3), more than a half of the patients (52.8%) have been treated with thiopurines (mostly with azathiopurine) at some point during their illness; other treatments such as biological ones and immunosuppressants (methotrexate, cyclosporine, tacrolimus) have been used less frequently (25.2%). In general, the treatment with thiopurines was more frequently used in CD patients.

The average daily dose of azathiopurine was 123.79 ± 36.5 mg (range: 50-250 mg), with an average usage time 72.16 ± 55.7 mo (range: 1-300 mo), and the accumulated dose for this time was 274.32 ± 233.5 g (range: 1.5-1350 g). With regards to mercaptopurine, the average daily dose was 74.7 ± 23.9 mg (range: 25-150 mg), with an average time under this thiopurine treatment of 23.37 ± 27.6 mo (range: 1-118 mo), and a total accumulated dose of 52.2 ± 63.5 g (range: 1.5-243 g). TPMT activity (tested

Table 3 Treatments *n* (%)

	Thiopurines (*n* = 429)	No thiopurines (*n* = 383)	*P* value
TPMT activity	283 (66)	-	
Low	5 (1.8)	-	
Intermediate	43 (15.2)	-	
High	235 (83)	-	
Azathioprine	341 (79.5)	-	
Mercaptopurine	60 (14)	-	
Azathioprine + Mercaptopurine	28 (6.5)	-	
Infliximab	112 (26.1)	7 (1.8)	< 0.001
Adalimub	50 (11.7)	3 (0.8)	< 0.001
Other immunosuppressants	29 (6.8)	4 (1)	< 0.001

Patients (98.2%) treated with thiopurines have intermediate or high activity. Most of the patients treated with thiopurines take azathioprine. TPMT: Thiopurine S-methyltransferase.

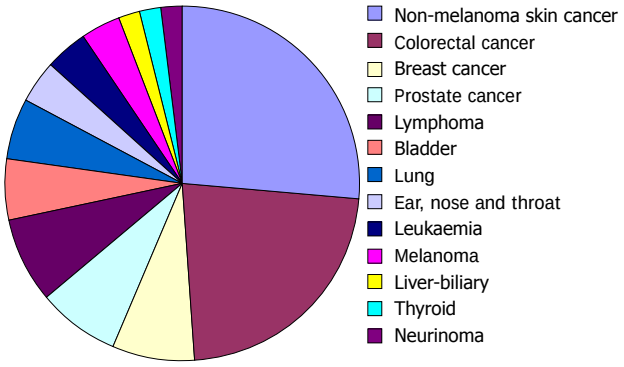

Figure 1 Types of neoplasms (*n* = 53). The most frequent neoplasms were non-melanoma skin cancer, colorectal cancer and haematological (lymphomas and leukaemia).

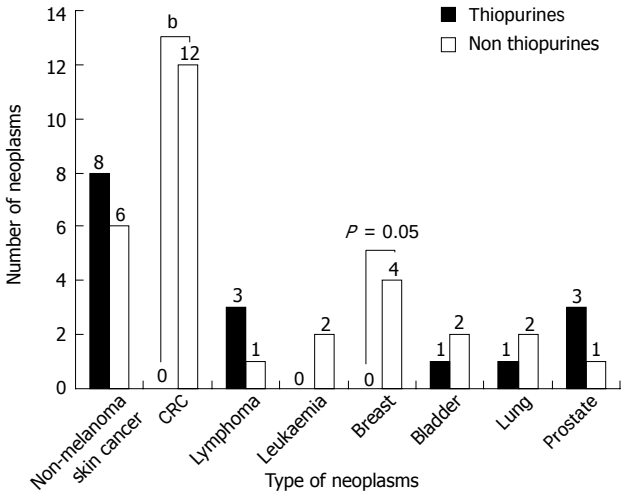

Figure 2 All the neoplasms, except for non-melanoma skin cancer, lymphomas and prostate, have been more common in patients not treated **with thiopurines, however, significant differences have only been identified in colorectal and breast cancer.** Non thiopurines *vs* thiopurines, [b]*P* value < 0.01 (OR = 0.96, 95%CI: 0.94-0.98); *P* value = 0.05 (OR = 0.99, 95%CI: 0.98-1).

by high-performance liquid chromatography: low < 5 IU/mL, intermediate 5-20 IU/mL, high > 20 IU/mL) has been tested, before using thiopurines, for 66% of the patients treated with these drugs, of which 98.2% had intermediate or high activity. The average usage time for infliximab was 29.09 ± 25.8 mo (range: 1-112 mo) and for adalimumab 25.4 ± 20.4 mo (range: 2-79 mo).

Fifty-one point nine percent of the women received thiopurines, as well as 71.4% of patients with CD and 33.1% of the patients with UC $P < 0.001$ (OR = 5.04, 95%CI: 3.74-6.79).

We found a total of 74 neoplasms in 67 patients (7 patients had 2 neoplasms), 21 of those neoplasms appeared before the diagnosis of IBD, and so they were excluded from the analysis, leaving 53 neoplasms which appeared in 48 patients (5 of which had 2 neoplasms) (Figure 1). Of these neoplasms, 37 (69.8%) appeared after the IBD diagnosis but before the use of thiopurines, while 16 (30.2%) were identified after the treatment with thiopurines had started.

Mean time from IBD diagnosis to malignancy finding was 117 ± 91.8 mo (110.96 ± 99.6 mo in patients with no thiopurine treatment, and 129.21 ± 75.7 mo in treated patients, $P > 0.05$), with a mean time from IBD diagnosis to tiopurine treatment of 49.45 ± 57.05 mo. Mean time from thiopurine initiation to malignancy diagnosis was 67.05 ± 53.7 mo. Mean follow up time was 146.25 ± 91.2 mo, 151.56 ± 98 mo in patients who did not received thiopurines and 141.35 ± 84 mo in patients who did ($P > 0.05$).

The most frequent neoplasms were non-melanoma skin cancer, colorectal cancer and haematological (lymphomas and leukaemia). In general, the neoplasms were less frequent in the group of patients treated with thiopurines except for non-melanoma skin cancer, lymphoma and prostate cancer; however, these differences reached statistical significance only in the cases of breast and colorectal cancer, which had been more frequent in patients not treated with thiopurines (Figure 2).

In the univariate analysis, we identified a higher incidence of neoplasms among patients not treated thiopurines (8.1% *vs* 4%) ($P = 0.013$, OR = 0.469, 95%CI: 0.255-0.861). However, when considering the two types of thiopurines separately [azathioprine (AZA) or 6-mercaptopurine (6-MP)] we did not find these differences: in AZA group, 4.3% of those treated with it developed a neoplasm in comparison to 7.2% of those not treated with AZA ($P > 0.05$); in the 6-MP group, 2.3% of those treated with it developed a neoplasm, in comparison to 6.4% of those not treated with 6-MP ($P > 0.05$). Moreover, we did not observe differences in the incidence of neoplasms in relation to other treatments (infliximab, adalimumab or other non-thiopurine immunosuppressants).

With regard to sex, 4.3% of the women have developed a neoplasm in comparison to 7.2% of the men ($P > 0.05$); however, we observed a higher incidence of neoplasms among women not taking thiopurines compared to the ones treated with this drugs; $P = 0.039$ (OR = 0.294, 95%CI: 0.093-0.930), these differences are not seen among men ($P > 0.05$).

With respect to the type of IBD, we have identified more neoplasms among patients with UC (7.9%) than

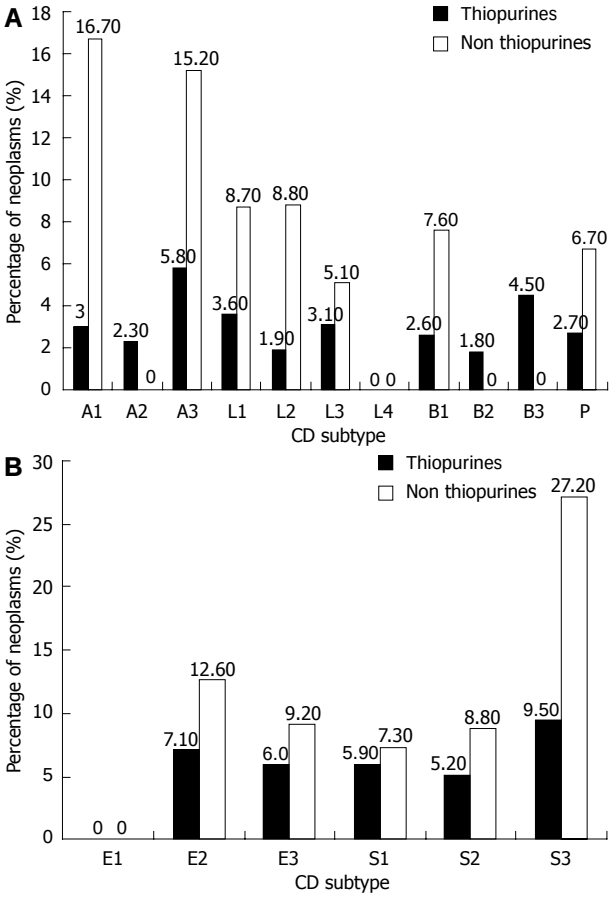

Figure 3 There seem to be no statistically significant differences in the appearance of neoplasms in any of the locations, behaviours or ages of diagnosis of the patients with Crohn's disease and ulcerative colitis in relation to thiopurines intake. A: Percentage of neoplasms according to Crohn's disease (CD) subtype; B: Percentage of neoplasms according to ulcerative colitis (UC) subtype.

among patients with CD (4.1%), $P = 0.021$ (OR = 0.494, 95%CI: 0.269-0.907). However, when we studied the emergence of neoplasms in relation to the treatment with thiopurines in each type of IBD (UC and CD), we found no differences in any of the groups ($P > 0.05$).

In patients with CD, after stratifying patients by age of diagnosis, location and disease pattern, we noted a higher number of neoplasms among patients not treated with thiopurines in all groups, without reaching statistical significance (Figure 3A). This also occurred in patients with ulcerative colitis when stratifying them regarding the extension of the disease and its severity (Figure 3B).

In the logistic regression analysis, we only identified the age of diagnosis of IBD as a risk factor ($P = 0.03$, OR = 1.34, 95%CI: 1.003-1.066).

DISCUSSION

For years, the aim of treating IBD has been to control the patient's symptoms, but as we have access to new treatments for this disease, the goal is becoming more ambitious, even trying to obtain mucosal healing. This is the reason for international guidelines to propose the use of stronger treatments in earlier stages and, although with this form of treatment we improve our control of the disease and its complications, we also increase the number of patients with treatments with potentially significant side effects (immunosuppressants, biological therapies, *etc.*). One of these undesirable effects is the development of a neoplastic disease which could be increased by the use of immunosuppressants and immunomodulators, given that these drugs block the natural containment mechanisms of neoplastic diseases as has been previously described in solid organ transplantation receptors.

On the other hand, in IBD as a chronic inflammatory disorder, there is a higher risk of neoplasms (it is known that there is a higher risk of colorectal cancer especially in more severe a long-lasting forms of the condition) and although the use of immunosuppressants such as thiopurines may reduce this risk by controlling the underlying inflammatory process, it is hard to establish whether the increased risk of malignancies in patients with IBD treated with thiopurines is due to the disease itself or to the treatment. Aiming to clarify this matter, there is an increasing number of publications suggesting a causative relationship between neoplasms affecting IBD patients and the treatment provided[1].

In our study, the incidence of neoplasms has been double among patients not treated with thiopurines despite having used them for a prolonged period of time (an average of 72.16 mo for AZA and 23.37 mo for MP) and with high doses (average accumulated dose of 274.3 g of AZA and 52.2 g of MP), which could be due to an improved control of the inflammatory process preventing the appearance of dysplasia and its subsequent progression to neoplasm.

The most frequent neoplasms have been skin ones, excluding melanoma, of which we have identified 14 cases (8 in the thiopurines group and 6 in the non-thiopurines group, $P > 0.05$). Although there are few studies published, the risk of developing this type of neoplasm is estimated to be higher in patients who are treated with thiopurines, especially in relation to the duration of the treatment and the accumulated dose of thiopurines. In the Long *et al*[19] study published in 2010 a relation was established between the risk of developing skin cancer in 50000 patients with IBD, suggesting that there is an increased risk of non-melanoma skin cancer in patients with IBD (RR = 1.64, 95%CI: 1.51-1.78) and the use of thiopurines increases this risk in direct relation to the duration of the treatment (OR = 3.56, 95%CI: 2.81-4.5 at 90 d; OR = 4.27, 95%CI: 3.08-5.92 at 1 year). The risk with other biological treatments was also increased (OR = 2.18, 95%CI: 1.07-4.46). In another study[20] in which 9618 patients with IBD were enrolled, the risk of developing non-melanoma skin cancer was also higher among those who were treated with thiopurines. Furthermore, in a recent paper[21] including 1084 patients, the risk of developing non-melanoma skin cancer was estimated to be 5 times higher among those who were treated with thiopurines, especially in Caucasian patients (12 times

higher). On the other hand, nearly all the studies refer to exposure to sunlight and UVA as a risk factor for this type of neoplasm, recommending to patients treated with thiopurines avoiding this exposure. Indeed, in our study, even though the absolute number of patients affected with this type of cancer is higher in the group of patients treated with thiopurines, there were no significant differences, which could be due to the low number of cases. Despite these data the risk/benefit balance is in favor of using thiopurines, given the low aggressiveness of these neoplasms. However, these patients must be advised to use sunscreen and avoid prolonged exposure to sunlight.

In our study, the second most common neoplasm was colorectal cancer, which was only identified in the group of patients which were not treated with thiopurines (and had not been treated with biological drugs either), of which most are men with UC. It is a well-known fact that patients with uncontrolled IBD have a higher risk of developing this type of neoplasm, especially in extensive UC with a long-term course. It is known that the use of treatments such as 5-aminosalicylates which help to control the inflammatory activity of the IBD reduce the incidence of colorectal cancer[22-26]. The effect of the thiopurines is hard to establish given that many studies refer to a neutral risk[27-31], however, in the 2009 study by Beaugerie *et al*[32] with 19438 patients included, they observed a seemingly protective effect derived of the use of thiopurines, an effect which has also been seen in another study published in the same year by Andrews *et al*[33]. Our results show a lower risk of colorectal cancer in patients with IBD treated with thiopurines ($P < 0.001$, OR = 0.961, 95%CI: 0.942-0.981), probably due to an improved control of the disease and a reduction of the sustained colonic inflammation. The fact that in our study colorectal cancer is more frequent in patients with UC, may be due to the fact that in these patients the inflammation of the colon increases its risk as well as the fact that CD patients received thiopurines more frequently, which would have played a protective role in relation to colorectal cancer by improving control over the inflammatory process.

Among the 812 patients, 5 lymphomas have been identified, of which 1 was diagnosed prior to the CD diagnosis, thus, it has been excluded from the analysis. Of the 4 remaining, there has been 1 case identified among the patients not treated with thiopurines and 3 among those treated with thiopurines, without significant differences. There are contradictory data regarding the appearance of lymphomas in patients with IBD among different studies[34-38]. In the paper by Beaugerie *et al*[39] published in Lancet in 2009, with 19486 patients, an increased risk of developing intestinal lymphoma was identified in patients with IBD, which suggests that this disease *per se* entails a higher risk of developing lymphoma. The authors observed an increased risk of lymphoma among the patients on thiopurines therapy (HR = 5.28, 95%CI: 2.01-13.9, $P = 0.0007$) which decreased to a similar risk in patients never treated with these drugs when they were suspended; however, in a subgroup of patients who stopped taking thiopurines, inflammatory activity was greater and the risk of lymphoma was lower, concluding that the risk was associated to thiopurines and not to inflammatory activity. In other studies[40], there are even cases describing lymphoma regression in CD after suspending treatment with Azathioprine. In a 2005 meta-analysis[41] there was a RR of 4 in the patients with IBD who received thiopurines in relation to those who did not take them. However, the absolute risk of lymphoma continues to be low and is estimated in 300-1400 cases-year of treatment, although the subgroup of elderly patients with IBD who would comprise a higher risk of this malignancy.

Moreover, there is a clear relationship, already known from other studies[42-46], between the Epstein-Barr virus (EBV) infection and the development of lymphoma. In our series only one patient had a positive serology to EBV.

T cells hepatosplenic lymphoma is a highly aggressive variant of lymphoma, described in patients who were co-treated with thiopurines and infliximab[47-50], although there are other published cases of patients treated with TNF inhibitor monotherapy[51-53] or azathioprine alone[54-57], from which we deduce that the increased risk of developing this special form of lymphoma may be due to the IBD itself, its treatment (monotherapy or combined treatment) or a combination of both. For thiopurines, the cases published occur after 1-2 years of treatment. In our study we have not observed any case of this nature due to its low incidence.

Another malignant haematological disorder frequently associated with IBD and thiopurines is acute myeloid leukaemia (AML). We have identified two cases of AML, both of which have been in patients not treated with thiopurines (neither have they been treated with Infliximab or Adalimumab) which entails a 0.5%. In the study by Caspi *et al*[58], the risk of presenting this entity for patients with IBD has been established at 5.3 times higher than in the general population, and it has been mainly related to the dose and the duration of the treatment[59,60], increasing when this exceeds 5 years or 600 g of accumulated AZA doses. On the other hand, in other studies there is a subgroup of patients in which the accumulated dose is low and the risk of AML continues to be high, this leads us to think of an increased susceptibility to this drug within a subgroup of patients, which could be explained by the TPMT activity, as was clarified in the paper by Relling *et al*[61] and Bo *et al*[62] in which the low TPMT activity was related to the AML risk and secondary myelodysplastic syndromes.

Therefore, despite the existence of contradictory data, there is a higher risk of malignant haematological disorders in patients with IBD and this risk would be higher, although only slightly, in patients treated under thiopurines therapy (depending on the total accumulated doses, duration of the treatment, TPMT activity and the aggressiveness of the treatment measured through metabolites or toxicity such as leucopenia); however, this

risk is overcome by the benefit of its use. In our study we observed no significant differences in these neoplasms incidence. However, we observed all the cases of leukemia neither in patients treated with thiopurines nor with TNF inhibitors, and most of the lymphomas appeared in patients treated with thiopurines. These differences with regard to the published data could be explained by to the low number of cases observed and the high TPMT activity found. Although some studies establish a relationship between the risk of developing these neoplasms with the levels of active thiopurines metabolites, this measurement is unavailable in the University Hospital Virgen de las Nieves, (Granada-Spain), thus, we are unable to provide more data.

Another important neoplasm observed in our series is breast cancer, which appeared in 4 cases, all of which occurred in women not treated with thiopurines ($P = 0.05$, OR = 0.99, 95%CI: 0.98-1). There are little data regarding the occurrence of this neoplasm in patients treated with thiopurines or with IBD and they don't seem to have a higher risk compare to the general population[63,64]. However, there are more data regarding the development of other gynecological neoplasms such as cervical cancer. As most of them are related to the infection by human papillomavirus types 16 and 18, we could hypothesize that the use of immunosuppressants would increase the risk of this type of neoplasms; however, this has not been proved in the initial studies[37,65]. Indeed, other studies showed an increase incidence in cervical dysplasia in patients with IBD[66,67] and subsequent papers showed a higher risk of cervical cancer in relation to the duration of the treatment with azathioprine, combined use of corticoids or the use of tobacco[68-71]. In conclussion, the risk of breast or cervical cancer in patients with IBD is similar to the general population, and it seems not affected by thiopurines.

Among the risk factors identified in other studies, the accumulated doses of thiopurines, the usage time, the TPMT activity and the use of tobacco have been recognized. None of those factors has been found as a risk factor for any of the neoplasms described in our series. Regarding tobacco, we are unable to provide data as this information is lacking in our database. However, it is an obvious risk factor for the development of neoplastic pathology and it might have played a role.

In conclusion, in our study neoplasms were more frequent among the patients who have not been treated with thiopurines, as well as in patients diagnosed with UC (the treatment with thiopurines was more usual among CD patients), regardless of the sex. Considering the type of neoplasm, we observed significant differences in colorectal and breast cancer, for which the use of thiopurines has a protective role. In the rest of neoplasms we did not observe any difference, but the lymphomas and non-melanoma skin cancer had a higher incidence among patients treated with thiopurines, as occurs in the previously published studies. Due to this, we can conclude that the benefit provided by thiopurines use surpasses the risk.

COMMENTS

Background

Inflammatory bowel disease (IBD) is a chronic condition with bouts of bowel inflammation, resulting in abdominal pain, diarrhea, weight loss, and bleeding among other symptoms. Pathogenesis is mostly unknown, but autoimmunity is involved, and immunosupressants like thiopurines (azathioprine and mercaptopurine) are one of the available therapeutic options. However, these drugs have the theoretical potential to facilitate the development of other diseases controlled by the immune system, as infections or neoplasms. Therefore, it is essential to determine whether thiopurines therapy has an influence on neoplasms development, and if this effect is more intense that the benefits for IBD.

Research frontiers

A variety of drugs are used for IBD therapy, and changes have to be done depending on disease control or adverse events. Because of this, it is very difficult to determine whether the risk of neoplasm is attributable to a given drug, its combination with other, the time under treatment, doses or the disease itself. Moreover, intervening diseases, therapies, smoking and the severity of the disease itself could also have a role in neoplasms development.

Innovations and breakthroughs

Early thiopurines therapy provides a better control of IBD symptoms and prevents its complications. Nevertheless, a higher risk of neoplasms has been suggested in some papers after an early immunosuppressants introduction, although in other studies addressing this same issue this effect is not clear. They present a group of 812 IBD patients, 52.8% under thiopurines treatment, in which they have studied malignancies development.

Applications

Their results suggest that thiopurine treatment does not increase the risk of neoplasms in IBD patients. They have shown to be safe and effective drugs for disease control.

Terminology

IBD is a chronic sistemic condition that affects chiefly the gastrointestinal tract. The disease consists in inflammatory changes that involve the target organs. Thiopurines (azathioprine and mercaptopurine) are drugs known as immunosupresants, with an effect on the immune system and the inflammatory changes triggered by it.

Peer review

In this paper, the authors describe a retrospective analysis of 812 patients with inflammatory bowel diseases who were treated in a single center in Spain since 1996. The authors mainly compare malignancy incidences between patients treated with thiopurines and those who never received thiopurines.

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ORIGINAL ARTICLE

Histopathology of type C liver disease for determining hepatocellular carcinoma risk factors

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Abstract

AIM: To evaluate the histopathological findings of type C liver disease to determine risk factors for development of hepatocellular carcinoma (HCC).

METHODS: We studied 232 patients, who underwent liver biopsy for type C chronic liver disease between 1992 and 2009, with sustained virological response (SVR) after interferon therapy. The patients were divided into two groups according to the F stage 0 + 1 + 2 group (n = 182) and F3 + 4 group (n = 50). We prospectively observed and compared the incidence of HCC of the patients with SVR in the F0 + 1 + 2 and F3 + 4 groups. Then, the background factors and liver histopathological findings, including the degree of fibrosis, F stage, inflammation, necrosis, bile duct obstruction, fat deposition, and degree of irregular regeneration (IR) of hepatocytes, were correlated with the risk of developing HCC.

RESULTS: HCC developed in three of 182 (1.6%) patients in the F0 + 1 + 2 group, and four of 50 (8.0%) in the F3 + 4 group. The cumulative incidence of HCC in the former group was found to be significantly lower than in the F3 + 4 group (log rank test P = 0.0224). The presence of atypical hepatocytes among IR of hepatocytes in the F3 + 4 group resulted in a higher cumulative incidence of HCC, and was significantly correlated with risk of HCC development (RR = 20.748, 95%CI: 1.335-322.5, P = 0.0303).

CONCLUSION: Atypical hepatocytes among the histopathological findings of type C liver disease may be an important risk factor for HCC development along with progression of liver fibrosis.

Key words: Hepatocellular carcinoma; Irregular regeneration of hepatocytes; Liver fibrosis; Type C chronic liver disease; Histopathology of liver biopsy

Core tip: To evaluate the histopathological findings of type C liver disease to determine risk factors for the development of hepatocellular carcinoma (HCC), we studied 232 patients, who underwent liver biopsy, with sustained virological response after interferon therapy. We investigated in detail the histopathological findings, and analyzed the findings to determine the risk factors. Consequently, atypical hepatocytes among irregular regeneration of hepatocytes may be an important risk factor for HCC development, along with progression of liver fibrosis. Clarification of atypical hepatocytes as a risk factor of carcinogenesis may aid in the early diagnosis of HCC.

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Ogawa M, Tanaka N, Moriyama M. Histopathology of type C liver disease for determining hepatocellular carcinoma risk factors. *World J Gastroenterol* 2013; 19(30): 4887-4896 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/4887.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.4887

INTRODUCTION

At our facility, we maintain a database of hepatitis patients who were recruited with informed consent from the 1990s. We previously reported the natural progression of patients with type C chronic hepatitis (CH) and liver cirrhosis (LC) - both attributable to hepatitis C virus (HCV) infection - over the course of about 30 years. The incidence of hepatocellular carcinoma (HCC) among those with LC was 7% per annum[1,2]. Many other studies have reported the progression of liver disease and the risk of development of HCC with HCV infection[3-8].

In this study, we collected liver biopsy samples from type C chronic liver disease patients before interferon (IFN) therapy and examined in detail the histopathological findings of the liver tissue. According to the progression of liver fibrosis (F stage), we examined the factors that may contribute to the development of HCC. We further studied the association of the condition of the hepatocytes, present during regeneration of the liver parenchyma destroyed by infection, with the incidence of HCC. The irregular regeneration (IR) of hepatocytes, which occurs as a result of repeated cycles of necrosis and regeneration of the liver parenchyma in CH, was found to be important for the prognosis of HCC[9-15]. A few studies have sought to determine the association between the histopathological findings from liver biopsies and the risk of developing HCC[4]. Therefore, patients with type C chronic liver disease who underwent liver biopsy were investigated in detail for findings on the sites and degree of fibrosis and inflammation, necrosis, IR of hepatocytes, and fat deposition in the liver. These findings were analyzed to determine the risk factors for development of HCC.

MATERIALS AND METHODS

Study population

In this study, samples from 482 patients with type C liver disease who underwent liver biopsy between 1992 and 2009 were collected prior to the start of IFN therapy. Two hundred and thirty-two patients achieved a sustained virological response (SVR) to IFN therapy. We determined the F stage in the liver (stages 0-4, as described for histological scoring) by liver biopsy according to the method of Desmet *et al*[16], Knodell *et al*[17] and Ishak *et al*[18]. Then, 232 patients were divided into two groups according to the F stage: stages 0, 1 and 2 (F0 + 1 + 2 group, n = 182) and stages 3 and 4 (F3 + 4 group, n = 50). We prospectively observed and compared the incidence of HCC in the patients with SVR in the F0 + 1 + 2 and F3 + 4 groups. The observation period was from the time of diagnosis of SVR to the time of the final examination or of diagnosis of HCC. Then, the clinical background factors and the histopathological findings at the liver biopsy were correlated with the risk of developing HCC together with progressive liver fibrosis.

All of the patients were positive for serum HCV antibody (2nd generation ELISA; Dinabot, Tokyo, Japan) and HCV RNA in serum, and negative for serum hepatitis B surface antigen (HBsAg ELISA; Dinabot), anti-nuclear antibody (indirect immunofluorescence assay: IF, Special Reference Laboratory, Tokyo, Japan), anti-smooth muscle antibody (IF), and anti-mitochondrial antibody (IF). No heavy drinkers (more than 30 g of ethanol intake daily) were included.

We confirmed the positivity and measured the concentration of HCV RNA in the blood samples using the competitive reverse transcriptase-polymerase chain reaction and DNA probe methods (Special Reference Laboratory, Tokyo, Japan) or Amplicor monitoring method (Amplicor HCV Monitor, Roche Diagnostic K.K., Tokyo, Japan). Subjects without HCC were confirmed by abdominal computed tomography (CT) or ultrasonography within the 6 mo prior to initial liver biopsy. At the time of liver biopsy, we determined the patient's body mass index, serum aspartate aminotransferase (AST) level (U/L), serum alanine aminotransferase (ALT) level (U/L), serum γ-glutamyltransferase (GT) level (U/L), serum platelet count ($\times 10^4/\mu L$), and HCV genotype, and carried out a histopathological examination of the biopsy specimens. We recorded the patient's sex and age, period of observation (years), and incidence of HCC. All patients gave their consent to be included in this study according to the Declaration of Helsinki.

Administration of IFN

Initial IFN therapy varied according to the time period when the patients were treated. From 1992 to 2001, IFN monotherapy was administered using natural (n) IFN (n-IFNα: Sumiferon, Sumitomo Pharma Co., Osaka, Japan) in 155 cases, recombinant (r)-α2a (r-IFNα2a: Canferon, Takeda Pharmaceutical Co., Osaka, Japan) in 44 cases, and r-α2b (r-IFNα2b: Intron, Schering-Plough Co., Osaka, Japan) in 121 cases. From 2001 to 2007, we treated 68 patients with combination therapy of IFN-α2b and ribavirin (Rebetol; Schering-Plough Co.). From 2007 to 2009, we administered combination therapy of Peg-IFN-α2b (Peg Intron; Schering-Plough Co.) and ribavirin in 93 cases. In accordance with the protocol based on the medical insurance system in Japan, considering the HCV genotype, we administered IFN therapy for 6-12 mo. The following classification of outcomes was determined according to the effects of IFN therapy: SVR was achieved in patients who became negative for serum HCV RNA for > 6 mo after termination of IFN therapy.

Liver biopsy

Liver tissues were taken from patients by percutaneous

needle biopsy (MONOPTY, 14 or 16 gauge; C. R. Bard Inc., Tempe, AZ, United States) with the aid of an ultrasonic echo guide within the 6 mo preceding the start of IFN therapy. These tissue specimens were fixed in 2.5% formalin, embedded in paraffin, sectioned at 3-4 μm, and stained with hematoxylin and eosin (HE).

Histological scoring

The tissues obtained by liver biopsy were evaluated for the sites and degree of fibrosis, inflammatory reaction, and necrosis, as well as the degree of IR of hepatocytes, bile duct obstruction, and fat deposition in the liver.

The histopathological findings were scored using 5 grades: score 0, none of the above features; score 1, minimal detection (observed in less than one-third of the field); score 2, mild (observed in one-third to less than two-thirds of the field); score 3, moderate (observed in two-thirds or more of the field), and score 4, severe (diffusely in all fields). The samples were scored blindly by two pathologists (Moriyama and the author), and the final value was calculated as the average of their scores (mean ± SD).

Only minimal fibrosis was observed in the portal areas of normal livers. Pericellular fibrosis was defined as fibrosis around the hepatocytes, perivenular fibrosis as fibrosis around the central vein, and portal sclerosis as fibrosing expansion of the portal area. The degree of pericellular fibrosis, perivenular fibrosis, and portal sclerosis were evaluated and assigned a score from 0 to 4 as described above for histopathological scoring. According to the method of Desmet *et al*[16] and Knodell *et al*[17], we determined the F stage in the liver (stages 0-4, as described above for histopathological scoring), including bridging fibrosis.

In evaluating hepatocellular necrosis, focal necrosis was defined as necrosis of several hepatocytes with infiltration of inflammatory cells such as macrophages and lymphocytes. Bridging necrosis refers to necrosis connecting areas, such as two portal tracts, leading to extensive hepatocellular necrosis and a state of acute hepatic failure. The patients were further defined as with (Y) or without bridging necrosis (N).

Infiltration of lymphocytes is a characteristic of viral hepatitis. The degree of inflammatory cell infiltration into the liver parenchyma (parenchymal infiltration) and portal area (periportal infiltration) were assessed, and assigned a score from 0 to 4 as described above for histopathological scoring. The degree of lymphocytic infiltration into the entire hepatic lobule (lymphoid reaction) and portal area (portal lymphocyte infiltration) was also evaluated and assigned a score from 0 to 4 as described above for histopathological scoring.

We also investigated the histopathological abnormalities characteristic of IR of hepatocytes (Figure 1), which were evaluated in each sample according to the criteria by Uchida[9], Shibata *et al*[10], Ueno *et al*[11] and Moriyama *et al*[12]. IR comprises the following six elements: dysplastic change, map-like distribution, bulging, oncocytes, nodularity, and atypical hepatocytes. Based on these, we examined the liver biopsy samples for: presence and degree of dysplastic change defined as anisocytosis characterized by variability of the cells; presence and degree of map-like distribution, which is defined as distinct populations of hepatocytes with a homogeneous appearance within each population and separated from each other by a sharp outline; presence and degree of bulging, which is defined as expansive proliferation of hepatocytes compressing the surrounding parenchyma; presence of oncocytes with nonuniformity of the cytoplasm; presence and degree of nodality, defined as nodular appearance of hepatocytes; and presence of atypical hepatocytes characterized by cellular degeneration. Each finding of IR was evaluated and assigned a score from 0 to 4 as described above for histopathological scoring.

Bile duct obstruction was investigated according to the degree of cholangitis by lymphocytes (bile duct damage) and assigned a score from 0 to 4 as described above for histopathological scoring. Finally, the degree of fat deposition in liver (steatosis) was assessed according to the method by Brunt *et al*[19] and Matteoni *et al*[20], and assigned a score from 0 to 4 as described above for histopathological scoring.

Evaluation of the long-term outcome of patients

Patients with IFN therapy underwent abdominal ultrasonography every 3 or 6 mo and abdominal CT examination every 6-12 mo to check for the occurrence of HCC. When space occupying lesions (SOLs) were detected in the livers of the patients by dynamic CT, enhancement of SOLs was observed in the early phase of dynamic CT and the disappearance of SOL staining was observed in the late phase. A precise diagnosis was made by abdominal angiography. When SOLs in the liver were not enhanced in the early phase of dynamic CT, or if a precise diagnosis could not be made by abdominal angiography, tumor biopsy was carried out and a precise diagnosis was made on the basis of the pathological findings.

Statistical analysis

Sex, genotype, and bridging necrosis (Y/N) were compared using the χ^2 test for independence. The remaining parameters including the clinical characteristics at the time of liver biopsy and the liver histopathological findings are shown as mean ± SD and were compared using the Mann-Whitney *U* test. Cumulative incidence curves were determined with the Kaplan-Meier method and the differences between groups were assessed using the log-rank test. Analysis of risk factors for HCC occurrence was made using the Cox proportional hazard model and these were compared by multivariate analysis. These were performed using SPSS 11.0 software (SPSS Inc., Chicago, IL, United States). $P < 0.05$ was considered significant.

RESULTS

Clinical background factors at time of liver biopsy and liver histopathological findings in SVR patients according to F stage

Comparison of clinical background factors at the time of liver biopsy and liver histopathological findings in

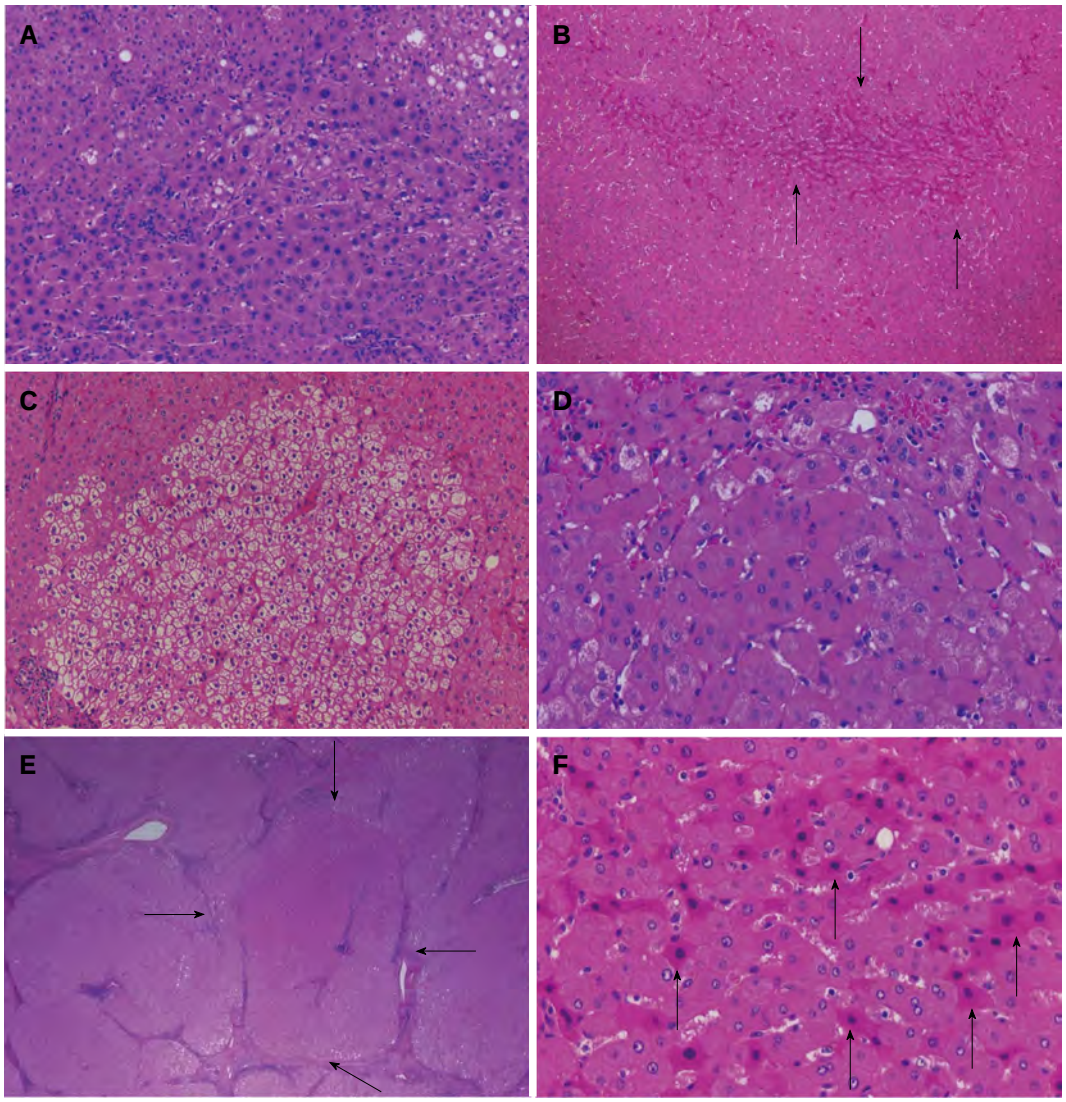

Figure 1 Features of irregular regeneration of hepatocytes. Microscopic views are shown of the biopsied liver tissues (F1 and F3 stage) of representative patients with hepatitis C virus infection. A: Dysplastic change; anisocytosis characterized by variability of cell size with focal dysplastic change [hematoxylin and eosin (HE); × 200]; B: Map-like distribution; distinct populations of hepatocytes with a homogeneous appearance within each population are separated from each other by a sharp outline (arrows) (HE; × 100); C: Bulging; expansive proliferation of hepatocytes compressing the surrounding parenchyma (HE; × 100); D: Oncocytes; oncocytic change of hepatocytes (HE; × 400); E: Nodularlity; nodular arrangement of the parenchyma (arrows) (HE; × 40); F: Atypical hepatocytes; degeneration of hepatocytes (arrows) (HE; × 400). These histopathological findings were scored using 5 grades: score 0, none; score 1, minimal (observed in less than one-third of the field); score 2, mild (observed in one-third to less than two-thirds of the field); score 3, moderate (observed in two-thirds or more of the field) and score 4, severe (diffusely in all fields). The findings were scored as the average (mean ± SD).

SVR patients according to the F stage (F0 + 1 + 2, n = 182 and F3 + 4, n = 50) was performed (Tables 1 and 2). As demonstrated in Table 2, the number of patients for each score was compared, then the presence of bridging necrosis (Y/N) was compared, and the remaining parameters are presented as mean ± SD. The findings demonstrated that there was a higher level of deterioration of the liver in the F3 + 4 group as compared to the F0 + 1 + 2 group; liver fibrosis was more progressive, age, AST, ALT, and α-fetoprotein (AFP) were higher, platelet count was lower, and inflammatory reaction was stronger.

Cumulative incidence of HCC between patients with SVR in the F0 + 1 + 2 and F3 + 4 groups

The cumulative incidence of HCC was compared between patients with SVR in the F0 + 1 + 2 and F3 + 4 groups by the Kaplan-Meier method (Figure 2A). HCC developed in seven of 232 (3.0%) patients, which included three of 182 (1.6%) patients in the F0 + 1 + 2 group, and four of 50 (8.0%) patients in the F3 + 4 group. The cumulative incidence of HCC in the former group was found to be significantly lower than in the F3 + 4 group (log rank test P = 0.0224).

Cumulative incidence of HCC in the F0 + 1 + 2 and F3 + 4 patients with or without atypical hepatocytes

Among the histopathological findings in the liver, the occurrence of the IR of hepatocytes is analyzed to determine the risk factors for development of HCC together with progressive liver fibrosis. In order to correct for the

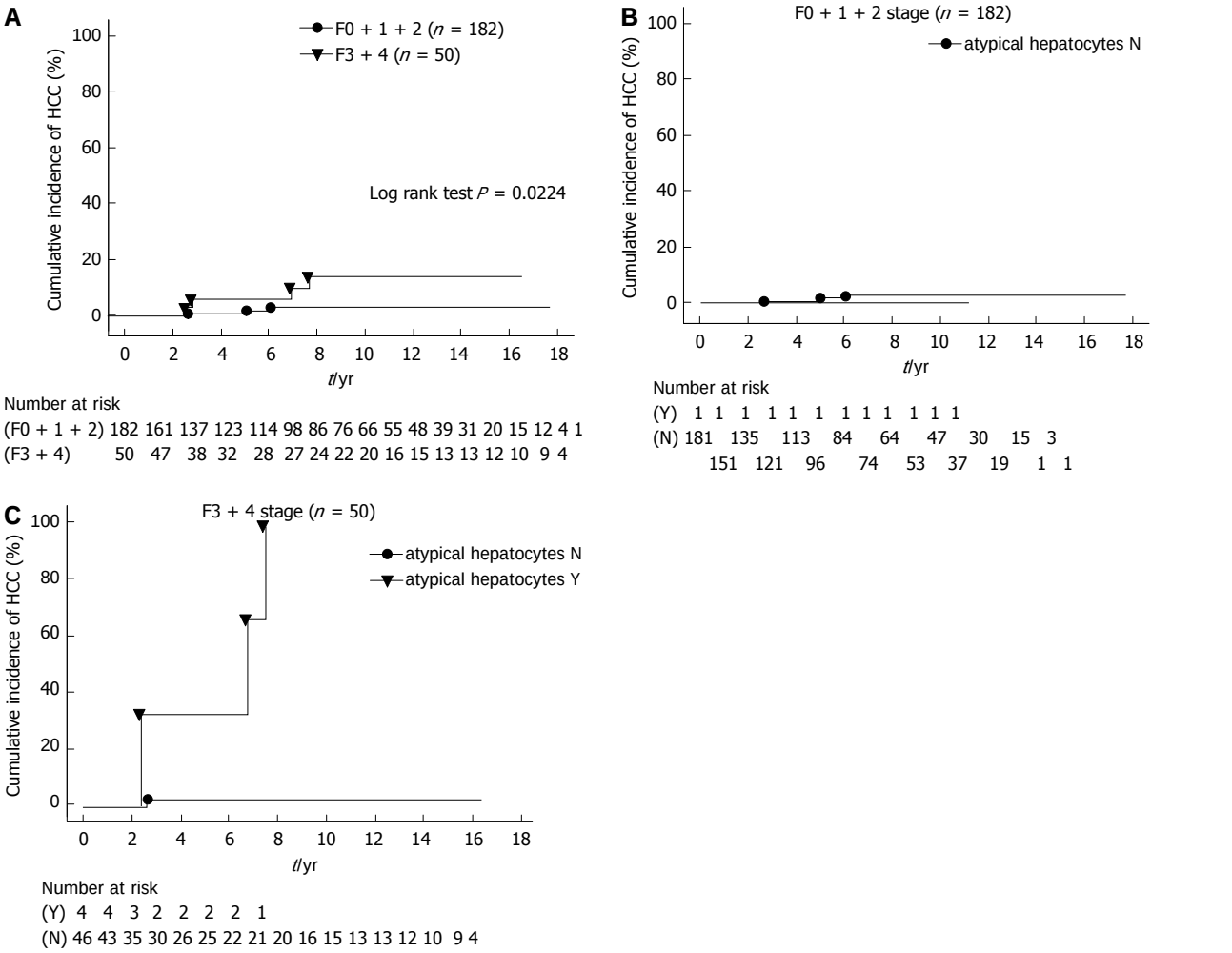

Figure 2 Comparison of the cumulative incidence of hepatocellular carcinoma. A: Between patients with sustained virological response in the F0 + 1 + 2 and F3 + 4 groups. The cumulative incidence of hepatocellular carcinoma (HCC) was compared between patients with sustained virological response in the F0 + 1 + 2 and F3 + 4 groups by the Kaplan-Meier method. HCC developed in seven of 232 (3.0%) patients, which included three of 182 (1.6%) patients in the F0 + 1 + 2 group, and four of 50 (8.0%) patients in the F3 + 4 group. The cumulative incidence of hepatocellular carcinoma in the former group was found to be significantly lower than in the F3 + 4 group (log rank test $P = 0.0224$). Values below the graphs show the numbers at risk; B and C: Between patients with sustained virological response in the atypical hepatocytes (N) and (Y) groups, according to the F0 + 1 + 2 and F3 + 4 groups. HCC developed in seven of 232 (3.0%) patients, of which three belonged to the F0 + 1 + 2 group and four to the F3 + 4 group. To correct for the atypical hepatocytes, the 232 patients were divided into two groups as follows: those with an absence (score 0) of atypical hepatocytes (N group, $n = 227$) and those with presence (score 1) of atypical hepatocytes (Y group, $n = 5$). The Y group included all cases with scores 1-4, but actually there were only cases with score 1. The cumulative incidence of HCC was compared in the F0 + 1 + 2 and F3 + 4 patients according to the atypical hepatocyte status by the Kaplan-Meier method. For the F3 + 4 patients, we found that there was a significantly lower cumulative incidence of HCC in the N group as compared to the Y group (log rank test, $P = 0.0001$). Values below the graphs show the numbers at risk.

IR of hepatocytes, dysplastic change, map-like distribution, bulging, oncocytes, nodularity, and atypical hepatocytes, the 232 patients were divided into two groups as follows: those with an absence (score 0) of IR of hepatocytes (N group) and those with presence (score 1-4) of IR of hepatocytes (Y group) according to the F0 + 1 + 2 and F3 + 4 groups. The cumulative incidence of HCC was compared with the IR of hepatocytes according to the F0 + 1 + 2 and F3 + 4 patients by the Kaplan-Meier method.

For example, to correct for the atypical hepatocytes, the 232 patients were divided into two groups as follows: those with an absence (score 0) of atypical hepatocytes (N group, $n = 227$) and those with presence (score 1-4) of atypical hepatocytes (Y group, $n = 5$). The Y group included all cases with score 1, but there were only cases with score 1. For the F3 + 4 patients, we found that there was a significantly lower cumulative incidence of HCC in the N group as compared to the Y group (log rank test, $P = 0.0001$, Figure 2B and C). However, there was no significant difference in the remaining IR of hepatocytes (data not shown). Thus, our findings demonstrate that with the progression of liver fibrosis, those patients with atypical hepatocytes among the IR of hepatocytes have a higher cumulative incidence of HCC.

Comparison of clinical background factors to determine association with development of HCC

In the F0 + 1 + 2 and F3 + 4 groups, the findings were analyzed to determine the risk factors for development of HCC using the Cox proportional hazard regression method. We compared the clinical background factors at

Table 1 Comparison of clinical background factors at the time of liver biopsy in sustained virological response patients, between F0 + 1 + 2 and F3 + 4 groups *n* (%)

Parameter		F0 + 1 + 2	F3 + 4	*P* value
Number	232	182	50	
Gender (male)	136 (58.6)	104 (57.1)	32 (64.0)	0.3832
Age (yr)	47.9 ± 12.2	45.9 ± 12.4	55.2 ± 8.3	0.0001
BMI	22.6 ± 3.3	22.7 ± 3.3	22.2 ± 3.6	0.5466
AST (U/L)	55.2 ± 45.9	49.8 ± 46.6	74.5 ± 37.6	0.0007
ALT (U/L)	75.5 ± 62.7	70.5 ± 63.0	93.5 ± 58.9	0.0216
γ-GT (U/L)	62.3 ± 71.8	61.4 ± 76.7	65.8 ± 50.4	0.7048
Platelet count ($\times 10^4/\mu L$)	18.8 ± 6.2	20.2 ± 6.0	13.9 ± 3.9	0.0001
Genotype type 1/2	108 (46.5)/ 124 (53.5)	86 (47.2)/ 96 (52.8)	22 (44.0)/ 28 (56.0)	0.6830
AFP (ng/mL)	7.7 ± 13.3	5.4 ± 8.6	15.5 ± 21.4	0.0008
Observation period (yr)	7.5 ± 4.9	7.4 ± 4.7	8.0 ± 5.5	0.4350

These 232 patients were divided into two groups according to the F stage: stages 0, 1, and 2 (F0 + 1 + 2 group, *n* = 182) and stages 3-4 (F3 + 4 group, *n* = 50). Comparison of clinical background factors at the time of liver biopsy in SVR patients according to the F stage (F0 + 1 + 2 and F3 + 4) was performed, and the data are shown. The clinical background factors demonstrated that there was a higher level of deterioration of the liver in the F3+4 group as compared to the F0 + 1 + 2 group; Age, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and α-fetoprotein (AFP) was higher, and platelet count was lower. The parameters are presented as mean ± SD. AST: Upper limit of the normal range is 38 U/L; ALT: Upper limit of the normal range is 44 U/L; γ-glutamyltransferase (GT): Upper limit of normal is 73 U/L; AFP: Upper limit of normal is 20.0 ng/mL; Platelet count: reference value is 15.0×10^4-$35.0 \times 10^4/\mu L$. BMI: Body mass index.

liver biopsy of the patients with SVR in the F0 + 1 + 2 and F3 + 4 groups (Table 3). Univariate analysis using the Cox proportional hazard regression method was applied to these parameters to determine the clinical background factors that were associated with the development of HCC. None of the clinical background factors showed any significant associations with HCC. Blank values in the table signify that the bias was applied, but could not be statistically processed.

Comparison of liver histopathology to determine factors associated with development of HCC

We compared the histopathological findings of liver biopsy in patients with SVR in the F0 + 1 + 2 and F3 + 4 groups (Table 3). Univariate analysis using the Cox proportional hazard regression method was applied to these parameters to determine the liver histopathological findings that were associated with the development of HCC. Significant correlations were found for map-like distribution (RR = 3.082, 95%CI: 1.066-8.913, *P* = 0.0378) and atypical hepatocytes (RR = 42.055, 95%CI: 4.303-411.0, *P* = 0.0013) in the F3 + 4 patients. In the F0 + 1 + 2 patients, none of the liver histopathological findings showed any significant associations with HCC. Blank values in the table signify that the bias was applied, but could not be statistically processed.

Multivariate analysis using the Cox proportional hazard regression method was applied to the parameters that could be of statistical significance in Table 3 in each of the F stage groups, and the findings are shown in Table 4. In the F0 + 1 + 2 group, none of the factors showed any significant correlation with the development of HCC. In the F3 + 4 group, map-like distribution (RR = 1.687, 95%CI: 0.404-7.053, *P* = 0.4734) was not found to have any correlation while the presence of atypical hepatocytes (RR = 20.748, 95%CI: 1.335-322.5, *P* = 0.0303) was shown to be significantly associated with HCC development.

DISCUSSION

In Japan, ≥ 90% of cases of LC associated with HCC are attributable to hepatitis B or C virus infection. Many studies of hepatitis C have reported risk factors for HCC other than the virus, such as the progression of fibrosis, male sex, increasing age, consumption of a large amount of alcohol, and excess iron in the liver[7,21-23]. It has been reported that the occurrence of HCC may be suppressed by reducing the degree of inflammation, regardless of the presence or absence of HCV[24-26]. Furthermore, among the liver histopathological findings, it has been reported that the progression of liver fibrosis (F stage) constitutes a risk factor for carcinogenesis[4]. Therefore, we sought to identify the liver histopathological findings, other than the F stage, that are correlated with the risk of carcinogenesis. Previously, we have reported that the degree of IR is related to factors other than liver fibrosis that contribute to liver carcinogenesis. It has been reported that the cumulative incidence of HCC was significantly lower in cases in which the degree of IR had improved after IFN therapy than in cases without IFN therapy[11,12]. If the degree of inflammation and necrosis in the liver were also improved by IFN therapy, this would be reflected by an improvement in the histopathological findings, particularly the degree of IR and liver fibrosis, and by a reduction in the incidence of HCC. Moreover, it is generally accepted that HCV may be a direct cause of HCC[27]. Thus, by considering the differences in liver histopathological findings in patients with SVR following viral clearance, it was possible to eliminate the direct effects of the virus on HCC progression. If the degree of inflammation and necrosis in the liver was improved following elimination of the virus by IFN therapy, the development of HCC would be attributable, at least in part, to nonviral factors. Therefore, we investigated the risk factors in detail, including the degree of liver IR and the histopathological findings of liver biopsy specimens, and prospectively examined the findings separately according to the degree of liver fibrosis in SVR patients.

In type C chronic disease, it is said that active inflammation persists in the liver, with the formation of lymphoid follicles, varying degrees of liver parenchyma failure, and IR of hepatocytes, ultimately leading to the formation of small regenerative nodules. In other words, IR of hepatocytes, which occurs in the liver as a result of repeated cycles of necrosis and regeneration, is recognized as a population of hepatocytes that differ from normal ones in size, appearance, and arrangement of

Table 2 Comparison of liver histological findings at the time of liver biopsy in sustained virological response patients, between F0 + 1 + 2 and F3 + 4 groups

Parameter	Score					F0 + 1 + 2	F3 + 4	*P* value
	0	1	2	3	4			
Irregular regeneration								
Dysplastic change	109	50	49	21	3	0.769 ± 0.998	1.660 ± 1.081	0.0001
Oncocytes	211	16	4	1	0	0.066 ± 0.290	0.300 ± 0.647	0.0003
Map-like distribution	160	37	22	13	0	0.396 ± 0.756	0.960 ± 1.142	0.0001
Nodular arrangement	216	13	1	2	0	0.027 ± 0.164	0.320 ± 0.713	0.0001
Bulging	206	18	6	2	0	0.104 ± 0.400	0.340 ± 0.688	0.0022
Atypical hepatocytes	227	5	0	0	0	0.005 ± 0.074	0.080 ± 0.274	0.0012
Inflammatory cell infiltration								
Peri-portal	0	65	127	35	5	1.819 ± 0.652	2.260 ± 0.828	0.0001
Parenchymal	0	37	149	44	2	1.978 ± 0.585	2.300 ± 0.678	0.0010
Portal lymphocyte	0	5	103	121	3	2.456 ± 0.562	2.780 ± 0.507	0.0003
Portal lymphoid reaction	0	6	47	78	101	3.132 ± 0.850	3.360 ± 0.802	0.0904
Fibrosis								
F stage	5	128	49	34	16			
Portal sclerosis	113	81	32	6	0	0.615 ± 0.783	1.020 ± 0.795	0.0014
Pericellular fibrosis	89	90	41	11	1	0.802 ± 0.870	1.260 ± 0.853	0.0011
Perivenular fibrosis	63	66	65	25	0	1.236 ± 1.016	1.243 ± 0.895	0.9691
Bridging necrosis Y (presence)						0/228	4/228 (1.7%)	0.0020
Bile duct damage	123	71	26	9	2	0.597 ± 0.861	1.000 ± 0.904	0.0041
Steatosis	96	46	68	18	1	0.944 ± 0.990	1.429 ± 1.118	0.0035

Comparison of liver histological findings in sustained virological response patients according to the F stage (F0 + 1 + 2 and F3 + 4) was performed, and the data are shown. The histological findings demonstrated that there was a higher level of deterioration of the liver in the F3 + 4 group as compared to the F0 + 1 + 2 group; liver fibrosis was more progressive and inflammatory reaction was stronger. The number of patients for each score was compared, then the presence of bridging necrosis (Y/N) was compared and the remaining parameters are presented as mean ± SD. Scoring: score 0, none; score 1, minimal (observed in less than one third of the field); score 2, mild (observed in one third to less than two thirds of the field); score 3, moderate (observed in two thirds or more of the field) and score 4, severe (diffusely in all fields).

the nucleus. As a result, nodular lesions often are detectable in the livers of patients with type C chronic disease. These nodular lesions may be classified into two types: dysplastic nodules and early HCC. In addition, dysplastic nodules are divided into low-grade and high-grade dysplastic nodules or borderline lesions[28]. Fatty changes may be seen in 40% of high-grade dysplastic nodules, but are not observed in the low-grade ones, and regenerative large nodules are observed at high frequency in early HCC[29,30].

We compared the cumulative incidence of HCC between patients with low (F0 + 1 + 2) and high (F3 + 4) degree of liver fibrosis, and it was found that the former group had a significantly lower rate than the F3 + 4 group, suggesting that as fibrosis progresses in the liver, HCC is more likely to occur. It is clear that liver fibrosis progresses and also contributes to an increased risk of the development of HCC. In this study, factors associated with the development of HCC were identified by comparing patients with SVR by multivariate analysis using the Cox proportional hazard regression method, which was applied to the clinical and histopathological parameters. We found that among all the investigated factors, the presence or absence of atypical hepatocytes among IR of hepatocytes may be an important risk factor for HCC development along with progression of liver fibrosis.

Moreover, patients with progressive liver fibrosis and atypical hepatocytes among the IR of hepatocytes also had a higher cumulative incidence of HCC. Therefore, our results clearly demonstrate the occurrence of atypical hepatocytes in progressive liver fibrosis as a risk factor of HCC. This finding is also considered important for determination of a patient's therapeutic options.

HCC, stemming from LC induced by HCV infection, and other nonviral liver diseases, such as nonalcoholic steatohepatitis (NASH)[31-33] and autoimmune hepatitis[34], may develop through carcinogenic mechanisms based on inflammation[35,36]. Accordingly, we consider that the atypical hepatocytes may also contribute to the development of HCC in these nonviral diseases. Thus, during the assessment of the histopathological findings from liver biopsies, attention should be paid to the liver fibrosis, and additionally the presence or absence of atypical hepatocytes according to the type of liver disease.

In conclusion, among the histopathological findings in the liver of type C chronic disease, the occurrence of atypical hepatocytes in the IR of hepatocytes is significantly correlated with the risk of developing HCC together with progressive liver fibrosis. We believe that clarification of this finding as a risk factor of carcinogenesis may aid in the early diagnosis of HCC, and it would be meaningful to perform liver biopsy in patients with progression of liver fibrosis. Thus, by treating patients for both hepatitis C infection and atypical hepatocytes, we may attain an increase in the survival rate and a lower incidence of HCC.

Table 3 Factors associated with the development of hepatocellular carcinoma, identified by comparing patients with sustained virological response by univariate analysis using Cox proportional hazard regression method (n = 232)

Parameter	F0 + 1 + 2			F3 + 4		
	RR	95%CI	*P* value	RR	95%CI	*P* value
Clinical background factors[1]						
Gender (male)	1.200	0.109-13.252	0.8815	-	-	-
Age (yr)	1.308	0.985-1.737	0.0635	1.123	0.959-1.314	0.1494
BMI	1.121	0.693-1.814	0.6415	0.987	0.549-1.772	0.9644
AST (U/L)	0.996	0.964-1.028	0.7903	1.003	0.971-1.037	0.8540
ALT (U/L)	0.992	0.965-1.020	0.5678	0.996	0.972-1.020	0.7494
γ-GT (U/L)	0.996	0.975-1.018	0.7169	1.001	0.977-1.025	0.9436
Platelet count (× 10^4/μL)	1.004	0.837-1.204	0.9634	1.088	0.855-1.384	0.4936
Genotype type 1	-	-	-	0.285	0.030-2.752	0.2781
AFP (ng/mL)	0.989	0.867-1.129	0.8698	0.995	0.919-1.077	0.9046
Histological findings						
Irregular regeneration[2]						
Dysplastic change	1.920	0.720-5.124	0.1926	1.330	0.543-3.256	0.5322
Oncocytes	-	-	-	1.788	0.510-6.265	0.3639
Map-like distribution	-	-	-	3.082	1.066-8.913	0.0378
Nodularity	-	-	-	-	-	-
Bulging	-	-	-	-	-	-
Atypical hepatocytes	-	-	-	42.055	4.303-411.029	0.0013
Infiltration						
Peri-portal	3.248	0.537-19.655	0.1995	0.477	0.110-2.067	0.3225
Parenchymal	3.028	0.389-23.604	0.2902	0.420	0.092-1.921	0.2634
Portal lymphocyte	2.085	0.217-20.014	0.5241	1.213	0.177-8.333	0.8443
Portal lymphoid reaction	2.362	0.345-16.168	0.3812	3.364	0.484-23.407	0.2202
Fibrosis						
F stage						
Portal sclerosis	0.551	0.078-3.884	0.5500	1.293	0.444-3769	0.6373
Pericellular fibrosis	0.963	0.243-3.822	0.9576	0.534	0.117-2.440	0.4184
Perivenular fibrosis	0.627	0.168-2.344	0.4878	0.429	0.076-2.412	0.3370
Bridging necrosis Y	-	-	-	-	-	-
Bile duct damage	0.678	0.113-4.061	0.6707	1.305	0.374-4.555	0.6766
Steatosis	1.064	0.343-3.306	0.9142	1.660	0.623-4.421	0.3106

We compared the clinical background factors and histological findings at liver biopsy of the patients with sustained virological response in the F0 + 1 + 2 and F3 + 4 groups, data of which are shown. Univariate analysis using the Cox proportional hazard regression method was applied to these parameters to determine the clinical background factors that were associated with the development of hepatocellular carcinoma (HCC). [1]None of the clinical background factors showed any significant associations with HCC. Blank in the table signifies that the bias was applied, but could not be statistically processed; [2]Significant correlations were found for map-like distribution (RR = 3.082, 95%CI: 1.066-8.913, P = 0.0378) and atypical hepatocytes (RR = 42.055, 95%CI: 4.303-411.0, P = 0.0013) in the F3 + 4 patients. None of the liver histopathological findings showed any significant associations with HCC in the F0 + 1 + 2 patients. Blank values signify that the bias was applied, but could not be statistically processed. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AFP: α-fetoprotein; BMI: Body mass index; GT: glutamyltransferase.

Table 4 Factors associated with the development of hepatocellular carcinoma, identified by comparing patients with sustained virological response by multivariate analysis using Cox proportional hazard regression method

F3 + 4 parameter	RR	95%CI	*P* value
Map-like distribution	1.687	0.404-7.053	0.4734
Atypical hepatocytes	20.748	1.335-322.530	0.0303

Multivariate analysis using the Cox proportional hazard regression method was applied to the parameters in each of the F stage groups. In the F0 + 1 + 2 group, none of the factors showed any significant correlation with the development of hepatocellular carcinoma (HCC). In the F3 + 4 group, map-like distribution (RR = 1.687, 95%CI: 0.404-7.053, P = 0.4734) was not found to have any correlation while the presence of atypical hepatocytes (RR = 20.748, 95%CI: 1.335-322.5, P = 0.0303) was shown to be significantly associated with HCC development.

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COMMENTS

Background

A few studies have sought to determine the association between thehistopathological findings from liver biopsies and the risk of developing hepatocellular carcinoma (HCC). The authors studied 232 patients who underwent liver biopsy, with a sustained virological response (SVR) after interferon (IFN) therapy. They investigated in detail the histopathological findings and analyzed the findings to determine the risk factors.

Research frontiers

It has been reported that progression of liver fibrosis (F stage) constitutes a

risk factor for carcinogenesis. Therefore, the authors sought to identify the liver histopathological findings, other than F stage, that are correlated with the risk of carcinogenesis. If the degree of inflammation and necrosis in the liver were improved following elimination of the virus by IFN therapy, the development of HCC would be attributable, at least in part, to nonviral factors. Then, they investigated the risk factors in detail, including the degree of liver irregular regeneration (IR) and the histopathological findings of liver biopsy specimens, and prospectively examined the findings separately according to the degree of liver fibrosis in SVR patients.

Innovations and breakthroughs

Atypical hepatocytes among IR of hepatocytes may be an important risk factor for HCC development along with progression of liver fibrosis.

Applications

The authors believe that clarification of this finding as a risk factor of carcinogenesis may aid in the early diagnosis of HCC, and it would be meaningful to perform liver biopsy for patients with progression of liver fibrosis. Then, by treating patients for both hepatitis C infection and atypical hepatocytes, they may attain an increase in the survival rate and a lower incidence of HCC.

Terminology

IR of hepatocytes, which occurs as a result of repeated cycles of necrosis and regeneration of the liver parenchyma in chronic hepatitis, is divided into six elements: dysplastic change; anisocytosis characterized by variability of cell size with focal dysplastic change; map-like distribution; distinct populations of hepatocytes with a homogeneous appearance within each population, which are separated from each other by a sharp outline; bulging; expansive proliferation of hepatocytes compressing the surrounding parenchyma. Oncocytes: oncocytic change of hepatocytes. Nodularlity: nodular arrangement of the parenchyma. Atypical hepatocytes: degeneration of hepatocytes.

Peer review

In this study, the authors analyzed the risk factors for HCC associated with type C chronic liver disease. They found that the presence of atypical hepatocytes was significantly correlated with risk of HCC development, and therefore concluded that atypical hepatocytes among the histopathological findings of type C liver disease may be an important risk factor for HCC development along with progression of liver fibrosis.

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ORIGINAL ARTICLE

Infective severe acute pancreatitis: A comparison of ^{99m}Tc-ciprofloxacin scintigraphy and computed tomography

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Abstract

AIM: To evaluate ^{99m}Tc-ciprofloxacin scintigraphy compared with computed tomography (CT) for detecting secondary infections associated with severe acute pancreatitis (SAP) in swine.

METHODS: Six healthy swine were assigned to a normal control group (group A, n = 6). SAP was induced in group B (n = 9) and C (n = 18), followed by inoculation of the resulting pancreatic necroses with inactive *Escherichia coli* (*E. coli*) (group B) and active *E. coli* (group C), respectively. At 7 d after inoculation, a CT scan and a series of analyses using infecton imaging (at 0.5, 1, 2, 3, 4 and 6 h after the administration of 370 MBq of intravenous infecton) were performed. The scintigrams were visually evaluated and semi-quantitatively analyzed using region of interest assignments. The differences in infecton uptake and changes in the lesion-background radioactive count ratios (L/B) in the 3 groups were recorded and compared. After imaging detection, histopathology and bacterial examinations were performed, and infected SAP was regarded as positive. The imaging findings were compared with histopathological and bacteriological results.

RESULTS: In group A, 6 animals survived without infection in the pancreas. In group B, 7/9 swine survived and one suffered from infection. In group C, 15/18 animals survived with infection. Hence, the number of normal, non-infected and infected SAP swine was 6, 6 and 16, respectively. The sensitivity, specificity, accuracy, positive predictive value and negative predictive value of the infecton method were 93.8% (15/16), 91.7% (11/12), 92.9% (26/28), 93.8% (15/16) and 91.7% (11/12), whereas these values for CT were 12.5% (2/16), 100.0% (12/12), 50.0% (14/28), 100.0% (2/2) and 46.2% (12/26), respectively. The changes in L/B for the infected SAP were significantly different from those of the non-infected and normal swine (P < 0.001). The mean L/B of the infectious foci at 0.5, 1, 2, 3, 4 and 6 h was 1.17 ± 0.10, 1.71 ± 0.30, 2.46 ± 0.45, 3.36 ± 0.33, 2.04 ± 0.37 and 1.1988 ± 0.09, respectively. At

3 h, the radioactive counts (2350.25 ± 602.35 k) and the mean L/B of the infectious foci were significantly higher than that at 0.5 h (P = 0.000), 1 h (P = 0.000), 2 h (P = 0.04), 4 h (P = 0.000) and 6 h (P = 0.000).

CONCLUSION: ^{99m}Tc-ciprofloxacin scintigraphy may be an effective procedure for detecting SAP secondary infections with higher sensitivity and accuracy than CT.

Key words: Pancreatitis; Infection; Radionuclide imaging; Ciprofloxacin; X-ray computed tomography

Core tip: We successfully used a specific inflammatory agent, ^{99m}Tc-ciprofloxacin, which non-invasively detected secondary infections in an infective severe acute pancreatitis (SAP) model with higher sensitivity and accuracy than computed tomography. This method may be an effective tool for accurately diagnosing and assessing the severity of secondary infections in human SAP patients in the future. To our knowledge, there have been no previous studies that have compared the differential diagnosis of non-infectious and infectious SAP using ^{99m}Tc-ciprofloxacin imaging and histopathological and biological methods.

Wang JH, Sun GF, Zhang J, Shao CW, Zuo CJ, Hao J, Zheng JM, Feng XY. Infective severe acute pancreatitis: A comparison of ^{99m}Tc-ciprofloxacin scintigraphy and computed tomography. *World J Gastroenterol* 2013; 19(30): 4897-4906 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/4897.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.4897

INTRODUCTION

Infection of pancreatic or peripancreatic necroses occurs in 30%-70% of patients with severe acute pancreatitis (SAP)[1,2]. This disease is often accompanied by a late deterioration of organ function or generalized systemic illness[3], which is the leading cause of SAP-related deaths, with mortality rates of more than 30%[4-6]. Infected pancreatic necrosis in patients with clinical signs and symptoms of sepsis is an important indication for interventional therapy including surgery and drainage[7,8], whereas patients with sterile necrosis should be managed conservatively and undergo intervention only under certain circumstances[9].

However, until recently, the differential diagnosis of sterile and infectious SAP has remained a challenging issue. Effective biomarkers that will enable the localized diagnosis of infected pancreatic necrotic tissue are still under development and need to be confirmed in studies of larger patient cohorts[10,11]. Ultrasonography, computed tomography (CT) and magnetic resonance imaging have been used widely in the evaluation of pancreatitis, and CT is usually the first choice, however, these techniques have limitations in detecting secondary infections in cases of SAP *i.e.*, unusual gas bubbles and typical manifestations of an abscess on the images. White blood cell (WBC) imaging is regarded as the principal nuclear medicine method for the imaging of infection and inflammation[12]. However, it is also difficult to distinguish between infective and sterile inflammatory conditions using this method[13]. Fine-needle aspiration has contributed to making a definitive diagnosis of infected pancreatic necrosis, but is an invasive procedure and there are difficulties in applying this technique to critically ill patients[10].

It is therefore essential to develop a sensitive and specific imaging methodology that will non-invasively detect secondary infections in SAP patients. Over the past few decades, a number of radiopharmaceuticals have been developed to investigate infective and non-infective inflammatory disorders[13-16]. In this regard, ^{99m}Tc-ciprofloxacin may be one of the most promising agents in the field of nuclear medicine[17,18]. This radiochemical combines the advantages of a ^{99m}Tc label and the broad-spectrum bacteria-localizing capability of ciprofloxacin, which has a higher sensitivity and specificity for bacterial infections than WBC scans[14,17-22].

We speculate that ^{99m}Tc-ciprofloxacin may have efficacy in the diagnosis of SAP secondary infections. In our study, a SAP secondary infection model was developed in swine as previously reported[23]. The features and effectiveness of ^{99m}Tc-ciprofloxacin scintigraphy in the diagnosis of secondary bacterial infection in this infective SAP animal model were then evaluated and compared with contrast-enhanced CT, and with histopathological and bacteriological testing.

MATERIALS AND METHODS

This study was approved by the Animal Care Committee of Changhai Hospital. Healthy female Taihu swine (Experimental Animal Center of the Second Military Medical University, Shanghai, China), weighing 20-25 kg were acclimatized for one week before the start of the experiments. The animals had no access to food for 1 d and to water for 4 h prior to the start of the experiment.

Preparation of the SAP animal model

Six healthy swine were assigned to group A as normal controls. SAP was induced in 27 animals as previously reported[23], and these animals were randomly assigned to group B (n = 9) and group C (n = 18). Two days after the onset of SAP, 4 mL of inactive *Escherichia coli* (*E. coli*) and active (10^8/mL) *E. coli* were inoculated into necrotic foci of the pancreas in group B and C swine by CT-guided puncture, respectively (Table 1). Imaging examinations were performed 7 d after inoculation. The swine received ketamine hydrochloride (0.1 mL/kg) before imaging examinations and received 2 mL pentobarbital (Bioszune Life Sciences, Beijing, China) solution (3% w/v) at 20-min intervals during the examinations.

Radiopharmaceuticals

^{99m}Tc-ciprofloxacin was prepared by mixing 2 mg

Table 1 Results of secondary bacterial infection in a severe acute pancreatitis animal model

	n	Inoculation	Survival number	Pathologic diagnosis and biological results	
				Infection (bacteria)	Non-infection
Group A	6	No	6	0	6
Group B	9	Inactive *E. coli*	7	1 (*S. aureus*/*E.coli*)	6
Group C	18	Active *E. coli* (10^8/mL)	15	15 (14 *E. coli* and 1 *S. aureus* /*E.coli*)	0

E. coli: *Escherichia coli*; *S. aureus*: *Staphylococcus aureus*.

ciprofloxacin (Radiopharmaceuticals Laboratory of Beijing Normal University, China), 500 μg stannous tartrate, and 370 MBq freshly eluted sodium pertechnetate; then placed for 15 min at room temperature. Radiochemical purity was determined with a simple thin-layer chromatography technique, using 1-mm filters (Xinhua Group Co., Ltd., Hangzhou, China) in methyl ethyl ketone. ^{99m}Tc-ciprofloxacin remained at the base, and free pertechnetate moved with the solvent front. The Rf values of ^{99m}Tc-ciprofloxacin and $^{99m}TcO^{4-}$ were 1.0 and 0.0, respectively. The radiochemical purity and labeling rate of the radiopharmaceutical preparations were found to be greater than 90% at 6 h.

^{99m}Tc-ciprofloxacin scintigraphy and data analysis

^{99m}Tc-ciprofloxacin was administered into the ear vein of the swine. Abdominal imaging was then performed using a dual-head single photon emission CT (SPECT) scanner (Philips, Forte, Netherlands). The energy peak was controlled at 140 KeV with a 15% window. Each animal underwent a ^{99m}Tc-ciprofloxacin scan at 0.5, 1, 2, 3, 4 and 6 h after the injection of radiolabeled ciprofloxacin. Multi-position graphic information with a total of 64 tomographic images was acquired continuously. The radioactivity counts for each frame were 300 k and the matrix size was 64 pixel × 64 pixel. Following acquisition, filter back projection reconstructions were performed.

The ^{99m}Tc-ciprofloxacin scintigrams were visually evaluated by three experienced nuclear medicine physicians in a blind fashion based on CT anatomic images. Sequential images captured from 0.5-6.0 h were mandatory for inclusion and interpretation. The scans were read independently, and any disagreements in interpretation were discussed and a consensus was reached on a majority basis. They were considered positive for infection when the pancreatic necrosis and peripancreatic tissue had a higher radionuclide uptake with a clear edge than the surrounding tissue, and negative for infection when the pancreatic necrosis and peripancreatic tissue had no significant radionuclide uptake. Diagnostic results were compared to bacterial culture and smear results. Semi-quantitative analysis was performed by determining the radioactivity counts of the pancreas, liver, spleen, renal, intestinal track, muscle and infectious foci in the pancreas using region of interest techniques, and the radioactivity of the muscle at the level of the pancreatic body was considered to be background. The measured values were averaged by three physicians. The lesion-to-background (L/B) ratios were then scored. The L/B curves changed with time in the groups and the optimal imaging time for diagnosis of infective SAP was thereby investigated.

CT scan and imaging analysis

CT was performed using a Sensation 64 scanner (Siemens Medical Solutions, Forchheim, Germany) 15 min after the SPECT scan. CT scanning (plain plus enhanced) was performed using the following parameters: a 3 mm slice thickness, 120 kV, 110 mAs, a 512 × 512 matrix, and 1.5 mL/kg of contrast material (Ultravist 300 mg I/mL; Schering AG, Germany) at a rate of 2 mL/s. The images were read by the same three nuclear medicine physicians and a consensus was reached on a majority basis according to the following criteria: visible gas bubbles scattered within the pancreatic necrosis or peripancreatic fluid on the CT images were considered positive[24].

Pathologic study, bacterial culture and smear testing

After image examination, the animals were euthanized to remove the pancreas, and fluid was aspirated from the injection area or necrotic focus for bacterial culture or smear testing. Tissue samples were stained with hematoxylin and eosin (HE), and observed for evidence of pathologic changes to the pancreas. The criteria for diagnosing SAP secondary infection were as follows: (1) the appearance of the isolated pancreatic specimen was consistent with the pathologic diagnosis of SAP, whereby acute purulent inflammatory foci were present; and (2) the result of bacterial cultures from the necrotic area were positive or the presence of infection was confirmed using a smear. Diagnoses were made independently by a senior pathologist with no prior knowledge of the specimens.

Statistical analysis

Quantitative data were expressed as the mean ± SD. The sensitivity, specificity, accuracy, positive predictive value (PV^+), and negative predictive value (PV) of each imaging diagnosis were calculated. The effect of group and time on L/B was analyzed using two factor-repeated measure analysis of variance, the comparisons for the changes of L/B over time among groups were analyzed using one factor-repeated measure analysis of variance, the comparisons at the different time points in the same group and the comparisons among groups at the same time point were subjected to the Bonferroni test. Comparison of two rates was subjected to the χ^2 test. SPSS 10.0 software (SPSS, Chicago, IL, United States) was used for analysis and $P < 0.05$ was considered statistically significant.

RESULTS

In group A, all six swine survived. In group B, 1 animal was excluded due to a main pancreatic duct intubation

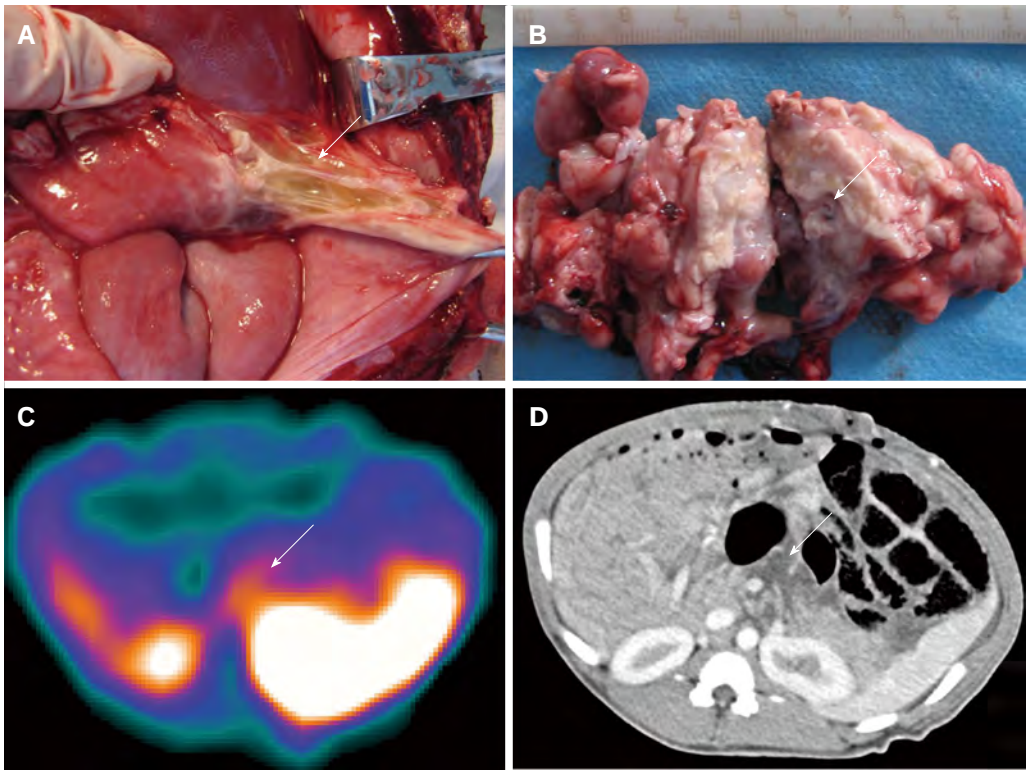

Figure 1 **^{99m}Tc-ciprofloxacin scintigraphy has higher sensitivity and accuracy in the detection of bacterial infections than computed tomography (the swine came from group C)**. A: The evidence of secondary infections in the peripancreatic fluid were confirmed by pathological examination and by bacterial smear or culture testing; B: Pancreatic necrosis was confirmed by pathological examination and by bacterial smear or culture testing; C: ^{99m}Tc-ciprofloxacin scintigraphy at 3 h demonstrated accumulation of radioactivity in the peripancreatic fluid and pancreatic necrosis (L/B = 3.37), indicating a true positive result; D: Enhanced computed tomography analysis showed low-density necrosis and peripancreatic fluid collection (arrow) without any indication of infection.

failure and another died of asphyxiation during anesthesia. In group C, one animal was also excluded from further analysis due to a main pancreatic duct intubation failure and two animals died of disease progression after the onset of SAP. Thus, in groups B and C, 7/9 and 15/18 were subjected to imaging analysis, respectively (Table 1).

Pathology findings

In group A, none of the swine showed infectious foci in the pancreas (Table 1). In group B, one of the seven animals (1/7) showed a focus with a *Staphylococcus aureus*/*E. coli* mixed infection. Light microscopy analysis of HE-stained sections revealed liquefactive necrosis in the center of this infectious focus. The remaining 6 animals in group B showed no bacterial infection (Table 1). In group C, 15 swine showed successful induction of a secondary infection. Bacterial culture and smear analysis of the necrotic foci in the pancreas showed that 14 SAP swine were infected with *E. coli* alone and 1 with a mixture of *E. coli* and Streptococcus, and showed intestinal perforation caused by this SAP secondary infection after paunching (Table 1). A total of 16 foci were found and yellow liquid flowed out of the cross-sections (Figure 1A and B). One animal had two cystic lesions in the pancreatic body and tail, with diameters of 19 and 5 mm, respectively. HE staining of infectious foci in group C showed liquefactive necrosis in most parts of the focal center, structureless substances in the fat cytoplasm, and coagulative necrosis in part of the foci.

Visual analysis

In group A, ^{99m}Tc-ciprofloxacin scintigraphy revealed high radionuclide uptake in the kidneys, liver and spleen with excretion to the urinary bladder. No activity was observed in the area of the pancreas, normal bone marrow, muscle or gastrointestinal tract at any time point (Figure 2A). The CT images showed that the pancreatic parenchyma of all 6 animals were homogeneous and uniformly enhanced after contrast administration (Figure 2A).

In group B, no radionuclide uptake in the pancreatic areas was detected by SPECT in 5 of 7 animals at any time point (Figure 2B) and these swine were therefore diagnosed as negative for secondary infection (Table 2). Mild uptake in the pancreatic area was evident in one animal and the L/B was 2.15 at 3 h after administration, indicating a positive diagnosis of secondary infection (Table 2). However, pathology only displayed significant proliferation of granulated tissue at the edge of the necrotic area, no infectious focus was found in this animal by either pathologic or bacterial examinations (Figure 3A-C). Radionuclide uptake in the pancreatic area was detected in one animal, which was subsequently found to be infected with a mixture of *S. aureus* and *E. coli* in the pancreatic necrosis (Table 2). CT images revealed the pancreas had enlarged markedly and that the gastroin-

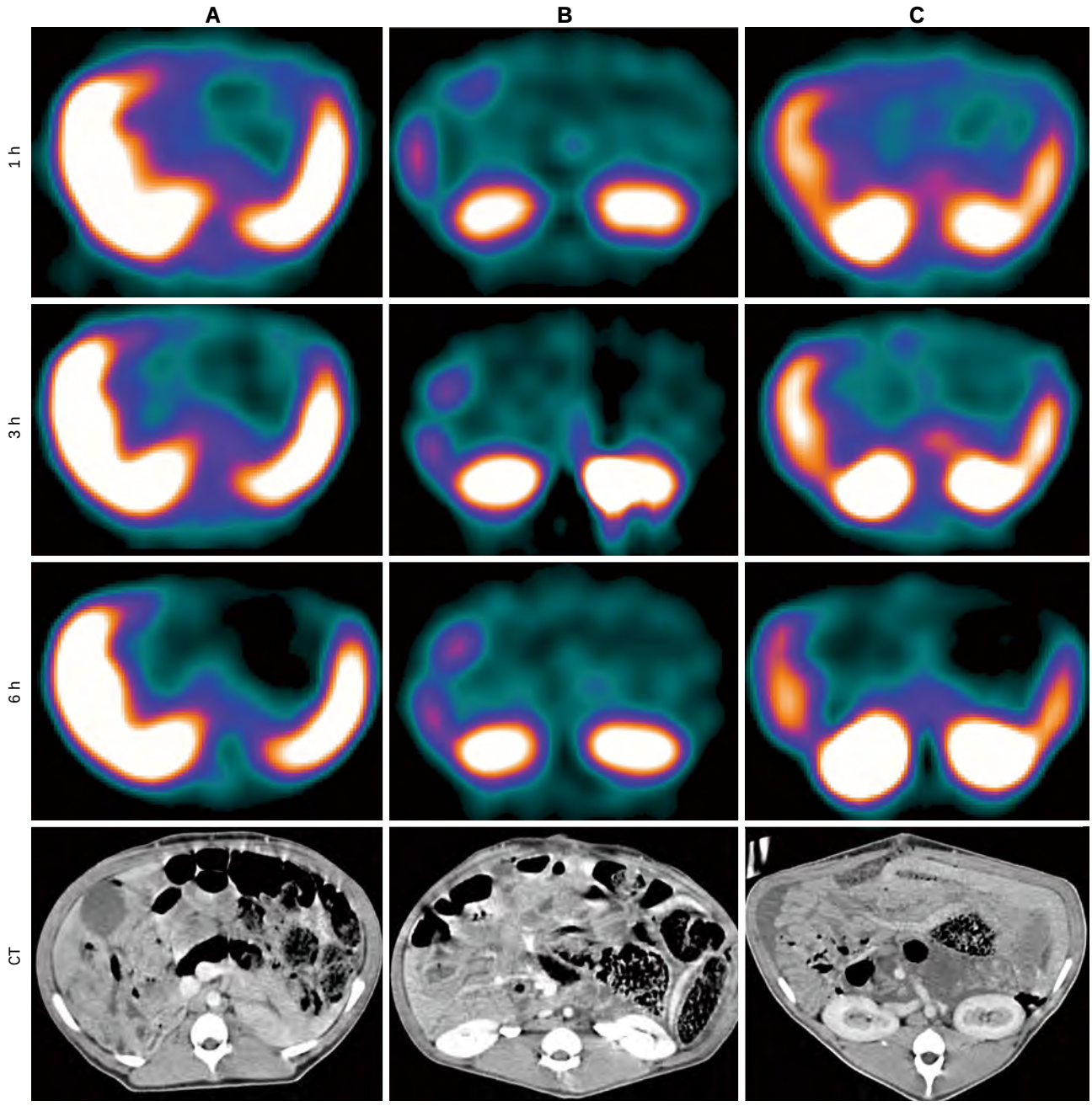

Figure 2 **^{99m}Tc-ciprofloxacin and computed tomography imaging of a normal swine pancreas and both non-infected severe acute pancreatitis swine and an animal with secondary infection associated with severe acute pancreatitis.** A: No uptake indicating no infection, and a homogenous pancreatic parenchyma is evident in the normal pancreas by computed tomography imaging; B: A lower accumulation of ^{99m}Tc-ciprofloxacin in the pancreatic necroses of non-infected severe acute pancreatitis (SAP) swine; C: A higher accumulation of this agent in SAP swine with a secondary infection focus is evident.

testinal tract was expanded and associated with effusion. Focal or pathy hypoattenuated areas within the pancreatic parenchyma were observed in all animals in group B and there were no signs of gas bubbles (Figures 2B and 3D).

In group C, one animal was interpreted as negative by scintigraphy, which was proven to be a misdiagnosis by pathological examination, and by bacterial culture and smear testing. The remaining 14 animals in this group showed radioactive accumulations in the pancreatic area and were diagnosed as positive for secondary infection (Figures 1C and 2C), which was confirmed in each case by pathologic examination and bacterial culture (Table 2). One animal showed irregular patches of radioactivity accumulation around the pancreatic area due to intestinal perforations (Figure 4A). The animal with two lesions in the pancreatic body and tail was found to have a bigger focus in the pancreatic body, while smaller lesions were unclear.

CT images revealed that the pancreas was enlarged with irregular patchy and round-like cystic low-density necrotic areas, and effusion around the pancreas (Figures 1D and 2C). One animal had two round-like cystic lesions in the pancreatic body and tail, with diameters of 19 and 5 mm, respectively, without significant enhancement after

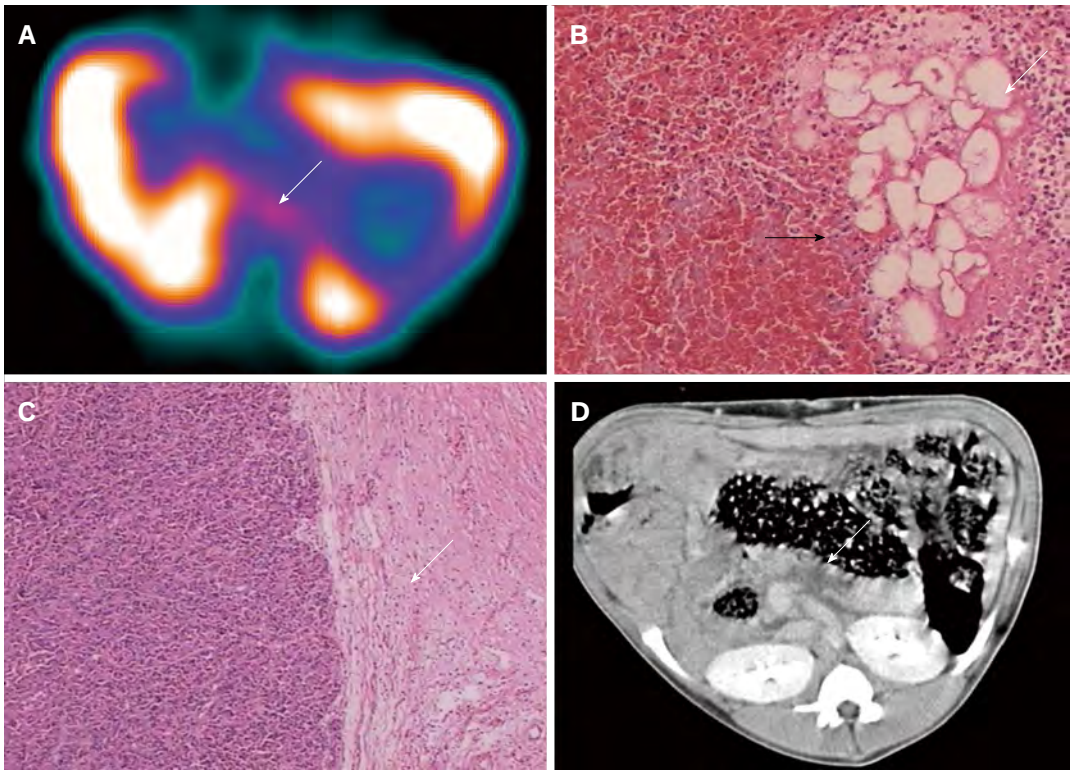

Figure 3 False-positive case of ^{99m}Tc-ciprofloxacin scintigraphy (the swine came from group B). A: ^{99m}Tc-ciprofloxacin scintigraphy (arrow) is indicative of secondary infection (L/B = 2.15); B: However, light microscopy analysis showed pancreatic tissue and fat tissue necrosis (arrow), surrounding a marked hyperplastic area in granulated tissue (black arrow); C: Fibrous tissue (arrow), but no associated bacterial infection; D: Computed tomography image showed focal hypoattenuated areas within the pancreatic parenchyma (arrow) without gas bubbles.

Table 2 Pathologic diagnosis, ^{99m}Tc-ciprofloxacin and computed tomography imaging analysis of secondary bacterial infection in a severe acute pancreatitis animal model

	Pathologic diagnosis	SPECT		CT	
		Infection	Non-infection	Infection	Non-infection
Group A	Infection	0	0	0	0
	Non-infection	0	6	0	6
Group B	Infection	1	0	0	1
	Non-infection	1	5	0	6
Group C	Infection	14	1	2	13
	Non-infection	0	0	0	0

SPECT: Single photon emission computed tomography; CT: Computed tomography.

contrast administration and were diagnosed as pseudocysts. Gas bubble signs were found in 2 swine, and one showed intestinal perforations and gas bubbles scattered throughout the necrotic area and in the peripancreatic fluid (Figure 4B), and the other showed gas bubbles in the pancreatic necrosis.

Quantitative analysis

Based on our histopathological and biological results, the number of normal, non-infected and infected SAP swine was 6, 6 and 16, respectively (Table 1). It was calculated that ^{99m}Tc-ciprofloxacin scintigraphy had a sensitivity of 93.8% (15/16), a specificity of 91.7% (11/12), an accuracy of 92.9% (26/28), a PV+ of 93.8% (15/16), and a PV of 91.7% (11/12) for detecting secondary bacterial infection associated with SAP (Table 3), and these values for CT were 12.5% (2/16), 100.0% (12/12), 50.0% (14/28), 100.0% and 46.2% (12/26), respectively. Of these parameters, sensitivity, accuracy and PV were significantly lower than those of ^{99m}Tc-ciprofloxacin scintigraphy ($P < 0.01$) (Table 3).

^{99m}Tc-ciprofloxacin scintigraphy results at different time points

In infected SAP swine, the infectious foci in the pancreatic tissues showed no radionuclide uptake at 0.5 h, mild uptake at 1 and 2 h, and peak radioactivity counts at 3 h (2350.25 ± 602.35 k), and then gradually decayed from 4-6 h. The change was different in the kidney, liver, spleen, gastrointestinal tract and muscle (Figure 5A). The L/B in 6 normal swine, 6 non-infected SAP and 16 infected SAP animals at 0.5, 1, 2, 3, 4, 6 h after the administration of ^{99m}Tc-ciprofloxacin are presented in Figure 5B. There were significant differences in the L/B changes over time among the three study groups ($F = 95.66$, $P < 0.001$). These changes in the infected SAP animals differed significantly from those in the non-infected SAP ($F = 88.63$, $P = 3.1e^{-16}$) and normal swine ($F = 63.61$, $P = 8.2e^{-13}$). In contrast, no significant differences were found between the non-infected SAP and normal groups ($t = 1.17$, $P = 0.251$). The L/B ratio at 3 h after the administration of

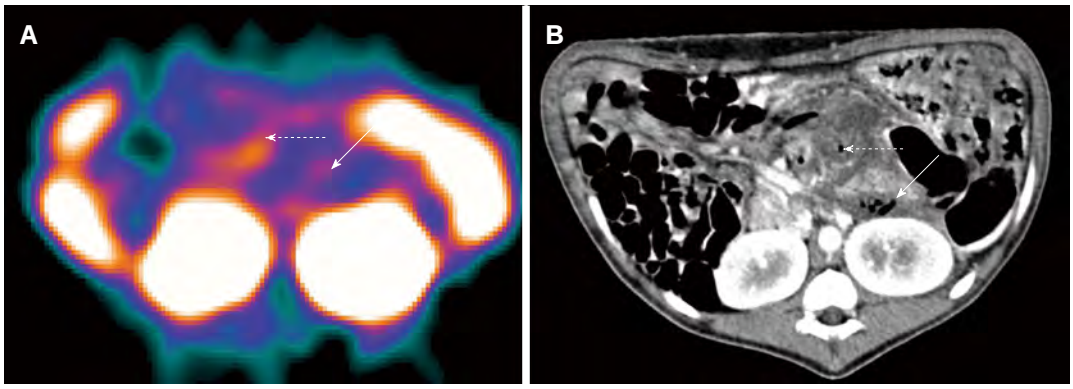

Figure 4 Swine with severe acute pancreatitis and secondary bacterial infection (from group C). A: The highest level of radioactivity accumulation was found by ^{99m}Tc-ciprofloxacin scintigraphy at 3 h (L/B = 3.42); B: Multiple bubbles scattered in the necrotic area and peripancreatic fluid were demonstrated on computed tomography images. Intestinal perforation caused by severe acute pancreatitis was found at autopsy.

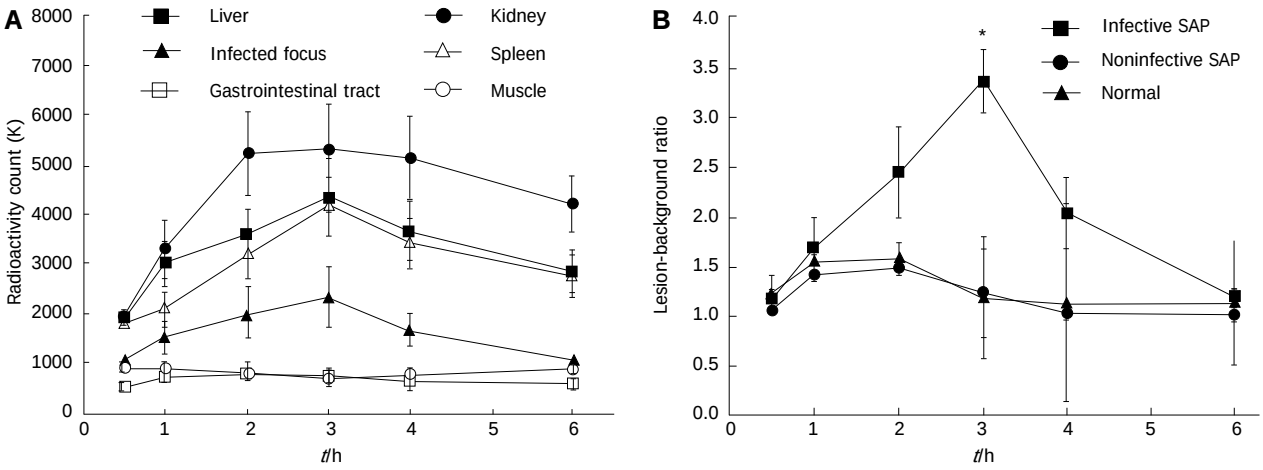

Figure 5 **^{99m}Tc-ciprofloxacin scintigraphy results at different time points.** A: Curves showing radioactivity count changes over time in the abdominal tissues of swine with infected severe acute pancreatitis (SAP) are shown. These curves show high uptake in the kidneys and moderate uptake in the liver and spleen. No activity was observed in the areas of the muscle or gastrointestinal tract. The radioactive uptake by infectious foci increased gradually over time, reached a peak at 3 h, and then gradually decayed; B: Lesion-background ratio change curves for the normal pancreas, and non-infected and infected SAP pancreas in the swine model are shown. The curves show that the optimal lesion-background ratio occurred at 3 h after administration of Infecton in positive SAP animals.

Table 3 Results of imaging diagnosis for secondary infection of animals with severe acute pancreatitis *n* (%)

	Imaging results	Pathologic results		Sensitivity	Specificity	Accuracy	PV+	PV-
		+	-					
Infection	+	15	1	15 (93.8)[b]	11 (91.7)	26 (92.90)[b]	15 (93.8)	11 (91.7)[b]
	-	1	11					
CT	+	2	0	2 (12.5)	12 (100.0)	14 (50.0)	2 (100.0)	12 (46.2)
	-	14	12					

[b]$P < 0.01$ *vs* computed tomography (CT). +: Infection; -: Non-infection; PV+: Positive predictive value; PV-: Negative predictive value.

^{99m}Tc-ciprofloxacin in the infected SAP swine reached 3.36 ± 0.33, which was significantly higher than at all other time points (*P* values were $1.1e^{-35}$, $3.5e^{-27}$, $3.9e^{-13}$, $4.2e^{-21}$ and $2.5e^{-35}$, respectively).

DISCUSSION

Secondary infection of pancreatic necrotic tissue is accepted as one of the most important prognostic indicators of disease severity and outcomes in SAP cases[4-6]. Early diagnosis is the key to improved treatment outcomes and reduced mortality. Although CT plays an important role in the diagnosis of SAP, it can not detect the sites of secondary infection with sufficient sensitivity as it can only do so if gas bubbles appear within the lesion, which occurs infrequently. Indeed, Bhansali *et al*[25]

reported previously that bubbles were detectable in only 16% of SAP patients with secondary infections using CT imaging (21/131 cases). Our present experimental results indicate that the sensitivity of CT is too low (12.5%, 2/16) to accurately diagnose a SAP secondary infection.

The novel radiopharmaceutical used in our scintigraphy analysis is based on the 4-fluoroquinolone broad spectrum antibiotic ciprofloxacin. Following intravenous injection, ciprofloxacin is widely distributed in the body and is excreted *via* the kidneys. The mode of action of ciprofloxacin is mediated *via* inactivation of the bacterial DNA gyrase, which results in the retention of this agent at the sites of active bacterial infection[18]. Sierra *et al*[26] analyzed the mechanism of intracellular accumulation of ^{99m}Tc-ciprofloxacin in *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains, and found that ^{99m}Tc-ciprofloxacin was equally accumulated intracellularly in all tested strains, while $^{99m}TcO^{4-}$ did not show accumulation in any of the strains. The absence of intracellular $^{99m}TcO^{4-}$ indicated that the entire radioactivity detected in the ^{99m}Tc-ciprofloxacin assay was due to the accumulation of the radiopharmaceutical compound, rather than free $^{99m}TcO^{4-}$.

^{99m}Tc-ciprofloxacin has been widely tested in clinical studies and shown to be taken up by a wide range of live (but not dead) bacteria *in vitro* and *in vivo* [12,13,17,18]. It has also been reported that the specificity of ^{99m}Tc-ciprofloxacin scintigraphy reached 85%-96% in detecting both bone and joint bacterial infections[27-29] and showed a sensitivity and specificity of 91.7% and 75%, respectively, in the diagnosis of acute bacterial cholecystitis[19]. In our present study, the sensitivity, specificity and accuracy of ^{99m}Tc-ciprofloxacin were found to be 93.8%, 91.7% and 92.9%, respectively. The accuracy was higher than that of CT. We found that ^{99m}Tc-ciprofloxacin scintigraphy had some important advantages over WBC imaging which have also been reported by other studies. Firstly, the technique used for preparation is easy, and can be carried out without withdrawing blood, purifying leucocytes, labeling and reinjecting the radiolabeled cells, compared to WBC imaging. Secondly, no adverse effects were reported in response to intravenous administration of ^{99m}Tc-ciprofloxacin. Thirdly, both the radiochemical purity and labeling rate were found to be over 90% within 6 h at room temperature. Hence, ^{99m}Tc-ciprofloxacin is an ideal specific targeting agent for the detection of bacterial infection[17,27-29].

In infected SAP cases, the radioactivity levels in the bacterial foci were higher than in the surrounding SAP, muscle or soft tissues. Pronounced focal accumulation was evident at 3 h after administration, when both the radioactivity counts and lesion-background ratios were at peak levels. This suggests that ^{99m}Tc-ciprofloxacin scintigraphy is a suitable diagnostic test for SAP patients with a suspected secondary infection. However, both false-positive and false-negative results still arise in ^{99m}Tc-ciprofloxacin scintigraphy. In our present analyses, one false-negative result was found in a group C animal for which the pathology showed a pancreatic necrosis with a mild infection. This may suggest that the detectable uptake of ^{99m}Tc-ciprofloxacin has a severity of infection threshold. One false-positive result was also found in an animal showing marked hyperplasia of granulated and fibrous tissue at the edge of the pancreatic necrosis, which suggested that radioactive uptake may be related to granulated tissue repair at the edge of necrotic foci, as well as an increase in blood perfusion or capillary permeability[30].

We also found that SPECT visualization has disadvantages, including low resolution and poor display of anatomic structure. It can not display the shape and area of pancreatic necrotic foci, nor display the non-infected foci with peripancreatic fluid or pseudocysts. However, while the clinical use of SPECT-CT is widely accepted, the resolution and capability for displaying anatomical details of the SPECT-CT scanner are much improved[31]. Incorporating SPECT with CT in one scanner, which has the advantages of the two imaging techniques, makes it possible to evaluate and diagnose the infected foci with indefinite anatomical localization and low radioactive uptake.

In summary, ^{99m}Tc-ciprofloxacin scintigraphy has a higher sensitivity and accuracy in the detection of bacterial infections than CT. Moreover, this agent is not taken up in a normal pancreas and non-infected SAP, which could be highly useful in the detection of infectious SAP. This method may therefore become an effective tool in the future for accurately diagnosing and assessing the severity of secondary infections in human SAP patients. Undoubtedly, it is very important for clinicians to develop treatment programs and improve the efficacy of SAP. ^{99m}Tc-ciprofloxacin scintigraphy, ^{18}F-FDG PET and diffusion-weighted imaging (DWI) have been applied widely for the diagnosis of infection, but they have advantages and limitations[32-36]. In the future, we will evaluate the efficacy of these commonly used techniques in the diagnosis of secondary infection of SAP.

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COMMENTS

Background

Secondary infection is one of the most challenging problems in the treatment of severe acute pancreatitis (SAP). Infected pancreatic necrosis in patients with the clinical signs and symptoms of sepsis is an important indication for interventional therapy including surgery and drainage, whereas patients with sterile necrosis should be managed conservatively and undergo intervention only under certain circumstances. Conventional scintigraphic and radiologic methods have limitations in the detection of secondary infection of SAP. It is therefore essential to develop a sensitive and specific imaging methodology that will non-invasively detect secondary infections in SAP patients. However, to their knowledge, only a few authors have applied traditional radionuclide imaging agents for the diagnosis of pancreatitis associated with infection. The utility of infecton in the detection of SAP secondary infection is still unclear.

Research frontiers

In the area of SAP, one of the research hotspots is the detection of second-

ary infection of pancreatic necrosis. Over the past few decades, a number of radiopharmaceuticals have been developed to investigate infective and non-infective inflammatory disorders. In this regard, ^{99m}Tc-ciprofloxacin may be one of the most promising agents in the field of nuclear medicine. This radiochemical combines the advantages of a ^{99m}Tc label and the broad-spectrum bacteria-localizing capability of ciprofloxacin, which has a higher sensitivity and specificity for bacterial infections than white blood cell scans.

Innovations and breakthroughs

The authors successfully used a specific inflammatory agent, ^{99m}Tc-ciprofloxacin, which non-invasively detected secondary infections in an infective SAP model with higher sensitivity and accuracy than computed tomography (CT). To our knowledge, there have been no previous studies that have compared the differential diagnosis of non-infectious and infectious SAP using ^{99m}Tc-ciprofloxacin imaging and histopathological and biological methods.

Applications

This method may be an effective tool in the future for accurately diagnosing and assessing the severity of secondary infections in human SAP patients. Undoubtedly, it is very important for clinicians to develop treatment programs and improve the efficacy of SAP.

Terminology

SAP is defined as necrosis involving at least 30% of the pancreas as visualized by contrast-enhanced CT, with greater involvement indicating greater severity of necrosis. It is a special type of acute pancreatitis with more complications and high mortality. The 4-fluoroquinolone broad spectrum antibiotic, ciprofloxacin, was labeled with $^{99m}TcO^{4-}$. The mode of action of ciprofloxacin is mediated *via* the inactivation of bacterial DNA gyrase, which results in the retention of ^{99m}Tc-ciprofloxacin at the sites of active bacterial infection.

Peer review

This is a well-presented manuscript describing a good research protocol on the use of ^{99m}Tc-ciprofloxacin in the detection of infection in severe acute pancreatitis in an animal model. The single photon emission CT (SPECT) images, with high background activity in the nearby organs, are indistinct, as is to be expected at this resolution. As the authors themselves have pointed out, SPECT-CT hybrid imaging would provide better images and localize the abnormal tracer activity more precisely to the focus of infection. The results are interesting and suggest that ^{99m}Tc-ciprofloxacin scintigraphy may therefore become an effective tool in the future for accurately diagnosing and assessing the severity instances of secondary infections in human SAP patients.

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ORIGINAL ARTICLE

Pancreatitis in patients with pancreas divisum: Imaging features at MRI and MRCP

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Abstract

AIM: To determine the magnetic resonance cholangiopancreatography (MRCP) and magnetic resonance imaging (MRI) features of pancreatitis with pancreas divisum (PD) and the differences *vs* pancreatitis without divisum.

METHODS: Institutional review board approval was obtained and the informed consent requirement was waived for this HIPAA-compliant study. During one year period, 1439 consecutive patients underwent successful MRCP without injection of secretin and abdominal MRI studies for a variety of clinical indications using a 1.5 T magnetic resonance scanner. Two experienced radiologists retrospectively reviewed all the studies in consensus. Disputes were resolved *via* consultation with a third experienced radiologist. The assessment included presence and the imaging findings of PD, pancreatitis, and distribution of abnormalities. The pancreatitis with divisum constituted the study group while the pancreatitis without divisum served as the control group. MRCP and MRI findings were correlated with final diagnosis. Fisher exact tests and Pearson × 2 tests were performed.

RESULTS: Pancreatitis was demonstrated at MRCP and MRI in 173 cases (38 cases with and 135 cases without divisum) among the 1439 consecutive cases. The recurrent acute pancreatitis accounted for 55.26% (21 of 38) in pancreatitis patients associated with PD, which was higher than 6.67% (9 of 135) in the control group, whereas the chronic pancreatitis was a dominant type in the control group (85.19%, 115 of 135) when compared to the study group (42.11%, 16 of 38) (χ^2 = 40.494, P < 0.0001). In cases of pancreatitis with PD, the dorsal pancreatitis accounted for a much higher percentage than that in pancreatitis without PD (17 of 38, 44.74% *vs* 30 of 135, 22.22%) (χ^2 = 7.257, P < 0.05).

CONCLUSION: MRCP and MRI can depict the features of pancreatitis associated with divisum. Recurrent acute pancreatitis and isolated dorsal involvement are more common in patients with divisum.

Key words: Pancreas divisum; Pancreatitis; Diagnosis; Magnetic resonance imaging; Magnetic resonance cholangiopancreatography

Core tip: We reviewed 1439 cases of abdominal magnetic resonance imaging (MRI) and magnetic resonance cholangiopancreatography (MRCP). There were 122 cases of pancreas divisum (PD) and 38 of them were diagnosed as pancreatitis. The pancreatitis associated with PD was usually distributed in dorsal pancreas and presented as recurrent acute type. MRCP in combination with MRI can accurately detect ductal and paren-

chymal abnormalities of pancreas. Therefore, MRCP and MRI should be referred to as primary diagnostic tools for pancreatitis with PD whereas endoscopic retrograde cholangiopancreatography can be reserved for those who require therapeutic interventions.

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INTRODUCTION

Pancreas divisum (PD) is the most common developmental anatomic variant of pancreatic duct with a reported incidence of 4%-14% in the population at autopsy series, 3%-8% at endoscopic retrograde cholangiopancreatography (ERCP), and 9% at magnetic resonance cholangiopancreatography (MRCP)[1-5]. This abnormality occurs when the dorsal and ventral pancreas anlage fails to fuse during the 6th-8th week of gestation. PD is characterized not only by the anatomical morphology but also by the physiology in which the majority of pancreatic juice drains through the duct of Santorini into the duodenum at orifice of minor papilla while the minority (about 10%) drains through the (ventral) duct of Wirsung into the duodenum at major papilla[1]. Although the clinical significance still remains controversial, there seems to be an association between PD and chronic abdominal pain and recurrent acute pancreatitis. Moreover, the timely and appropriate therapeutic interventions such as minor papillotomy or stent placement in the dorsal pancreatic duct or surgical procedures can benefit the patients with symptomatic PD remarkably from reducing the pressure in the main pancreatic duct[6].

The manifestations of acute and chronic pancreatitis at magnetic resonance imaging (MRI) and magnetic resonance (MR) cholangiopancreatography (MRCP) have been well described in previous studies[7-10], however, to our knowledge, there is no published literature on imaging features of pancreatitis in patients with PD using MRI together with MRCP without secretin injection. Although ERCP is considered as a gold standard of diagnosis, prior studies have shown that there is a great correlation between MRCP and ERCP in detecting PD[11,12]. Currently, the multidetector computed tomography (MDCT) has been reported to be valuable in the detection of PD[13]. As a noninvasive approach, MRCP can be used much more extensively than ERCP when radiation is in consideration and can always be performed together with MRI, which can depict the morphologic changes in detail[9,14]. Therefore, since MRI and MRCP can be employed to establish a diagnosis non-invasively, including for patients who are unable to undergo diagnostic ERCP, the ERCP can be reserved for those who require therapeutic intervention.

Therefore, the purpose of this study was to retrospectively evaluate the imaging features of pancreatitis in patients with PD at MRI and MRCP without injection of secretin and to describe the differences of MR imaging between pancreatitis with and without divisum.

MATERIALS AND METHODS

Patient population and proof of diagnosis

During one year period, a total of 1439 consecutive patients (age range, 16-95 years; 698 men and 741 women) consecutively underwent successful abdominal MRI and MRCP without injection of secretin in our institution for a variety of clinical indications. Among them, 173 cases were finally diagnosed as pancreatitis based on clinical presentations, laboratory values, and imaging findings. Of the 173 cases, 38 cases associated with PD constituted the study group in this study. A total of 42 times of ERCP examination and interventional therapy were performed in 21 cases in the study group. Among them, minor papillotomy and temporary transpapillary stent placement in main pancreatic duct (n = 15) through minor papilla, stent placement in common bile duct (CBD) (n = 3), and surgery of Puestow procedure (n = 1) were performed. Eighteen patients were male and 20 were female, with a mean age of 43.6 years (range, 20-79 years). The remaining 135 cases of pancreatitis without PD (66 male and 69 female) served as control group, aged from 19 to 85 years with a mean age of 53.4 years. We obtained the institutional review board approval and waiver of informed consent for this retrospective HIPPA-compliant study.

The recurrent acute pancreatitis was defined in this study as a clinical setting in which the clinical or/and serologic features were characteristic of acute pancreatitis with a history of recurrence at least 2 times. The clinical data, which included symptoms at presentation, history of previous episodes of pancreatitis, associated symptoms involving other systems, and laboratory findings, were reviewed for all of the 173 cases of pancreatitis in this study.

Imaging techniques

All the MR studies including coronal MRCP and axial MRI were performed with a 1.5-T MR imager (Magneton Vision; Siemens, Erlangen, Germany) using a phase array body coil. The MRCP images were initially obtained and axial MR imaging followed. The pancreaticobiliary tract was localized with a thick-slab (40 mm) half-Fourier RARE image in coronal-oblique (25 degrees) and axial planes, which necessitated an acquisition time of 7 s. The thin-slab MRCP acquisitions were obtained at various angles that allowed optimal visualization of the bile and pancreatic ducts; the number of thin-slab acquisitions per patient ranged from 3 to 15 (mean, 7 acquisitions). Both the thick-slab and thin slab images were obtained during breath hold. The half-Fourier RARE parameters included repetition time ms/echo time ms (effective) of ∞/95.0;

echo train length, 128; flip angle, 150 degrees; section thickness, 3.0 mm with no gap; field of view, 270 mm × 270 mm; number of signals acquired, 1; matrix, 240 × 256 and acquisition time, 20 s. Fat saturation and shim adjustments were used in all cases.

After MRCP, conventional axial MR imaging was conducted to examine the abdomen. MR imaging sequences included unenhanced T1-weighted breath-hold spoiled gradient echo (148/5 ms; flip angle, 70 degrees; section thickness, 10 mm; gap, 30%), unenhanced T2-weighted breath-hold fast SE (3500/138 ms; section thickness, 8 mm; gap, 25%), unenhnaced in phase and out-of-phase T1-weighted gradient recalled echo, unenhanced and double-phased dynamic contrast-enhanced T1-weighted fat suppression (200/4.4 ms; flip angle, 70 degrees; section thickness, 8 mm; gap, 20%) 30 and 60 s after beginning of intravenous administration of the contrast materials. Gadopentetate dimeglumine (Magnevist; Berlex Laboratories, Wayne, NJ, United States) was administered intravenously using an automatic injector at a dose of 0.1 mmol per kilogram of body weight as a bolus followed by a normal-saline flush.

Imaging analysis

All the images in this study were reviewed retrospectively using interactive picture archiving and communicating system (PACS) workstations by two experienced (10-15 years of practice) abdominal radiologists (Wang D and Yu J) in consensus. The disputes were resolved *via* consultation with a third experienced abdominal radiologist (Fulcher AS). During the reading, the following items were taken into account: (1) classification of PD, complete PD or incomplete PD; (2) distribution of pancreatitis in the pancreas (ventral, dorsal or ventral plus dorsal pancreas); (3) morphologic changes including pancreatic parenchyma (enlargement or atrophy of pancreas), and pancreatic duct changes (side branch ectasia, pancreatic ductal dilatation and strictures, and intraductal calculi); (4) signal intensity abnormalities on unenhanced or enhanced images, including necrosis or cystic changes in pancreas; (5) changes outside of the pancreas, *i.e.*, peripancreatic stranding, fluid collections, and involvement of the adjacent organs and vessels, *etc*; and (6) abscess inside pancreas and outside of pancreas. The classifications and distributions of pancreatitis were compared between the study group and control group. After careful analysis of the abovementioned findings, the imaging features of pancreatitis associated with PD were established.

Statistical analysis

Pearson χ^2 and Fisher exact probability test were introduced for classification and distribution of pancreatitis in patients with PD in the study group compared with those in the control group. A P value less than 0.05 was considered to indicate a statistically significant difference.

Table 1 Comparison of classification of pancreatitis with and without pancreas divisum *n* (%)

	Acute pancreatitis	Chronic pancreatitis	Recurrent acute pancreatitis	Total
Pancreatitis with PD	1 (2.63)	16 (42.11)	21(55.26)	38
Pancreatitis without PD	11 (8.14)	115 (85.19)	9 (6.67)	135
Total	12 (6.93)	131 (75.72)	30(17.34)	173

PD: Pancreas divisum.

RESULTS

Clinical features of the pancreatitis superimposed on PD

The classifications of pancreatitis in the 38 cases with PD in the study group included: recurrent acute pancreatitis in 21 cases (all cases with abdominal pain, 5 with gallstones, 1 with jaundice, and 16 with hyperlipasemia and hyperamylasemia), chronic pancreatitis in 8 cases (all cases with abdominal pain, 3 with mild serologic enzymes elevation, and 1 with gallstones) and the other 8 cases revealed with chronic pancreatitis incidentally at MRI and MRCP primarily for detecting hepatic lesions or biliary abnormalities, and acute pancreatitis in 1 case with worsening abdominal pain and serum lipase elevation of more than 1000 U/LH (normal, 23-300 U/LH). The gallstone pancreatitis was the dominant type in the control group (75.6%, 102/135). The other etiologies included intrapancreatic calculi (11.1%, 15/135), pancreatic ductal strictures (5.9%, 8/135), and autoimmune pancreatitis (4.4%, 6/135); and no distinct etiologic factors were found in 4 of the 135 cases (3%). The recurrent acute pancreatitis accounted for 55.26% (21 of 38) in pancreatitis with PD, which was higher in percentage than 6.67% (9 of 135) in the control group, whereas the chronic pancreatitis was a dominant type in the control group (85.19%, 115 of 135) when compared to the study group (42.11%, 16 of 38) ($\chi^2 = 40.494$, $P < 0.0001$) (Table 1). The pancreatitis in patients with PD accounted for 21.96% (38 of 173) in the total population of pancreatitis in this study.

Imaging features of the pancreatitis superimposed on PD

Ductal and parenchymal changes of pancreas: Pancreatic duct in patients with PD and without PD both showed dilatation (6 *vs* 65), irregularity (16 *vs* 86), dilatation with irregularity (8 *vs* 80), focal stricture (6 *vs* 31), intrapancreatic duct calculi (2 *vs* 15), and side branch ectasia (36 *vs* 116) (Figure 1). In the study group, 7 cases of the isolated dorsal pancreatitis showed dilatation of main pancreatic duct all the way proximally to minor papilla. Two of recurrent cases each had a santorinicele of 5 mm (Figure 2) and 15 mm, respectively. Totally, 38 segments of duct of Santorini, 32 of duct of Wirsung, and 36 of duct in the body and in the tail as well were visualized

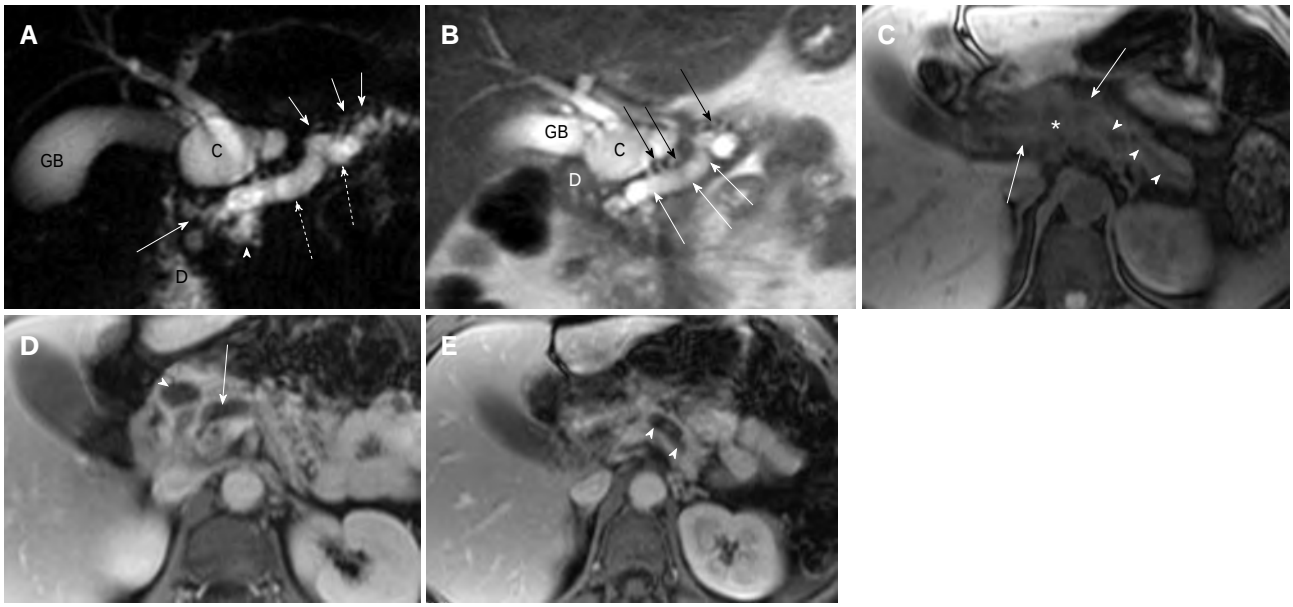

Figure 1 Magnetic resonance cholangiopancreatography and magnetic resonance imaging of recurrent acute pancreatitis involving the entire pancreas in a 44-year-old woman with several episodes of abdominal pain. A: Coronal oblique thick-section rapid acquisition with relaxation enhancement magnetic resonance (RARE-MR) cholangiogram [infinite/1100 (effective), 40-mm section thickness] shows severe dilatation of pancreatic duct (dotted arrows) with stricture (solid arrow) just before entering the duodenum (D) and side branch ectasia (short arrows) as well. There is a pseudocyst (C) formation in pancreatic parenchyma. The gallbladder (GB) is distended and the common bile duct is dilated (arrowhead) as well; B: Thin-section half-Fourier RARE-MR cholangiogram [infinite/95 (effective), 3-mm section thickness] shows remarkable dilatation of dorsal pancreatic duct (white arrows) with severe side branch ectasia (black arrows). The pseudocyst (C) is formed in the pancreatic head region immediately adjacent to the duodenum (D) and GB; C: Axial precontrast T1WI SPGR shows the dilatation of pancreatic duct (arrowheads) and the pseudocyst (star) formation in the pancreatic neck were not easily appreciated. The signal intensity of the dorsal pancreas (arrows) is dramatically decreased; D: Axial postcontrast T1WI SPGR shows delayed enhancement of the dorsal pancreas and the wall of the pseudocysts (arrowhead). Dilatation of the pancreatic duct (arrow) was noted; E: Axial postcontrast T1WI SPGR shows delayed enhancement of the dorsal pancreas and the dilatation of the pancreatic duct (arrowheads).

Table 2 Caliber measurement in four portions of pancreatic duct at magnetic resonance-cholangiopancreatography

	Santorini	Wirsung	Body	Tail
Acute pancreatitis (mm)	4.5	2.4	3.5	2.2
Recurrent pancreatitis (mm)	3.81 ± 1.02	2.40 ± 1.360	4.62 ± 3.49	3.37 ± 1.99
Chronic pancreatitis (mm)	4.06 ± 1.26	2.11 ± 0.597	3.74 ± 2.01	3.24 ± 2.35
Mean (mm)	4.00 ± 1.17	2.22 ± 0.906	4.11 ± 2.72	3.27 ± 2.14

Data are expressed as absolute numbers or mean ± SD.

Table 3 Comparison of distribution of pancreatitis with and without pancreas divisum *n* (%)

	Dorsal	Ventral	Entire	Total
Pancreatitis with PD	17 (44.74)	0 (0)	21 (55.26)	38
Pancreatitis without PD	30 (22.22)	4 (2.96)	101 (74.81)	135
Total	47 (27.17)	4 (2.31)	122 (70.52)	173

PD: Pancreas divisum.

clearly enough to be measured. The mean duct diameter was 3.00 ± 1.17 mm (SD) for the duct of Santorini, 2.22 ± 0.906 mm for the duct of Wirsung, 4.11 ± 2.72 mm for the body, and 3.27 ± 2.14 mm in the tail segments (Table 2). There were 3 severe chronic cases and 3 recurrent cases of pancreatitis showing the maximum of dorsal pancreatic ductal dilatation measured from 5 mm to 13.5 mm.

In the study group, pancreatic edematous enlargement (n = 3), peripancreatic stranding (n = 13), atrophy with T1 signal intensity decrease (n = 12), atrophy with normal signal (n = 12), only T1 signal decrease (n = 3), and small intrapancreatic necrosis (n = 4) were detected in pancreatitis patients with PD. Six intrapancreatic pseudocysts were detected in 6 cases with a size ranging from 5 to 20 mm while 4 extrapancreatic fluid collections and pseudocysts were formed in the other 3 cases sized 35 to 76 mm. Thirty-two cases (recurrent acute, n = 21; chronic pancreatitis, n = 8) demonstrated delayed enhancement of pancreas (Figure 3). There were no adjacent organ and vessel involvement, and no hemorrhage or abscess formation due to pancreatitis in patients with PD.

Classification of pancreas divisum and distribution of pancreatitis: In the study group, 35 cases were classified as complete PD and 3 cases as incomplete PD. Totally, 16 cases with complete divisum and 1 with incomplete divisum comprised dorsal pancreatitis in patients with PD. In cases of pancreatitis with PD, the dorsal pancreatitis accounted for a much higher percentage than in pancreatitis without PD (17 of 38, 44.74% *vs* 30 of 135, 22.22%) (χ^2 =7.257, P < 0.05) (Table 3, Figures 4 and 5). Fifteen cases of dorsal pancreatitis were classified as recurrent acute pancreatitis. According to the anatomical involvement of lesion, the 17 cases of dorsal pancreatitis

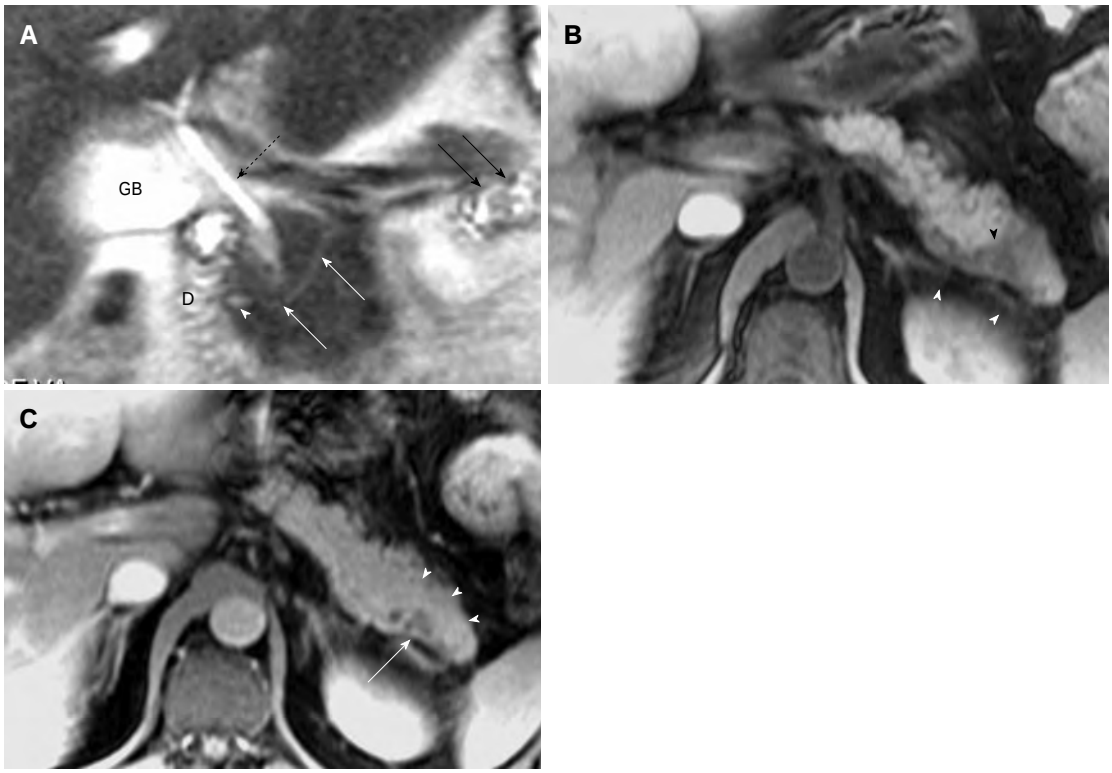

Figure 2 Magnetic resonance cholangiopancreatography and magnetic resonance imaging of recurrent acute pancreatitis with pancreas divisum only involving the pancreatic tail with a small santorinicele in a 44-year-old man with several episodes of abdominal pain. A: Thin-section half-Fourier rapid acquisition with relaxation enhancement magnetic resonance (RARE- MR) cholangiogram [infinite/95 (effective), 3-mm section thickness] shows mild dilatation of the duct of Santorini (white arrows) with a focal enlargement consistent with santorinicele (arrowhead) at the entrance into the duodenum (D) *via* minor papilla. The side branch ectasia (black arrows) are noted in the pancreatic tail consistent with pancreatitis. Distended gallbladder (GB) and the CBD (dotted black arrow) are noted. B: Axial precontrast T1WI SPGR shows swelling and decrease of signal intensity in the pancreatic tail (black arrowhead), and thickening of the left anterior renal fascia (white arrowheads). C: Axial postcontrast T1WI SPGR shows mild delayed enhancement of pancreatic tail (arrowheads) with focal cystic changes (arrow).

could be classified as complete dorsal involvement (n = 7), suproanterior portion of head and neck involvement (n = 3), dominant body involvement (n = 2), and dominant tail involvement (n = 5) with ductal stricture at body-tail junction resulting in upstream ductal dilatation in the tail.

DISCUSSION

Classic PD (type 1) is defined as complete failure of fusion of the ducts of Santorini and Wirsung; other fusion anomalies with dominant dorsal drainage include absence of duct of Wirsung (type 2) and the presence of a filamentous or tiny caliber communication between the dominant dorsal duct of Santorini and the duct of Wirsung (type 3, incomplete PD)[15]. In patient with complete PD, a larger amount secretion from the dorsal pancreas could exert a significant burden on the relatively smaller orifice of minor duodenal papilla causing elevation of endoluminal pressure in pancreatic duct, resulting in subsequent pancreatitis. It has been reported that the clinical implications of incomplete PD may be similar to those of complete PD though the precise physiology may differ from each other[16,17].

A prior study with ERCP showed that the highest incidence of PD associated with idiopathic acute pancreatitis reached 50% in a total of 58 cases, which is significantly higher than in both controls and the whole population[18]. The recurrent acute pancreatitis in our study accounted for 55.26% (21 of 38) of pancreatitis in patients with PD, which is higher than in control group (6.67%, 9 of 135) (P < 0.0001, Table 1). The recurrent acute pancreatitis is a clinical entity that is characterized by repeated episodes of pancreatitis, which evolves over time with recurrent attacks of acute pancreatitis in otherwise normal pancreas until interventional procedures were performed in this clinical setting[19]. The pancreatitis in patients with PD accounted for 21.97% (38 of 173) in total population of pancreatitis in this study, which is higher than in the results based on ERCP by Bernard *et al*[18] and Kamisava *et al*[20].

In Western countries, the incomplete PD is uncommon with a reported incidence of 0.13%-0.9%. However, there was a much higher prevalence of incomplete PD in the recent reports from Japan and Korea, indicating 48% and 52% of PD[16,17]. In the present study, the incomplete PD occurred in 7.9% (3 of 38) among the pancreatitis patients with PD. Partially, the fluctuation of the frequency of incomplete PD could result from the different techniques employed for the detection of PD, *i.e.*, the ERCP or MRCP, even for the same imaging modality, the techniques may be different with time due to intrinsic advances resulting in improved resolution[16,21-24].

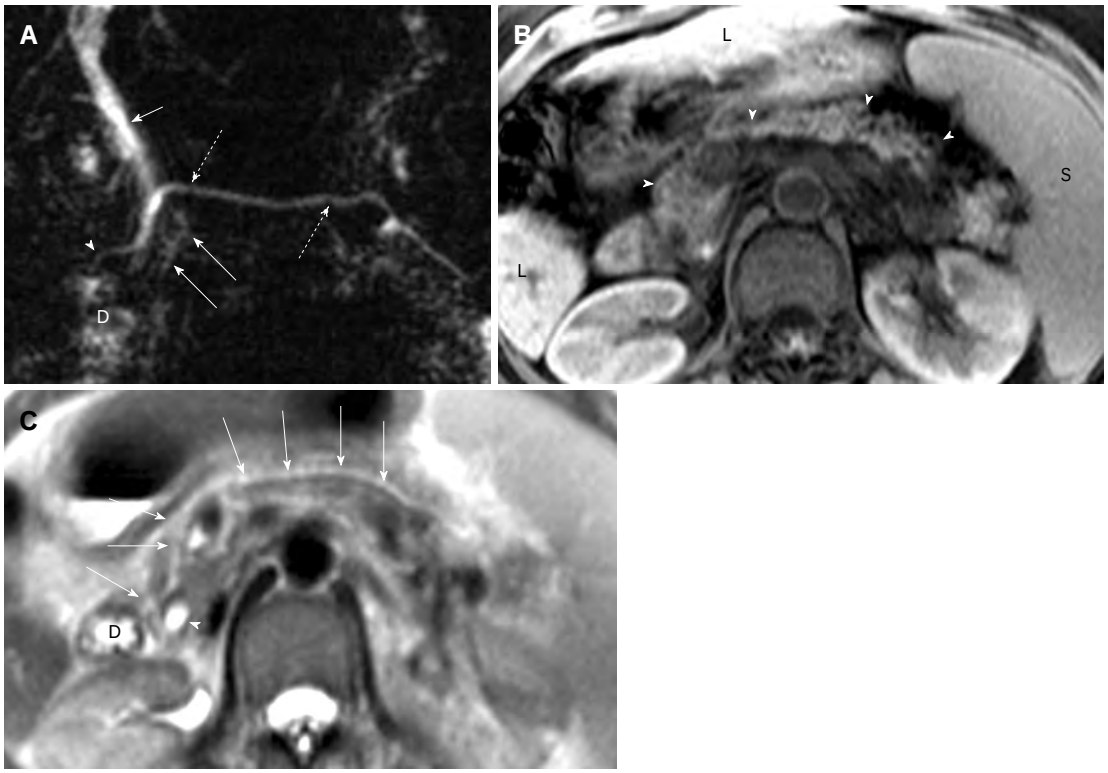

Figure 3 Magnetic resonance cholangiopancreatography and magnetic resonance imaging of incidental chronic pancreatitis in a 47-year-old woman with a history of liver cirrhosis. A: Coronal oblique thick-section rapid acquisition with relaxation enhancement magnetic resonance (RARE-MR) cholangiogram [infinite/1100 (effective), 40-mm section thickness] of the pancreaticobiliary ducts shows slight dilatation with irregularity and strictures in the main pancreatic duct (dotted arrows) and in the duct of Santorini (arrowhead) just before entering the duodenum (D) *via* the minor papilla. Several side branch ectasia (long arrows) arising from the ventral duct and a normal common bile duct (short arrows) are demonstrated; B: Axial T1WI with fat saturation shows severe atrophy of the entire pancreas parenchyma (arrowheads). The liver (L) cirrhosis and splenomegaly (S) are noted; C: Axial T2WI shows main pancreatic duct (solid arrows) continued by duct of Santorini entering the duodenum (D) anterior to the common bile duct (arrowhead).

In our study, 94.1% (16 of 17) of dorsal pancreatitis were detected in cases with complete PD. According to the pathophysiology of PD, the majority of pancreatic juice drained through minor papilla can result in endoluminal pressure elevation or obstruction with subsequent pancreatitis likely involving the dorsal pancreas and sparing the ventral pancreas instead.

Although the incidence of dorsal pancreatitis (44.74%, 17 of 38) in patients with PD was significantly higher than in patients without PD (30 of 135, 22.22%) ($P < 0.05$, Table 3) in this study and in the study with ERCP conducted by Kamisava *et al*[20], it is lower than the prior study performed with ERCP by Morgan *et al*[4]. The presence of pancreas divisum may reduce the severity of acute gallstone pancreatitis, as stone impaction at the major papilla only affects the ventral pancreas, a smaller portion (about 10%) of pancreas compared to the dorsal pancreas[25].

Among the isolated dorsal pancreatitis cases, severe strictures at the duct junction of body and tail were responsible for upstream ductal dilatation in tail with atrophy of the affected pancreas, which presented with recurrent acute pancreatitis clinically. The pancreatic parenchyma in pancreatitis associated with PD demonstrated a spectrum of abnormalities including low-signal-intensity of pancreas on T1-weighted fat-suppressed images due to edema or fibrosis, decreased and delayed enhancement after intravenous contrast administration, parenchymal atrophy or enlargement, and pseudocysts. Inflammation and fibrosis can diminish the proteinaceous fluid content in the pancreas, leading to the loss of the usual high signal intensity on T1-weighted fat-suppressed images; therefore, the pancreatitis in patients with PD can have some findings similar to the pancreatitis without PD as indicated in literature[8].

Although the ERCP is still considered as a gold standard for diagnosing PD, it is an invasive technique and expensive, particularly it has several drawbacks including failure to cannulate minor papilla[12,26], a high rate of complications such as ERCP-induced pancreatitis[26], radiation, and use of iodinated contrast medium. It was reported that as high as 35% of patients with pancreatitis had no abnormalities on ERCP[27]. A recent article reported that the MDCT could detect the PD *via* visualization of the Santorini duct[13]. However, MRCP together with MRI is a non-invasive technique without radiation; MRCP can always be done together with MRI in a single study, which can delineate the parenchymal morphology in detail[28]. Comparing to ERCP and MDCT, MRCP and MRI can be repeated more safely in the follow-up of the patients of pancreatitis with PD since patients in this subgroup are likely younger and more sensitive to radia-

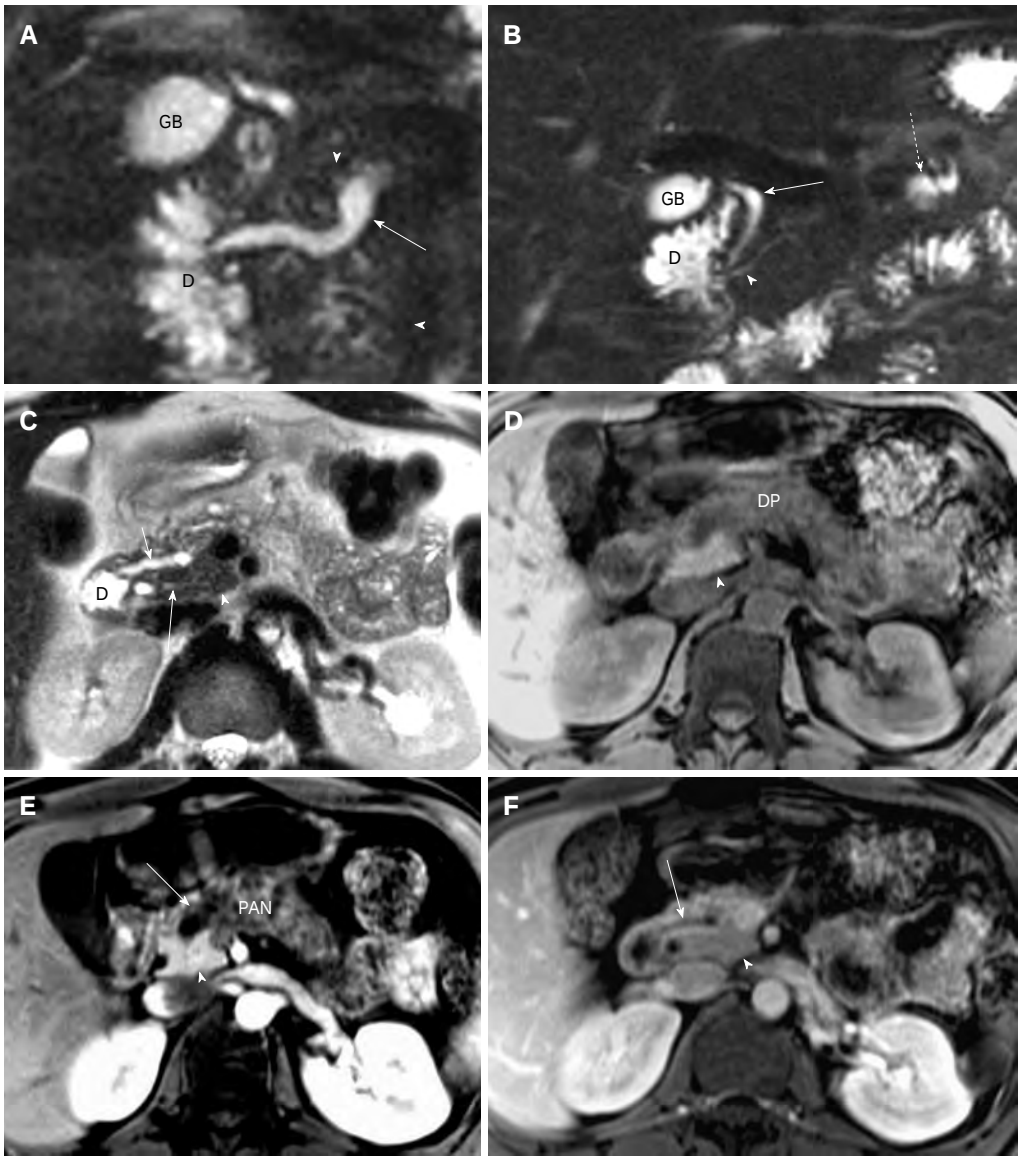

Figure 4 Magnetic resonance cholangiopancreatography and magnetic resonance imaging of recurrent acute pancreatitis only involving the dorsal pancreas in a 43-year-old man. A: Coronal-oblique, thin-section half-Fourier rapid acquisition with relaxation enhancement magnetic resonance (RARE-MR) cholangiogram [infinite/95 (effective), 3-mm section thickness] shows marked dilatation (1 cm in diameter) of duct of Santorini (arrow) with apparent side branch ectasia (arrowheads). The gallbladder (GB) and the duodenum (D) are demonstrated clearly; B: Coronal-oblique, thin-section half-Fourier RARE MR cholangiogram [infinite/95 (effective), 3-mm section thickness] shows normal common bile duct (arrow) and ventral duct (arrowhead) of pancreas. The cystic changes secondary to pancreatitis in the pancreatic tail (dotted arrow) is shown while the GB and duodenum (D) appear normal; C: Axial T2WI shows dilatation and irregularity of duct of Santorini (short arrow) which enters the duodenum (D) *via* minor papilla. The ventral duct (solid arrow) and the pancreatic uncinate (arrowhead) are normal in size and signal intensity while the anterior portion of pancreatic head is abnormal with elevation of signal intensity; D: Axial precontrast T1WI SPGR shows that the pancreatic uncinate (arrowhead) is normal in size and signal intensity and the dorsal pancreas (DP) is abnormal with decreased T1 signal intensity and the swelling of the parenchyma; E: Axial T1WI SPGR after administration of Gd-DTPA at arterial phase shows normal enhancement of the pancreatic uncinate (arrowhead) and the enhancement of dorsal pancreas (PAN) is remarkably compromised with duct dilatation (arrow) and cystic changes; F: Axial T1WI SPGR at portal venous phase after administration of Gd-DTPA shows normal wash-out of contrast material in pancreatic uncinate (arrowhead) and delayed enhancement of the anterior portion of pancreatic head with dilatation of duct of Santorini (arrow).

tion. MRCP with secretin stimulation can provide better visualization of pancreatic duct, resulting in higher sensitivity and specificity for diagnosis of the pancreatic abnormalities[29,30]. MRI can have the same accuracy as CT for pancreatitis at present. Additionally, MRCP is thought to depict the pancreatic duct in more physiologic states than under exogenous pressure such as ERCP. Therefore, MRCP and MRI can serve as a comprehensive diagnostic tool without radiation for the pancreatitis associated with PD whereas the ERCP can be reserved for those who require interventional procedures for therapeutic purpose.

This study had several limitations. One limitation was that not all cases (21 of 38) underwent ERCP procedure for reference or interventional management. Owing to the resolution of MRCP without secretin stimulation at present, there could be some compromises resulting in possible false-negative consequences in detection and characterization of PD and pancreatitis associated with

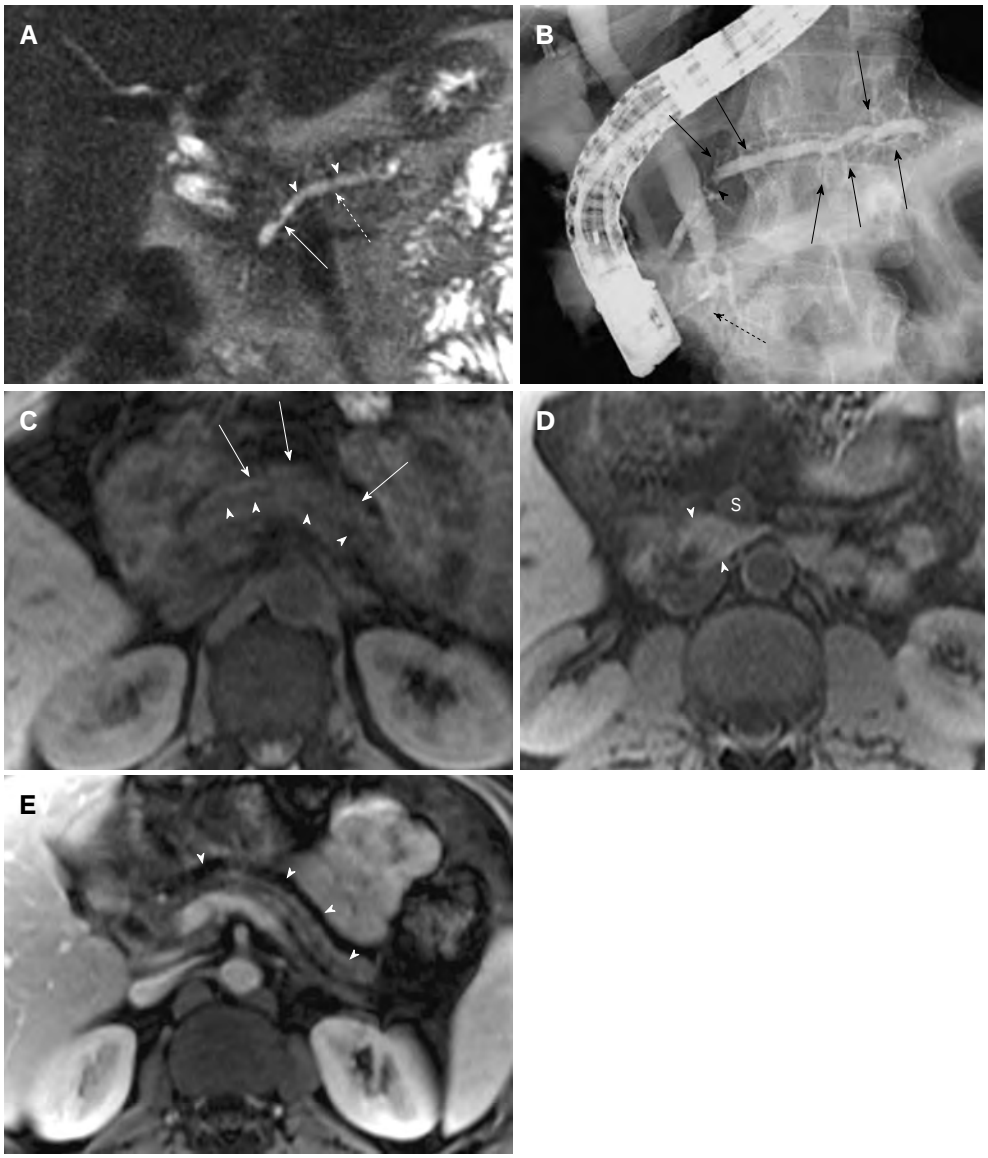

Figure 5 Magnetic resonance cholangiopancreatography and magnetic resonance imaging of recurrent acute pancreatitis only involving the dorsal pancreas, with endoscopic retrograde cholangiopancreatography correlation, in a 42-year-old man with several episodes of abdominal pain. A: Coronal-oblique, thin-section half-Fourier rapid acquisition with relaxation enhancement magnetic resonance (RARE-MR) cholangiogram [infinite/95 (effective), 3-mm section thickness] shows remarkable dilatation of the dorsal pancreatic duct (dotted arrow) with a conspicuous stricture (solid arrow) and severe side branch ectasia (arrowheads); B: Endoscopic retrograde cholangiopancreatography shows the dilatation of dorsal pancreatic duct and duct of Santorini with a well-seen stricture (arrowhead) and remarkable side branch ectasia (arrows) continual and proximal to the minor papilla. The intrapancreatic segment of common bile duct (C) is very narrow while the other part of the CBD is dilated. The ventral duct is normal in size (dotted arrow); C: Axial precontrast T1WI SPGR shows a marked decrease of signal intensity of dorsal pancreas (arrows) with ductal dilatation (arrowheads); D: Axial precontrast T1WI SPGR shows that the uncinate of pancreas is normal in size and signal intensity (arrowheads) is much higher than the superior mesenteric vein (S) and similar to the liver; E: Axial postcontrast T1WI SPGR shows the atrophy of dorsal pancreas with delayed enhancement (arrowheads) and dilatation of the allied pancreatic duct.

PD in some cases. Finally, the subjects enrolled into the study was based on the referring criteria for MRCP and MRI studies; the severity of pancreatitis or the classifications of pancreatitis might not exactly reflect the real profile of pancreatitis in patients with PD since most of severe and acute patients prefer CT because it is quicker than MRI in examination.

In conclusion, recurrent acute pancreatitis is more common in patients with divisum than in patients without divisum (21 of 38, 55.26% *vs* 9 of 135, 6.67%). In divisum patients, the dorsal pancreatitis accounts for a much higher percentage than in patients without PD (17 of 38, 44.74% *vs* 30 of 135, 22.22%). Therefore, MRCP and MRI could be a comprehensive diagnostic tool without radiation for the pancreatitis associated with PD whereas the ERCP can be reserved for those who require therapeutic interventions.

COMMENTS

Background

Pancreas divisum (PD) is the most common developmental anatomic variant

of pancreatic duct. Elevation of the intraluminal pressure of the pancreatic duct could result in pancreatitis. Magnetic resonance-cholangiopancreatography (MRCP) can always be performed together with magnetic resonance imaging (MRI), which can accurately detect both the ductal and the parenchymal abnormalities of the pancreas in detail.

Research frontiers

The pancreatitis in patients with PD could be different from the cases without PD both in clinical presentations and distribution of the abnormalities in pancreas since the congenital anomaly, however, the clinical significance remains controversial. To their knowledge, there is no published literature on imaging features of pancreatitis in patients with PD using MRI together with MRCP without secretin injection.

Innovations and breakthroughs

The pancreatitis associated with PD was usually distributed in dorsal pancreas and presented as recurrent acute type. MRCP in combination with MRI can accurately detect ductal and parenchymal abnormalities of pancreatitis in patients with PD.

Applications

The results of the present study indicated that repeated attacks of acute pancreatitis or isolated dorsal involvement of pancreas could imply a congenital PD in pancreas. MRCP and MRI should be referred to as a primary diagnostic tool for pancreatitis patients associated with PD whereas ERCP can be reserved for those who require therapeutic interventions.

Terminology

Recurrent acute pancreatitis was defined in this study as a condition in which the clinical or/and serologic features were characteristic of acute pancreatitis with a history of recurrence at least 2 times.

Peer review

Pancreas divisum is a common congenital anomaly of the pancreatic duct and possible cause of recurrent pancreatitis and chronic pancreatitis. But, there are still controversies in clinical significance as a cause of pancreatitis. This study revealed that recurrent acute pancreatitis is more common in divisum patients compared with those without divisum. The sample size was large to reach a conclusion and those results can help clinicians understand clinical significance of pancreas divisum. The imaging quality of presented figures is excellent and representative.

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ORIGINAL ARTICLE

Skp2-RNAi suppresses proliferation and migration of gallbladder carcinoma cells by enhancing p27 expression

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Abstract

AIM: To explore the role of S-phase kinase-associated protein-2 (Skp2) in gallbladder carcinoma and to identify whether depletion of Skp2 by Skp2-RNAi could attenuate proliferation and migration of gallbladder carcinoma.

METHODS: Skp2-RNAi was transduced into cells of the gallbladder carcinoma cell line GBC-SD, using a lentiviral vector. The effect of Skp2-RNAi on the proliferation, migration, invasion and cell cycle of GBC-SD cells was studied using *in vitro* assays for cell proliferation, colony formation, wound healing and cell cycle. The expression of Skp2 and p27 was detected by real-time polymerase chain reaction and Western immunoblotting. The effect of Skp2-RNAi on the proliferation of GBC-SD cells *in vivo* was investigated by tumorigenicity experiments in nude mice.

RESULTS: Lentivirus-mediated RNAi reduced the expression of Skp2 in cultured cells. The expression of the p27 protein increased along with the down-regulation of Skp2, although no significant difference was found in p27 mRNA expression. Flow cytometry revealed that Skp2-RNAi transfection significantly increased the proportion of cells in the S phase and significantly decreased the proportion of cells in the G_2/M phase. No significant difference in the frequency of cells in the G_0/G_1 phase was observed. The results from the cell proliferation, colony formation and wound healing assays revealed that Skp2-RNAi transfection markedly inhibited the proliferation and migration of GBC-SD cells *in vitro*. Additionally, tumorigenicity experiments showed that suppression of Skp2 significantly decreased the weights of the tumors (0.56 ± 0.11 and 0.55 ± 0.07 g in the control and Scr-RNAi groups *vs* 0.37 ± 0.09 and 0.35 ± 0.08 g in the Skp2-RNAi-L and Skp2-RNAi-H groups).

CONCLUSION: The expression of Skp2 in GBC-SD cells was inhibited following Skp2-RNAi transfection. Silencing of the *Skp2* gene inhibited proliferation, migration and invasiveness of GBC-SD cells by mechanisms dependent on enhanced expression of the p27 protein.

Key words: Gallbladder carcinoma; S-phase kinase-associated protein-2; p27; Gene therapy; Cell cycle

Core tip: The association between S-phase kinase-associated protein-2 (Skp2)/p27 and gallbladder carcinoma has rarely been reported. This study investigated the effects of Skp2-RNAi on *in vitro* and *in vivo* growth and the invasive potencies of gallbladder carcinoma cells. The authors proposed that the effects were due to the accumulation of the p27 protein following Skp2-depletion.

Zhang B, Ji LH, Liu W, Zhao G, Wu ZY. Skp2-RNAi suppresses proliferation and migration of gallbladder carcinoma cells by enhancing p27 expression. *World J Gastroenterol* 2013; 19(30): 4917-4924 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/4917.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.4917

INTRODUCTION

Primary gallbladder carcinoma is a common biliary malignancy. Its incidence is estimated to be approximately 1.2-10.6/100000, and this cancer accounts for almost 3% of all tumors[1]. Unfortunately, the majority of patients with primary gallbladder carcinoma have intermediate-advanced disease at presentation due, in part, to diagnostic difficulties and a high degree of malignancy. Thus, for these patients, the prognosis is extremely poor.

The cancer suppressor gene *p27* (wherein *p27* represents the gene and $p27^{(Kip1)}$ represents the protein) is a cyclin-dependent kinase inhibitor (CKI), which plays an important role in tumorigenesis and tumor development[2]. Altered expression of $p27^{(Kip1)}$ is closely associated with the prognosis in several types of human cancers[3,4]. It has been shown that the stability of $p27^{(Kip1)}$ can be enhanced by a specific proteasome inhibitor, which can further inhibit the growth of the tumor[5]. Over-expression of $p27^{(Kip1)}$ with an adenoviral vector (adenovirus-p27) can inhibit tumor growth and induce apoptosis[6,7]. In addition, the expression of p27 mRNA was determined to be constant during a normal cell cycle. The highest expression of $p27^{(Kip1)}$ was found during the G_0/G_1 phase of the cell cycle, and the lowest expression was throughout the S and M phases[8-10]. The expression of $p27^{(Kip1)}$ was found to be predominantly regulated by S-phase kinase-associated protein-2 (Skp2)[8,9].

Skp2 (wherein SKP2 represents the gene and Skp2 represents the protein) is an S-phase dependent protein kinase that was originally found by Rodriguez *et al*[11], constituting the F-box unit of the SCF-E3 ligase that specifically targets CKIs, such as $p21^{(Cip1)}$, $p27^{(Kip1)}$, $p57^{(Kip2)}$ and p130, for degradation[12]. Functional deletion of Skp2 leads to stabilization of CKIs, which can subsequently induce cell-cycle delay or arrest; conversely, the over-expression of Skp2 is frequently associated with a variety of human cancers[11,13]. Nelsen *et al*[14] reported that cotransfection of cyclin E and Skp2 synergistically promoted cell cycle progression in cultured primary hepatocytes in the absence of mitogen or in the presence of growth inhibitors. Furthermore, transfection of hepatocytes with cyclin E and Skp2 *in vivo* promoted abundant hepatocyte replication and hyperplasia of the liver. Hence, Skp2 is thought to be closely associated with cell cycle regulation, tumor emergence, tumor development and disease prognosis.

$p27^{(Kip1)}$ and Skp2 have been studied in many types of tumors[15-19]. The determination of an association between Skp2/$p27^{(Kip1)}$ and gallbladder carcinoma has been rarely reported[20,21]. In the current study, we constructed a lentiviral vector of Skp2-RNAi, and explored the role of Skp2/$p27^{(Kip1)}$ in the proliferation and metastasis of gallbladder carcinoma cells.

MATERIALS AND METHODS

Groups

The gallbladder carcinoma cell line (GBC-SD) cells (Shanghai Cell Library, China) were divided into four groups: (1) control group: without any treatment; (2) Scr-RNAi group (Scr-RNAi group): GBC-SD cells were transfected with a negative control RNA interference sequence (TTCTCCGAACGTGTCACGT) using lentivirus vectors (SunBio, United States); (3) for the Skp2-RNAi-Low group (Skp2-RNAi-L group); and (4) the Skp2-RNAi-High group (Skp2-RNAi-H group), the cells were transfected with an RNA interference sequence of Skp2 (AGGTC-TCTGGTGTTTGTAA) at a dose of 10 and 20 MOI, respectively.

Construction and identification of the RNAi lentivirus vector

The GBC-SD cells were plated and cultured in 24-well plates until cell fusion reached 40%-60%. Next, the appropriate amounts of lentivirus were added to the cells according to the different MOI values (2.5×10^4 TU/well in Skp2-RNAi-L group and 5×10^4 TU/well in Skp2-RNAi-H group). The transduction efficiency was assessed by fluorescence microscopy (Nikon, Japan) after 96 h. The cells were harvested 10 d following transduction.

The effect of the RNAi-Lentivirus on the expression of *Skp2* gene was assessed by determination of the mRNA and protein levels of Skp2 in the GBC-SD cells after infection with lentivirus for 5-7 d; real-time polymerase chain reaction (PCR) and Western immunoblotting were used for these assessments.

Cell proliferation assay

Cell proliferation ability was assessed with a methylthiazol tetrazolium (MTT) assay kit (SunBio, United States). The cells were inoculated into 96-well plates (1×10^4 cells per well). After incubation for 1, 2, 3, 4 and 5 d, 100 μL of sterile MTT (5 mg/mL, Sigma-Aldrich Corp, United States) was added to each well. The cells were further incubated at 37 ℃ for 4 h, and the reaction was stopped by adding 200 μL of dimethyl sulfoxide. After mixing for 10 min at room temperature, formazan production was determined by measurement of the optical density (OD) at 570 nm using an enzyme immunoassay analyzer (1420 multi-label counter).

Colony formation assay

Two hundred cells were prepared and plated into 35-mm culture plates for a period of 10 d. The resulting cellular clones were counted using an inverted microscope (BX45-72P15, Olympus, Japan). A cell clone was scored as positive following confirmation that the number of cells within the clone exceeded 50. The experiment was repeated 8 times.

Wound healing assay

Cells were inoculated into 6-well plates, and a 100-μL pipette tip was used to scribe a line across the cell monolayer. The cells that moved into the interspace of the wound line were counted 24 h later using a phase contrast microscope (BX45-72P15, Olympus, Japan). This assay was

repeated 8 times.

Cell cycle assay

Cells were seeded into a 6-well plate and harvested after infection for 10 d. After two washes in pre-cooled PBS, the cells were fixed in 70% alcohol. The percentage of cells in each stage of the cell cycle was determined by staining with propidium iodide (PI, Santa Cruz, United States). The cell cycle distribution was analyzed with a FAC-Scan Flow Cytometer (BD, United States), in accordance with the manufacturer's guidelines.

RNA extraction and real-time PCR

Total RNA (2 μg) was isolated and reverse-transcribed into cDNA. The cDNA samples (2 μL) were employed for real-time PCR in a total volume of 20 μL on a GeneAmp Thermal Cycler 9700 (ABI, United States). The reactions were incubated in 96-well optical plate at 94 ℃ for 4 min, followed by 35 cycles of 94 ℃ for 10 s, 57 ℃ for 15 s and 72 ℃ for 20 s, and a final extension reaction at 86.5 ℃ for 5 s. Melting-curve analysis was performed from 72 ℃ to 99 ℃ at a rate of 1 ℃ every 5 s. The average of the triplicate data obtained for each sample was employed to calculate the relative change in gene expression after normalization to β-actin mRNA. The primer sequences were as follows: Skp2: 5'-CCTAAGCAGCTGTCCCAGAC-3' (sense) and 5'-GTGTCAGTCGGCATTTGATG-3' (antisense); p27: 5'-ACCCAACAATACCACCGACC-3' (sense) and 5'-CCCGCCTAATCTGCACTGTG-3' (antisense); β-actin: 5'-CCAAGGCCAACCGCGAGAAGATGAC-3' (sense) and 5'-AGGGTACATGGTGGTGCCGCCAGAC-3' (antisense).

Western immunoblotting

After lysis with pre-cooled lysis buffer, 40 μg of protein extracted from the cells was loaded onto 10% SDS-PAGE gels, and the resolved proteins were transferred to a PVDF membrane over a 2-h period (Bio-Rad, United States). Next, the membrane was blocked in 5% non-fat milk for 1 h at room temperature and then probed overnight at 4 ℃ with antibodies against Skp2 (CST, United States, 1:1000 dilution) and p27 (CST, United States, 1:1000 dilution). After three washes with TBST (tris buffered saline with 0.5% Tween-20), the membrane was incubated with the appropriate secondary antibody (anti-mouse IgG, Santa Cruz, United States, 1:1000 dilution) for 2 h at room temperature. Following a further washing with TBST, the membrane's image was developed using enhanced chemiluminescence (ECL + plus™, Amersham, United Kingdom). β-actin was employed as an internal standard, and expressions of Skp2 and p27 were determined and normalized against the level of β-actin.

Tumorigenicity experiments in nude mice

Forty male nude mice weighing 18 to 21 g, provided by Shanghai Laboratory Animal Center (Chinese Academy of Science, China), were bred under aseptic conditions; the animals were housed in an area with a constant humidity of 60%-70% and a room temperature of 18 ℃-20 ℃. Animal maintenance, husbandry and experimental procedures were performed in accordance with the United States National Institute of Health Guidelines for the Use of Experimental Animals and approved by the Medical Animal Care and Use Committee of Renji Hospital (Shanghai, China). All of the mice were separated into four groups as described above: control, Scr-RNAi, Skp2-RNAi-L, and Skp2-RNAi-H groups. Lentivirus transfected cells from each group were administrated by subcutaneous injection (0.1 mL of a solution containing 1×10^4 cells/mL). The mice were examined every 4 d and were sacrificed 28 d after the initial subcutaneous injection. The tumors were resected and weighed.

Statistical analysis

All measurement data were expressed as the mean ± SD. The association analysis among the groups was performed using one-way analysis of variance with the SPSS17.0 statistical software package. Statistical significance was defined as having a P value less than 0.05.

RESULTS

Validation of Skp2-RNAi-lentivirus and expression of p27

The studies showed that Skp2-RNAi transfection could significantly reduce the level of Skp2 mRNA ($P < 0.05$, Figure 1A) and protein ($P < 0.05$, Figure 1B and C) in the Skp2-RNAi-L and Skp2-RNAi-H groups, compared with the control and Scr-RNAi groups, and that these alterations were closely related to the dosage of Skp2-RNAi. In addition, the expression of p27 was also detected in GBC-SD cells after infection with RNAi-Lentivirus. Expression of p27 mRNA did not change following the down-regulation of Skp2. Densitometric analysis of the immunoblot images showed that the ratios between the p27 protein in the Scr-RNAi, Skp2-RNAi-L and Skp2-RNAi-H groups and the p27 protein in the control group were 0.99, 1.52 and 1.93, respectively ($P < 0.05$, Figure 1). This result suggests that p27 was increased at the protein level but not at the mRNA level following Skp2-RNAi transfection. Expression of cyclin D1 and E mRNA and protein was unaltered (data not shown).

Skp2-RNAi inhibited cell growth

The effect of Skp2-RNAi on cell growth was evaluated using an MTT assay kit. As shown in Figure 2, the A values in the Skp2-RNAi-L and Skp2-RNAi-H groups were significantly higher than the values in the control and Scr-RNAi groups (0.94 ± 0.12 and 0.87 ± 0.11 *vs* 0.48 ± 0.06 and 0.41 ± 0.05, respectively, $P < 0.01$). This result suggests that cell growth was significantly inhibited along with the down-regulation of Skp2 by Skp2-RNAi.

Skp2-RNAi inhibited colony formation of GBC-SD cells

Cell colony formation was significantly decreased in the

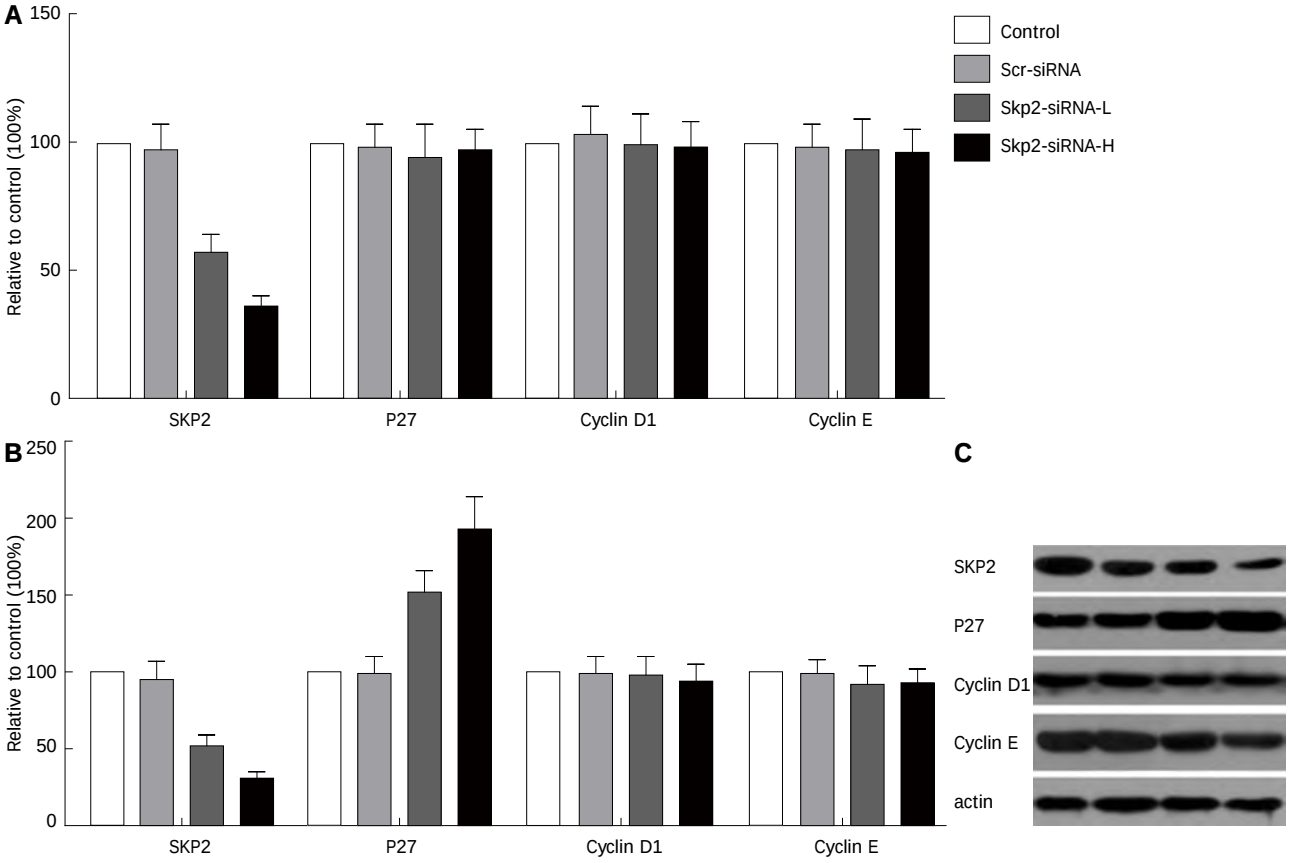

Figure 1 Detection of downstream gene and protein expression after kinase-associated protein-2-RNAi transfection. A: Expression of p27 mRNA did not change following down-regulation of S-phase kinase-associated protein-2 (Skp2); B and C: Protein expression of p27 was upregulated. Lane 1: Control; Lane 2: Scr-RNAi; Lane 3: Skp2-RNAi-L; Lane 4: Skp2-RNAi-H.

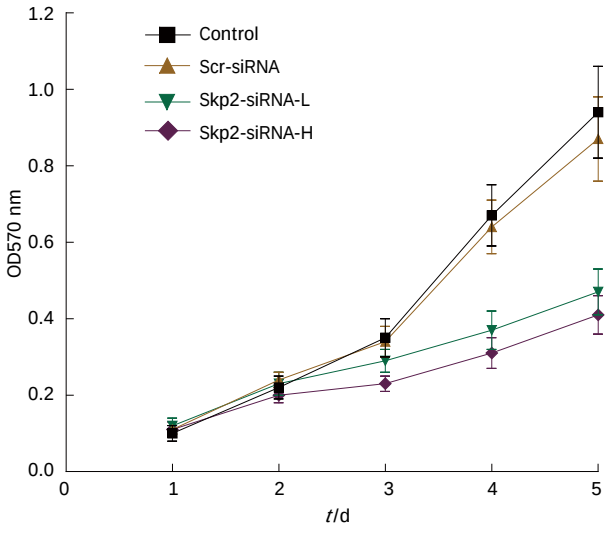

Figure 2 Proliferation curves for the gallbladder carcinoma cell line cells after the inhibition of kinase-associated protein-2 expression. Cell proliferation was significantly inhibited after down-regulation of S-phase kinase-associated protein-2 (Skp2) by Skp2-RNAi. GBC-SD: The gallbladder carcinoma cell line.

Skp2-RNAi-L and Skp2-RNAi-H groups, compared to that in the control and Scr-RNAi groups, (13.50 ± 5.90 and 7.25 ± 5.12 *vs* 51.25 ± 7.54 and 48.88 ± 11.93 cells per well, respectively, $P < 0.01$, Figure 3). Colony formation of GBC-SD cells was significantly inhibited after transfection with Skp2-RNAi, and those colonies formed were closely related to the dosage of Skp2-RNAi.

Skp2-RNAi suppressed migration ability of GBC-SD cells

The migrated cells in the control, Scr-RNAi, Skp2-RNAi-L and Skp2-RNAi-H groups were found to be 111.75 ± 19.96, 101.38 ± 14.32, 76.50 ± 13.15 and 63.16 ± 11.00 cells per mm^2, respectively. The migrated cells in the Skp2-RNAi-L and Skp2-RNAi-H groups were markedly decreased compared with the control and Scr-RNAi groups ($P < 0.01$, Figure 4).

Cell cycle changes

No significant difference was observed in the proportion of cells in the G_0/G_1 phase following inhibition of Skp2. However, in the Skp2-RNAi-L and Skp2-RNAi-H groups, the proportion of cells in S phase increased. Nevertheless, inhibition of Skp2 decreased the proportion of cells in the G_2/M phase as compared with the control and Scr-RNAi groups ($P < 0.05$, Figure 5).

Skp2-RNAi inhibited tumor growth in nude mice

Twenty-eight days after the mice were injected with carcinoma cells, the weights of the tumors in the control,

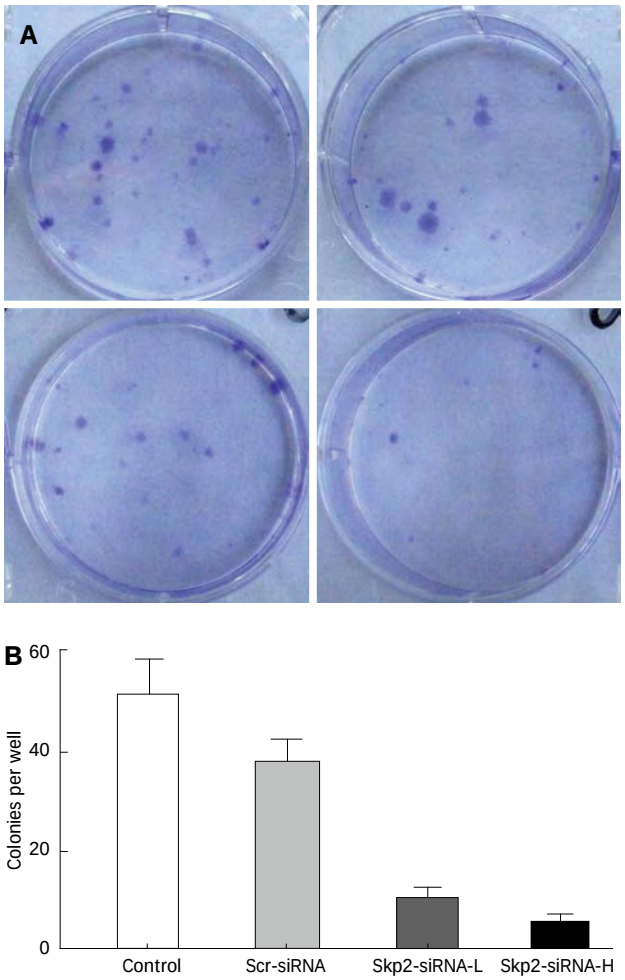

Figure 3 Colony formation assays after inhibition of kinase-associated protein-2 expression. A: Colony formation assays; B: Colony formation of the gallbladder carcinoma cell line (GBC-SD) cells was significantly inhibited after transfection with S-phase kinase-associated protein-2 (Skp2)-RNAi.

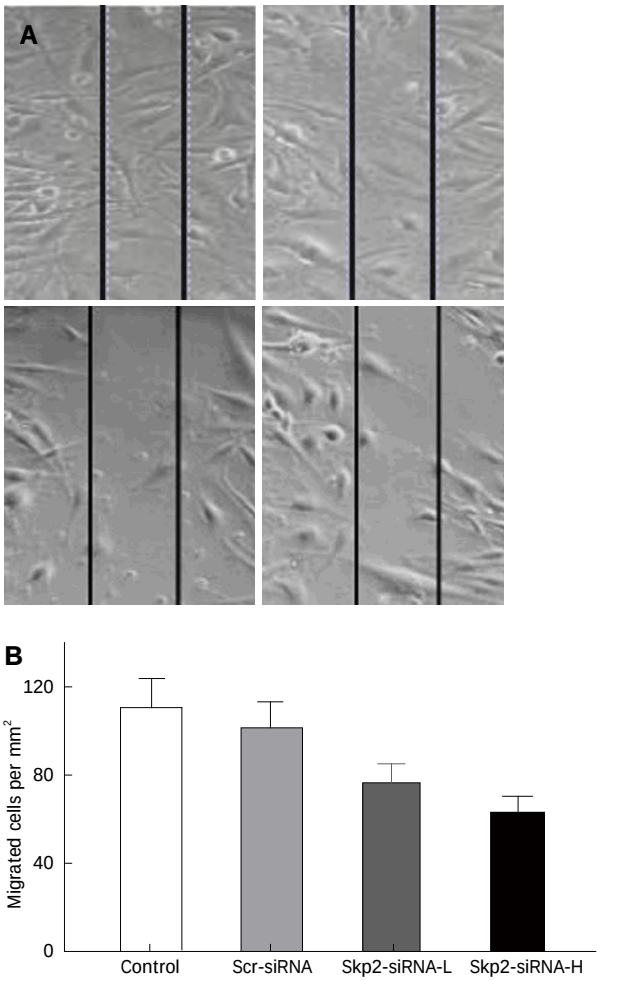

Figure 4 Results of the wound healing assay after inhibition of kinase-associated protein-2 expression. A: The wound healing assay after inhibition of kinase-associated protein-2 (Skp2) expression; B: The migrated cells in the two S-phase Skp2-RNAi groups were markedly decreased as compared with the two control groups.

Scr-RNAi, Skp2-RNAi-L and Skp2-RNAi-H groups were 0.56 ± 0.11, 0.55 ± 0.07, 0.37 ± 0.09 and 0.35 ± 0.08 g, respectively. Thus, treatment with Skp2-RNAi inhibited the growth of tumors as compared with both the control and Scr-RNAi groups ($P < 0.01$, Figure 6).

DISCUSSION

Gallbladder carcinoma was first described by Clemente *et al*[1]. Despite advances in hepatobiliary imaging techniques, the preoperative diagnosis of this condition remains a daunting task. Furthermore, the long-term survival remains dismal, not only because of the non-specific presentation of the disease and its similarity to benign biliary tract disorders, but also because of the malignant entity. Currently, the mean survival time of advanced stage gallbladder carcinoma is approximately 6 mo, and the 5-year survival rate is less than 5%[22]. Hence, the prognosis of gallbladder carcinoma remains poor despite improvements in surgical techniques. Moreover, the molecular mechanisms underlying the development of gallbladder carcinoma remain largely unknown.

Skp2 is an F-box substrate-recognition subunit of the SCF ubiquitin-protein ligase complex, which regulates progression of the cell cycle by targeting regulators such as $p27^{(Kip1)}$ for ubiquitin-mediated degradation. Decreased levels of $p27^{(Kip1)}$ are thought to be associated with highly aggressive tumors and related to a poor prognosis in a variety of cancers[21,23-25].

In the current study, Skp2 expression was inhibited in GBC-SD cells by transfection of a Skp2 specific vector, namely, Skp2-RNAi. Consequently, cells in the S-phase of the cell cycle were increased, whereas cells in the G_2/M phase were decreased. No significant difference was observed in the proportion of cells present in the G_0/G_1 phase; thereby, the cell cycle was blocked in the S phase. Cell growth was significantly decreased in several *in vitro* experiments, suggesting that silencing Skp2 could markedly reduce cell proliferation and the group-dependent capability to form colonies.

In most tumors, deletion or mutation of *p27* rarely occurs, and its transcription is negligibly changed. Our research found that suppression of Skp2 had no effect on the mRNA expression of p27, but it was found to upreg-

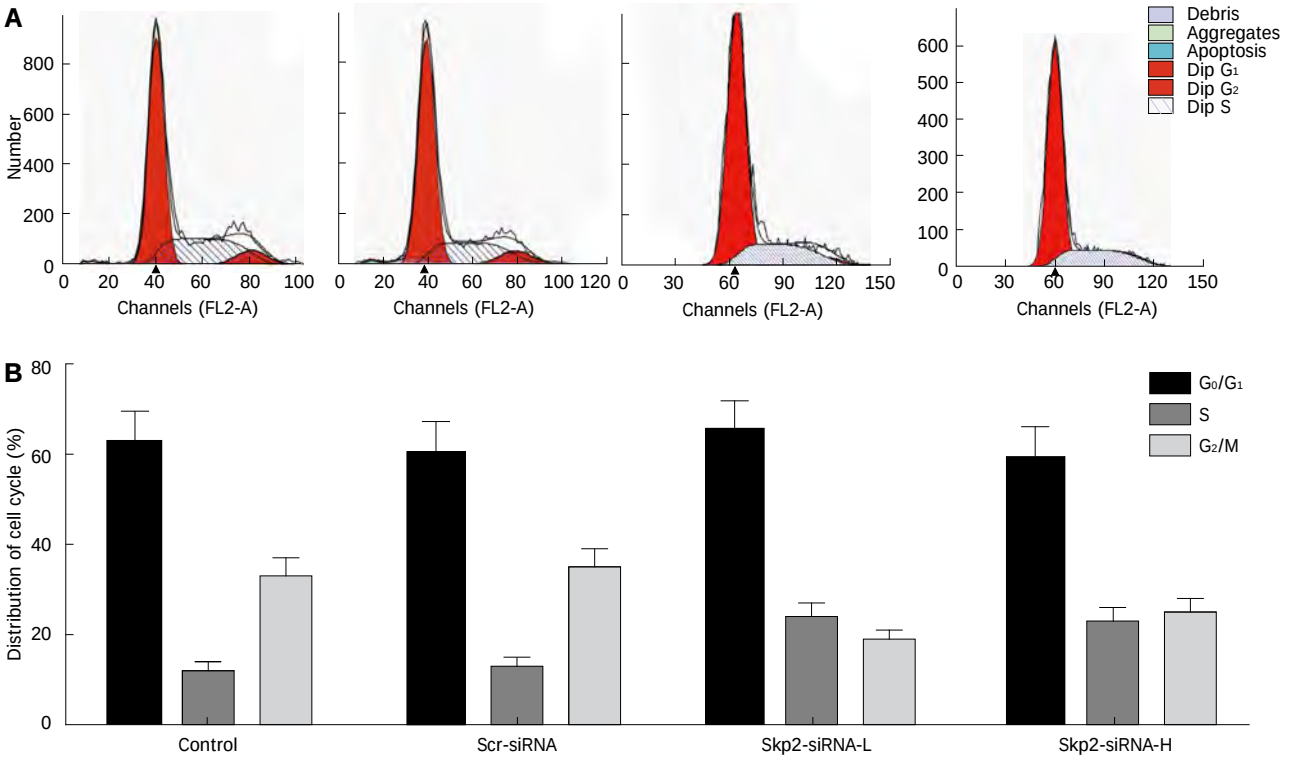

Figure 5 Proportion of cells in the cell cycle stages. A: Detection of the proportion of cells in the cell cycle stages after S-phase kinase-associated protein-2 (Skp2) expression was inhibited; B: The proportion of cells in the S phase of the cell cycle increased, and the proportion of cells in the G_2/M phase decreased in Skp2-RNAi-L and Skp2-RNAi-H groups.

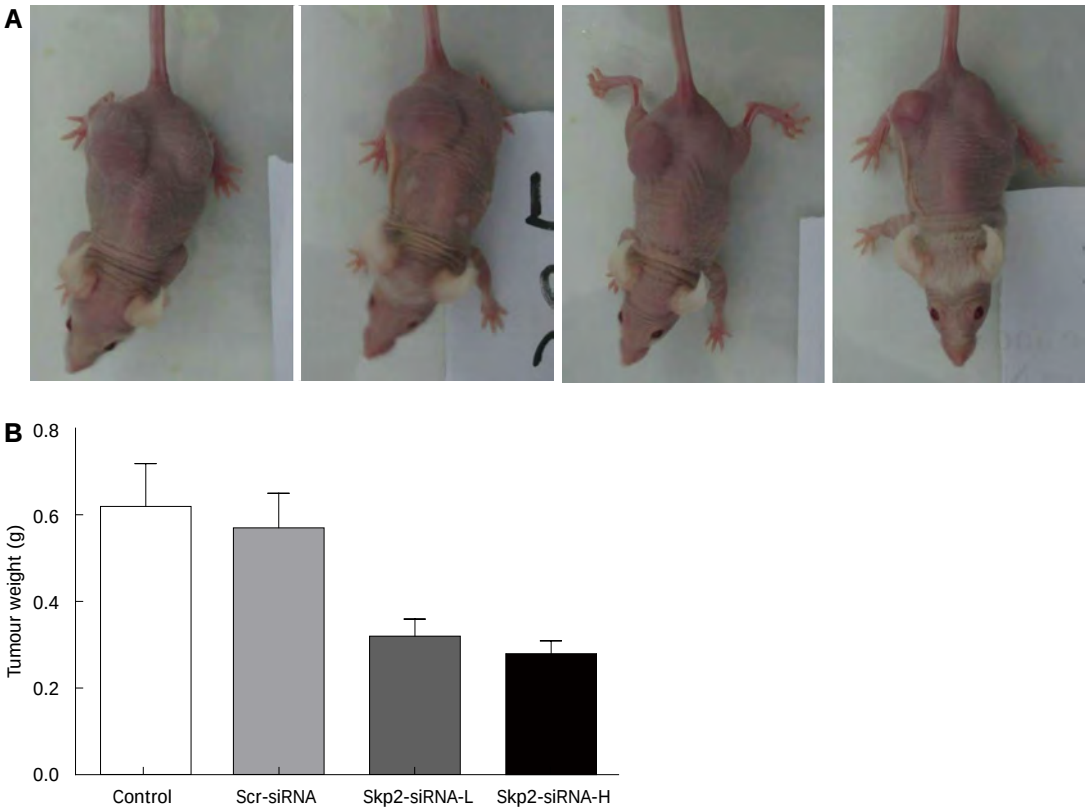

Figure 6 Tumorigenicity experiments in nude mice. A: Tumorigenicity experiments; B: Transfection with S-phase kinase-associated protein-2 (Skp2)-RNAi inhibited the growth of tumor cells.

ulate p27's protein expression. This observation suggested that regulation of gallbladder carcinoma proliferation by Skp2-siRNA is dependent on p27 protein expression, but not expression at the gene level. Nuclear polyubiquitination of $p27^{(Kip1)}$ is dependent on Skp2 and phosphorylation of $p27^{(Kip1)}$ at threonine 187. However, Hara *et al*[26] found that polyubiquitination activity was also detected in the cytoplasm of $Skp2^{(-/-)}$ cells, even with a threonine 187 to alanine 187 mutant of $p27^{(Kip1)}$ as the substrate. This outcome suggested that the polyubiquitination activity in the cytoplasm might contribute to an early phase of $p27^{(Kip1)}$ degradation in a Skp2-independent manner.

In addition to inducing the degradation of $p27^{(Kip1)}$ and promoting cellular proliferation, Skp2 also plays an important role in tumor invasiveness and metastasis. The numbers of migrated cells in the two Skp2-RNAi treated groups were found to be significantly fewer than those in the control groups. This observation suggested that Skp2-RNAi could inhibit the proliferation, migration and invasiveness of GBC-SD cells. We also studied the antitumor effect of Skp2-RNAi on nude mice in tumorigenicity experiments; the weights of the resulting tumors were decreased. This outcome suggested that treatment with Skp2-RNAi repressed the growth of metastatic tumors *in vivo*. Furthermore, the inhibition was shown to be positively associated with the dose of the lentivirus used. Hung *et al*[27] established Skp2-overexpressing stable transfectants in A549 human lung cancer cells and found that these stable transfectants exhibited increased migratory and invasive capabilities. Additionally, the expression of matrix metalloproteinase-2 (MMP-2) and MMP-9 were up-regulated and neutralization of these two MMPs using antibody-mediated approaches reduced cellular invasion. These data suggest that Skp2 promoted both tumor growth and metastasis and that enhanced expression of both MMP-2 and MMP-9 may have provided a contributory mechanism.

Moreover, Skp2 and $p27^{(Kip1)}$ have been shown to be useful indicators of prognosis[28-31]. Sanada *et al*[21] reported that Skp2 and $p27^{(Kip1)}$ were independent predictors of poor prognosis in patients with biliary tract cancers (BTCs). Discrepancies between *SKP2* DNA copy number and the level of Skp2 protein were observed, although a correlation was found between copy number and protein expression in some primary BTCs. Therefore, it is formally possible that Skp2 protein expression could be considered a more accurate prognostic marker for BTCs than *SKP2* gene copy number. Hashimoto *et al*[32] revealed that low levels of protein expression of $p27^{(Kip1)}$ and high Skp2 were associated with aggressive tumor behavior and that both $p27^{(Kip1)}$ and Skp2 could be considered useful markers in predicting the outcome of patients with intrahepatic cholangiocarcinomas. In an immunohistochemical study of 62 cases using tissue microarray, Li *et al*[20] confirmed that Skp2 over-expression represented the most significant independent adverse prognostic indicator in gallbladder carcinoma. Beyond the prognostic importance of Skp2/$p27^{(Kip1)}$, the development of drugs targeting Skp2 may provide novel molecular therapeutic approaches.

In summary, the results from our studies support the idea that Skp2 inhibitors and/or Skp2 regulatory sequences could provide a useful therapeutic protocol for the treatment of gallbladder carcinoma. In the future, the role of Skp2/$p27^{(Kip1)}$ in gallbladder carcinoma can be expected to be gradually unveiled.

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COMMENTS

Background

Primary gallbladder carcinoma is a common biliary malignancy, with a poor prognosis. p27 and S-phase kinase-associated protein-2 (Skp2) may play an important role in tumorigenesis and tumor development, and are closely associated with prognosis.

Research frontiers

Inhibition of Skp2 or over-expression of $p27^{(Kip1)}$ could inhibit tumor growth and induce apoptosis.

Innovations and breakthroughs

The authors explored the effect of Skp2-RNAi on GBC-SD cells, and found that suppression of the *Skp2* gene inhibited proliferation, migration and invasiveness of GBC-SD cells by mechanisms dependent on enhanced expression of p27 protein.

Applications

The results indicated that Skp2 inhibitors and/or Skp2 regulatory sequences such as Skp2-RNAi could provide a useful therapeutic protocol for the treatment of gallbladder carcinoma.

Peer review

The authors used several assays to explore the role of Skp2 in gallbladder carcinoma. The results indicated that Skp2-RNAi might provide a useful therapeutic protocol for the treatment of gallbladder carcinoma.

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ORIGINAL ARTICLE

Tumor necrosis factor-α mediates JNK activation response to intestinal ischemia-reperfusion injury

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Abstract

AIM: To investigate whether tumor necrosis factor-α (TNF-α) mediates ischemia-reperfusion (I/R)-induced intestinal mucosal injury through c-Jun N-terminal kinase (JNK) activation.

METHODS: In this study, intestinal I/R was induced by 60-min occlusion of the superior mesenteric artery in rats followed by 60-min reperfusion, and the rats were pretreated with a TNF-α inhibitor, pentoxifylline, or the TNF-α antibody infliximab. After surgery, part of the intestine was collected for histological analysis. The mucosal layer was harvested for RNA and protein extraction, which were used for further real-time polymerase chain reaction, enzyme-linked immunosorbent assay and Western blotting analyses. The TNF-α expression, intestinal mucosal injury, cell apoptosis, activation of apoptotic protein and JNK signaling pathway were analyzed.

RESULTS: I/R significantly enhanced expression of mucosal TNF-α at both the mRNA and protein levels, induced severe mucosal injury and cell apoptosis, activated caspase-9/caspase-3, and activated the JNK signaling pathway. Pretreatment with pentoxifylline markedly downregulated TNF-α at both the mRNA and protein levels, whereas infliximab pretreatment did not affect the expression of TNF-α induced by I/R. However, pretreatment with pentoxifylline or infliximab dramatically suppressed I/R-induced mucosal injury and cell apoptosis and significantly inhibited the activation of caspase-9/3 and JNK signaling.

CONCLUSION: The results indicate there was a TNF-α-mediated JNK activation response to intestinal I/R injury.

Key words: Tumor necrosis factor-α; Intestine; Mucosa; Apoptosis; c-Jun N-terminal kinase

Core tip: Ischemia-reperfusion (I/R) injury is a critical physiopathological phenomenon wherein further damage may occur when the blood supply of ischemic organs is recovered, and the mechanism of I/R remains unclear. This paper demonstrates that tumor necrosis factor-α (TNF-α) played a pivotal role in intestinal I/R injury, and pretreatment with the TNF-α inhibitor pentoxifylline or the TNF-α antibody infliximab remarkably attenuated I/R-induced injury by inhibiting TNF-α-mediated apoptosis and c-Jun N-terminal kinase (JNK) activation. The results of the study indicate there is a TNF-α-mediated JNK activation response to intestinal I/R injury.

Yang Q, Zheng FP, Zhan YS, Tao J, Tan SW, Liu HL, Wu B. Tumor necrosis factor-α mediates JNK activation response to intestinal ischemia-reperfusion injury. *World J Gastroenterol* 2013; 19(30): 4925-4934 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/4925.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.4925

INTRODUCTION

Intestinal ischemia-reperfusion (I/R) not only damages the local intestinal mucosa but also induces remote organic injury. I/R injury may occur in numerous situations, such as small bowel transplantation, strangulated hernias, neonatal necrotizing enterocolitis, cardiopulmonary bypass surgery and hypovolemic/septic shock[1]. A series of factors, including reactive oxygen species (ROS) production, calcium overload, neutrophil infiltration and cytokines release, are involved in I/R injury. Among these mediators, tumor necrosis factor-α (TNF-α), as an initial factor of the inflammatory reaction in I/R injury, is thought to play a pivotal role[2]. Prophylactic anti-TNF-α treatment may be an effective therapeutic strategy for preventing I/R-induced injury, as has been demonstrated by some studies[3]. TNF-α is thought to initiate three signaling pathways involved in cell injury and apoptosis: the apoptotic signaling pathway, the c-Jun N-terminal kinase (JNK) signaling pathway and the nuclear factor kappa-B (NF-κB) signaling pathway[4]. However, the role of TNF-α in I/R-induced injury and its mechanisms remain to be elucidated. Previous studies[5,6] demonstrated that apoptosis is a major mode of cell death caused by I/R, whereas the effect of TNF-α-mediated signaling pathways in cell apoptosis requires further exploration.

Pentoxifylline, a TNF-α inhibitor, has been investigated for a long time in I/R injury, but its effects on I/R-induced intestinal apoptosis and apoptotic pathways remain to be evaluated. Recent studies also have suggested that infliximab, a TNF-α antibody, attenuates I/R-induced injury[7]. However, it is still poorly understood whether infliximab alleviates the intestinal mucosal injury by downregulating apoptotic signaling or has acts *via* some other mechanisms.

The aims of this study were to determine: (1) whether inhibition of TNF-α ameliorates I/R-induced intestinal mucosal injury by suppressing cell apoptosis; (2) whether TNF-α is involved in a caspase-dependent apoptotic signaling in intestinal I/R injury; and (3) whether the JNK signaling pathways are activated by TNF-α in response to cell apoptosis in intestinal I/R injury.

MATERIALS AND METHODS

Animals and surgery

The experimental protocol and design were approved by the Sun Yat-sen University Animal Experimentation Committee and performed according to the Sun Yat-sen University Guidelines for Animal Experimentation. Male Sprague-Dawley rats (approximately 200-250 g) were used in this study. The animals were housed in wire-bottomed cages placed in a room illuminated from 8:00 AM to 8:00 PM (12:12-h light-dark cycle) and maintained at 21 ℃ ± 1 ℃. The rats were allowed access to water and chow ad *libitum*. The rats were anaesthetized for 3 h by intraperitoneal injection of 4% chloral hydrate (200 mg/kg). A laparotomy was performed under chloral hydras anesthesia, and the superior mesenteric artery (SMA) was occluded with a micro bulldog clamp. At the end of the ischemic period, the clamp was released, and three drops of lidocaine were applied directly onto the SMA to facilitate reperfusion. After the experiment, the animals were euthanized, and then, the entire small intestine was carefully removed and placed on ice. The oral 10-cm segment (duodenum) was removed, and the rest of the intestine was divided into two equal segments, representing the proximal (jejunum) and distal (ileum) segments. Each segment was rinsed thoroughly with physiological saline. Jejunal and ileal pieces, approximately 2 cm in length, were removed from the middle portion of each segment and fixed in 10% neutral-buffered formalin for the measurement of mucosal injury, terminal deoxynucleotidyl transferase-mediated dUDP-biotin nick-end labeling (TUNEL) assay, and immunohistochemistry of caspase-3. The remainder of the segment was opened longitudinally on the antimesenteric border to expose the intestinal mucosa. The mucosal layer was harvested by gentle scraping using a glass slide.

Experimental design and animal pretreatment

To investigate mucosal injury after I/R, the SMA was occluded for 60 min followed by 60-min reperfusion (I/R), and pretreatment with vehicle (1 mL of physiological saline) for 60 min prior to I/R. To evaluate the effect of pentoxifylline, a TNF-α inhibitor, on mucosal injury in the small intestine after I/R, the animals were pretreated with 50 mg/kg pentoxifylline (Sigma, St Louis, MI, United States), dissolved in 1 mL of physiological saline, by intraperitoneal injection 60 min prior to I/R. Similarly, an infliximab (Janssen Biotech, Horsham, PA) dose of 5 mg/kg dissolved in 1 mL of physiological saline was administered by intraperitoneal injection 60 min prior to I/R to evaluate the effect of infliximab pretreatment. In sham-operated (SO) rats, pretreatment was performed with vehicle for 60 min, and then, the SMA was isolated in a similar manner but was not occluded. Six rats were studied in each group.

RNA extraction and real-time polymerase chain reaction

RNA was extracted from 100 mg of mucosal scrapings using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) per the manufacturer's instructions. First-strand cDNA was synthesized from 1.5 μg of total RNA using a ReverTra Ace kit (Toyobo, Japan) per the manufacturer's instructions. An ABI Prism 7000 sequence detection system (Applied Biosystems, Bedford, MA) was then used for real-time polymerase chain reaction (PCR) experiments to quantitate the gene expression of TNF-α and β-actin for each sample. The reactions were performed in a 20-μL volume with TaKaRa Taq™ (TaKaRa, Japan).

The PCR conditions included a denaturation step at 94 ℃ for 5 min. Amplification was conducted for 35 cycles (denaturation at 94 ℃ for 30 s, annealing at 60 ℃ for 30 s, and extension at 72 ℃ for 30 s). The quantification was performed by using 7000 SDS instrument software (Applied Biosystems) for the relative quantification of gene expression. The primer sequences used were as follows: TNF-α forward primer, 5'-CACCACGCTCTTCTGTCT ACT-3'; TNF-α reverse primer, 5'-AGATGATCT-GAGTGTGAGGGTC-3'; β-actin forward primer, 5'-GAAATCGTGCGTGACATCAAAG-3'; and β-actin reverse primer, 5'-TGTAGTTTCATGGATGCCA-CAG-3'. The primers were supplied by Invitrogen. The results are expressed as the fold change in mRNA expression compared with the levels in sham-operated rats.

Purification of proteins

The mucosal scraping samples were immediately washed twice with ice-cold PBS (pH 7.4) and then homogenized in total protein extraction buffer. This extraction buffer consisted of 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.1% SDS, 5 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.5 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leuptin and 1.8 mg/mL iodoacetamide. The tissues were homogenized and lysed at 4 ℃ for 30 min and then centrifuged at 14000 *g* for 20 min at 4 ℃. The resulting supernatants were purified total proteins. The supernatants were divided into multiple samples and stored at -80 ℃. The protein concentrations were determined using a kit (Bio-Rad, Hercules, CA, United States).

Enzyme-linked immunosorbent assay

The TNF-α concentration of intestinal mucosa was measured using a commercial kit (eBioscience, San Diego, CA, United States), according to the manufacturer's instructions. Briefly, the enzyme-linked immunosorbent assay (ELISA) plates were coated with 100 μL/well of capture antibody diluted in coating buffer and incubated overnight at room temperature (RT). The plates were washed with wash buffer and blocked for 1 h at RT with 200 μL/well assay diluent. Then, the TNF-α standard and samples (100 μL) were pipetted into appropriate wells. Next, the plates were sealed and incubated at RT for 2 h. After washing, 100 μL of detection antibody was added to each well, sealed, and incubated for 1 h at RT. After washing, 100 μL of substrate solution was added to each well and incubated for 30 min at RT in the dark. Stop solution (2 mol/L H_2SO_4, 50 μL/well) was added, and the plates were read at 450 nm (570 nm correction) on a MicroPlate Reader (BioTek, Seattle, WA, United States). The results are expressed as pg TNF-α/mg protein.

Morphological analysis and mucosal injury score

After the animals were sacrificed, the tissue samples removed from the jejunum and ileum were immediately fixed in 10% neutral-buffered formalin, embedded in paraffin and sectioned. The sample sections were processed with hematoxylin-eosin staining and examined by light microscopy, according to the criteria described by Chiu *et al*[8] as follows: grade 0, normal mucosa; grade 1, development of subepithelial (Gruenhagen) spaces near the tips of the villi with capillary congestion; grade 2, extension of the subepithelial space with moderate epithelial lifting from the lamina propria; grade 3, significant epithelial lifting along the length of the villi with a few denuded villous tips; grade 4, denuded villi with exposed lamina propria and dilated capillaries; and grade 5, disintegration of the lamina propria, hemorrhage, and ulceration. Twenty visual fields at × 100 magnification were evaluated for each sample slide, and the final injury scoring was a gross assessment of the degree of mucosal damage. All slides were evaluated by two examiners in a blinded fashion.

TUNEL assay and apoptotic index analysis

The sample sections were used to detect cell apoptosis. The fragmented DNA of apoptotic cells was stained via the TUNEL method by using an in situ cell death detection kit (Roche, Switzerland). The apoptotic index was calculated in a minimum of 20 randomly selected crypts and analyzed in six separate samples. The apoptotic index was determined by dividing the number of apoptotic cells by the total number of cells in the crypt column and multiplying by 100.

Immunohistochemistry

Sample sections were used for caspase-3 immunohistochemical staining. The sections were deparaffinized and rehydrated prior to antigen retrieval by using EDTA (pH 8.0) for 3 min at 130 ℃. The sections were incubated for 10 min with 5% BSA prior to incubation with caspase-3 antibody (1:400, Cell signaling technology, Danvers, MA, United States) at 4 ℃ overnight. Subsequently, the sections were incubated at 37 ℃ for 30 min with anti-rabbit IgG (1:400; Santa Cruz Biotechnology, Santa Cruz, CA). Antigen-antibody complexes were visualized by staining with a DAB kit (Dako, Denmark). The slides were then counter-stained with hematoxylin for 1 min and mounted. The negative controls were created by omitting the primary antibody.

Western blotting analysis

Apoptotic proteins (caspase-9 and caspase-3) were analyzed by Western blotting. Equal quantities (20 μg) of lysates were electrophoresed in an SDS-PAGE gel and then transferred onto a nitrocellulose membrane (Bio-Rad). After blocking with PBS containing 0.1% polyoxyethylene sorbitan monolaurate (Tween 20, Sigma) and 5% skim milk for 1 h, the membrane was incubated with a rabbit polyclonal anti-TNF-α antibody (1:500; cell signaling technology), a mouse polyclonal anti-caspase-9 antibody (1:1000; cell signaling technology), a rabbit polyclonal anti-caspase-3 antibody (1:1000; cell signaling technology), a rabbit polyclonal anti-JNK antibody (1:1000; cell signaling technology), a rabbit polyclonal anti-p-JNK antibody (1:500; cell signaling technology), a rabbit polyclonal anti-c-Jun antibody (1:1000; Cell signaling technology), and a rabbit polyclonal anti-p-c-Jun antibody (1:500;

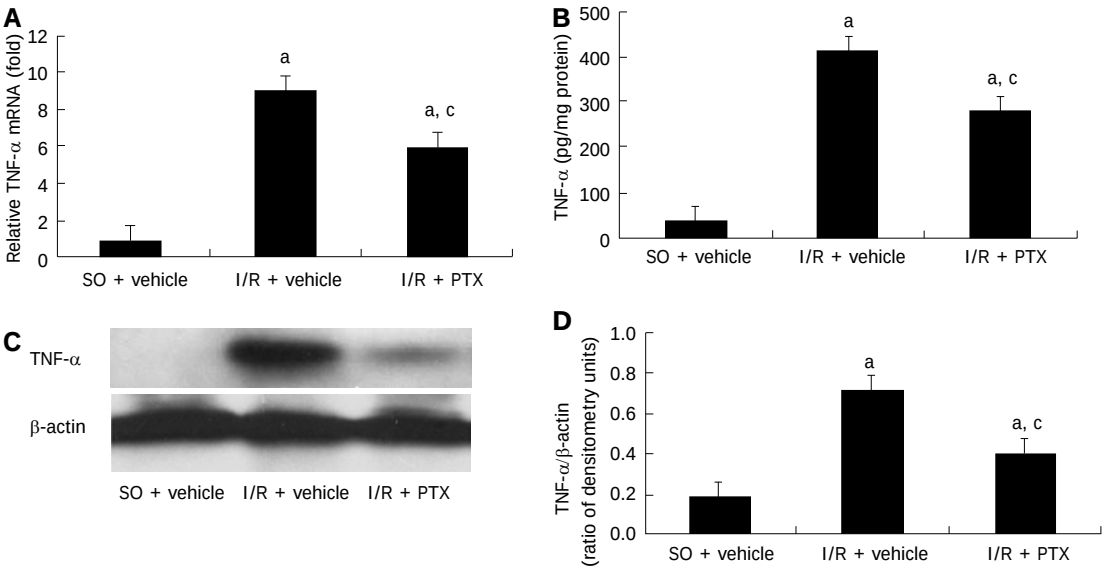

Figure 1 Ischemia-reperfusion induced intestinal mucosal tumor necrosis factor-α expression. A: Small intestinal mucosal tumor necrosis factor-α (TNF-α) mRNA expression; B: ELISA analysis of small intestinal mucosal TNF-α protein expression; C: Western blotting analysis of small intestinal mucosal TNF-α protein expression; D: The results of Western blotting analysis were expressed as a ratio to β-actin densitometry units. A TNF-α inhibitor, pentoxifylline (PTX), significantly suppressed the TNF-α mRNA and protein expression. Values are mean ± SE. Six rats were tested in each group. [a]*P* < 0.05 *vs* sham-operation (SO) rats pretreated with vehicle (SO + vehicle), [c]*P* < 0.05 *vs* ischemia-reperfusion (I/R) rats pretreated with vehicle (I/R + vehicle).

Cell signaling technology) at 4 ℃ overnight. Antigen-antibody complexes were detected with horseradish peroxidase-conjugated anti-rabbit IgG (1:6000; Santa Cruz Biotechnology) or anti-mouse IgG (1:6000; Santa Cruz Biotechnology). Detection of chemiluminescence was performed using ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, United States). The densitometric assessment of bands from the autoradiogram was performed using Image Gauge VDS (Fujifilm, Tokyo, Japan). Band intensities were quantified by measuring the absolute integrated optical intensity, which estimates the band in the lane profile. The results are expressed as the ratios of β-actin densitometry units.

Statistical analysis

Ranked data (mucosal injury scoring) are presented as the median and range, and the significance between groups was tested with the Wilcoxon rank test. Other results are expressed as the mean ± SE. Data were evaluated by one-way Analysis of Variance, and multiple comparisons were performed using the method of least significant difference *t* test. Differences were considered significant if $P < 0.05$.

RESULTS

I/R induced TNF-α expression in rat small intestines

Real-time PCR, ELISA and Western blotting analysis were conducted to evaluate the expression of TNF-α after intestinal I/R and the effect of pretreatment with pentoxifylline on the expression of TNF-α. The results are shown in Figure 1. A small amount of TNF-α was detected in sham-operated rats. Compared with the sham-operated rats, the amount of intestinal mucosal TNF-α at both the mRNA and protein levels was significantly increased after I/R, and this increase of TNF-α was markedly inhibited by pretreatment with pentoxifylline.

Suppression of TNF-α alleviated I/R-induced mucosal injury

The hematoxylin-eosin staining of jejunum sections is shown in Figure 2. Samples from sham-operated rats pretreated with the vehicle alone displayed an intact mucosal structure, whereas the intestinal I/R induced apparent mucosal damage, extensive epithelial layer damage, disintegration of lamina propria and hemorrhage. In contrast, pretreatment with pentoxifylline or infliximab attenuated the I/R-induced injury. In the ileum, the results were similar to those observed in the jejunum (data not shown). The mucosal injury score is shown in Table 1, and the results indicated that pretreatment with pentoxifylline or infliximab markedly attenuated I/R-induced intestinal mucosal injury, compared with I/R pretreatment with vehicle.

Repression of TNF-α attenuated mucosal cell apoptosis after intestinal I/R

The effect of TNF-α on mucosal cell apoptosis was investigated. The results of TUNEL staining of the jejunum indicated that few apoptotic cells were observed at the villus tips in the sham-operated rats, which is consistent with physiological apoptosis during the renewal of intestinal epithelium. Compared with the sham-operated rats, marked destruction of the jejunum structure and increased staining signal of apoptotic cells were observed in I/R rats. Pretreatment with either pentoxifylline or infliximab reduced the destruction of the structure in the jejunum and decreased the number of apoptotic cells (Figure 3A-D). The jejunum mucosal apoptotic index in each

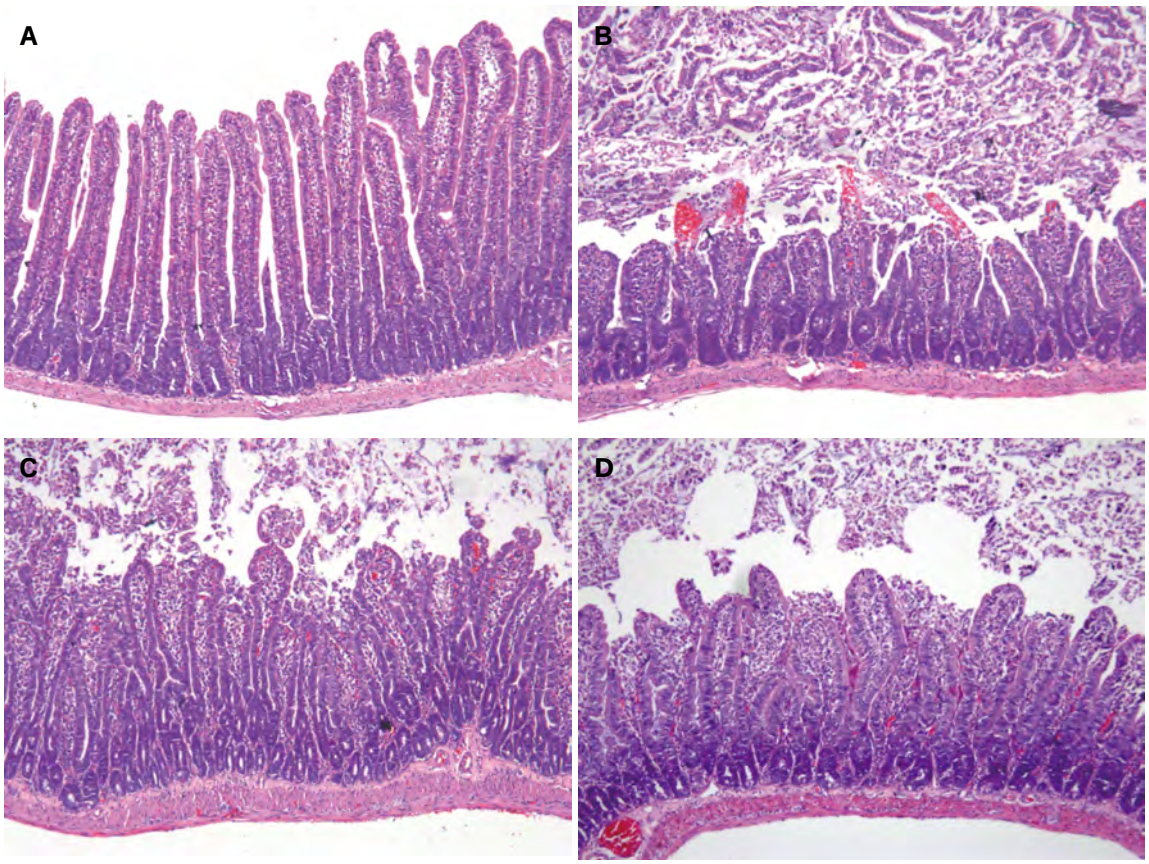

Figure 2 Suppression of tumor necrosis factor-α alleviated ischemia-reperfusion-induced small intestinal injury. A: Sham-operation pretreated with vehicle; B: Ischemia-reperfusion (I/R) pretreated with vehicle. C: I/R pretreated with a tumor necrosis factor-α (TNF-α) inhibitor pentoxifylline; D: I/R pretreated with a TNF-α antibody infliximab; Representative sections of jejunum for hematoxylin and eosin staining (× 100) were showed. Six rats were studied in each group, and a similar pattern was seen in six different rats in each group.

Table 1 Inhibition of tumor necrosis factor reduced mucosal injured scoring after intestinal ischemia-reperfusion

Group	Mucosal injured scoring	
	Jejunum	Ileum
SO rats: pretreated with vehicle	0.0 (0-1)	0.0 (0-1)
I/R rats: pretreated with vehicle	5.0 (4-5)[a]	4.5 (4-5)[a]
I/R rats: pretreated with PTX	3.0 (2-4)[a, c]	3.0 (2-4)[a, c]
I/R rats: pretreated with IFX	3.5 (2-4)[a, c]	3.0 (2-4)[a, c]

Values are expressed as the median (range). Six rats were tested in each group. [a]$P < 0.05$ *vs* sham-operated (SO) rats pretreated with vehicle, [c]$P < 0.05$ *vs* ischemia-reperfusion (I/R) rats pretreated with vehicle. PTX: Pentoxifylline; IFX: Infliximab.

group is shown in Figure 3E. Pretreatment with pentoxifylline or infliximab significantly attenuated the apoptotic index after intestinal I/R. The results in the ileum were similar to those observed in the jejunum (data not show).

To confirm the cell apoptotic death, immunohistochemistry staining of caspase-3 was performed. The results are shown in Figure 4. In the sections from the sham-operated rats, few caspase-3-positive cells were observed at the villus tips. Samples from I/R-treated rats displayed intense and extensive positive staining for caspase-3. The number of caspase-3-positive cells was markedly reduced in I/R rats pretreated with pentoxifylline or infliximab.

TNF-α mediated I/R-induced mucosal apoptosis via caspase activation in small intestine

Intestinal apoptotic proteins were analyzed by Western blotting assay, and the results are shown in Figure 5. In the sham-operated rats, only small amounts of cleaved caspase-9 and caspase-3 were detected in the small intestinal mucosa. In contrast, the expression of cleaved activated caspase-9 and caspase-3 were significantly increased after intestinal I/R. However, pretreatment with pentoxifylline or infliximab significantly suppressed the activation of those caspase proteins.

TNF-α mediated JNK activation response to intestinal I/R-induced injury

As shown in Figure 6, after intestinal I/R, the phosphorylation of JNK/c-Jun was significantly enhanced compared with that in sham-operated rats pretreated with the vehicle alone. Pretreatment with pentoxifylline markedly inhibited the phosphorylation of JNK/c-Jun, and pretreatment with infliximab also significantly decreased the activation of JNK/c-Jun. These results suggest that TNF-α mediated a JNK activation response to intestinal ischemia-reperfusion injury.

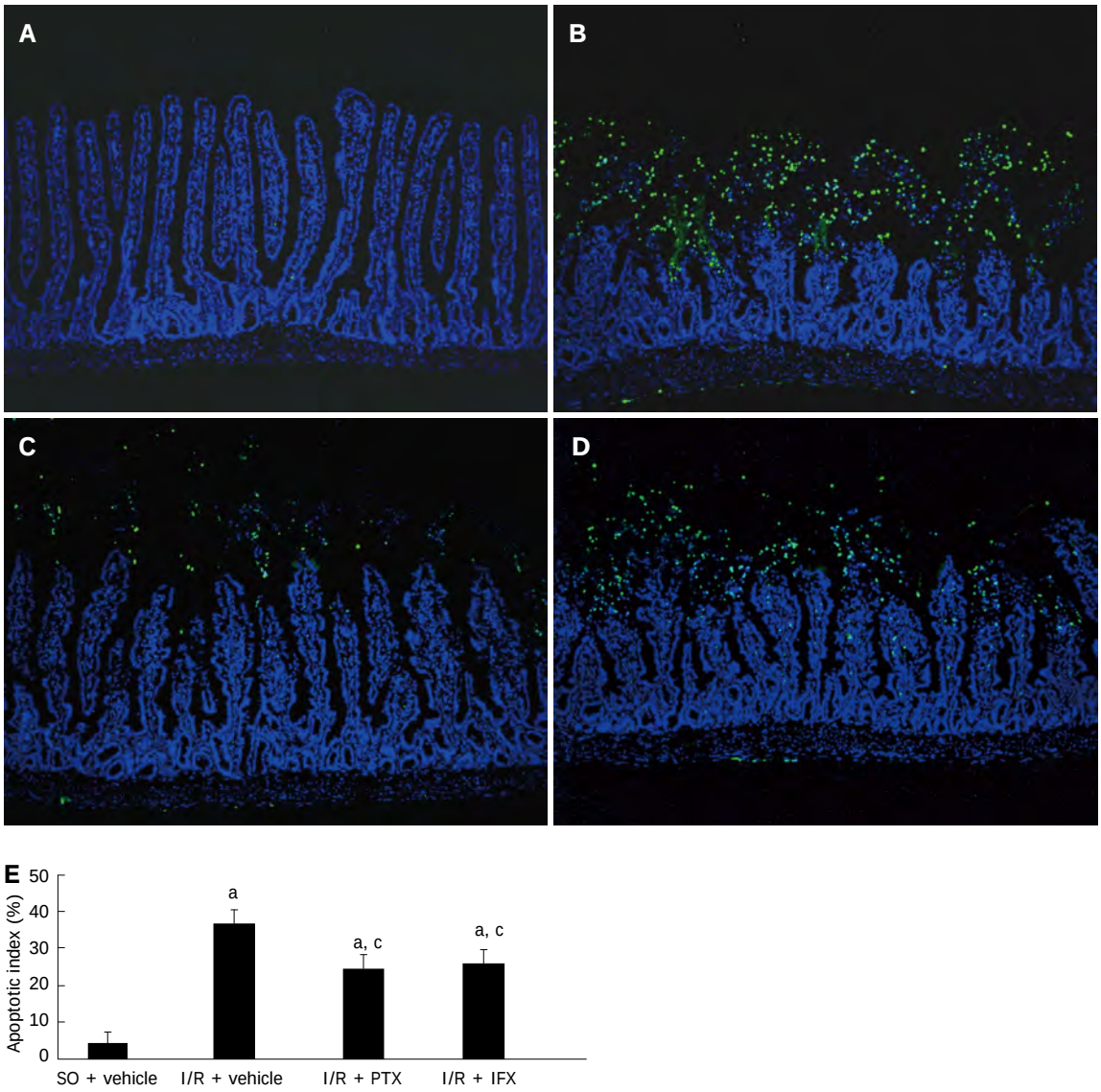

Figure 3 Suppression of tumor necrosis factor-α attenuated ischemia-reperfusion-induced intestinal mucosal apoptosis. A: Sham-operation (SO) pretreated with vehicle; B: Ischemia-reperfusion (I/R) pretreated with vehicle; C: I/R pretreated with a tumor necrosis factor-α (TNF-α) inhibitor pentoxifylline (PTX); D: I/R pretreated with a TNF-α antibody infliximab (IFX); E: The apoptotic index was calculated by counting a minimum of 20 randomly selected villi and crypts in the sections following terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining. The index was obtained by dividing the TUNEL positive cells by the total number of cells. Values are mean ± SE. Six rats were tested in each group. $^{a}P < 0.05$ *vs* SO rats pretreated with vehicle (SO + vehicle); $^{c}P < 0.05$ *vs* I/R rats pretreated with vehicle (I/R + vehicle). Apoptosis was assessed by TUNEL immunofluorescence staining. Mid-jejunum sections of rats were stained by TUNEL (green), with nuclei counterstained by 4',6-diamidino-2-phenylindole dihydrochloride (blue). Magnifications: × 100. Six rats were studied in each group, and a similar pattern was seen in six different rats in each group.

DISCUSSION

In tissue I/R injury, TNF-α is believed to be an early mediator. TNF-α is produced by a variety of cells, including macrophages, neutrophils, endothelia cells, nature killer cells and T/B lymphocytes[9,10]. The increased TNF-α may, in turn, augment the activation and action of those cells in I/R injury. TNF-α is involved in ROS production and the release of inflammation factors, such as interleukin-1, platelet-activating factor, and intercellular adhesion molecule[11]. TNF-α also mediates the injury of endothelia cells and the infiltration of neutrophils[12,13] and plays a pivotal role in I/R injury[14]. Subsequent studies have indicated that TNF-α mediates the injury induced by I/R, while inhibiting its function or expression supplied a protective effect[3]. However, some studies have demonstrated that TNF-α might be a protective factor in I/R-induced injury[15-17]. Because TNF-α has pleiotropic functions, researchers believe that after its interaction with TNF-receptor-1 and TNF-receptor-2, TNF-α activates pathways that favor both cell survival and apoptosis depending on the cell type and biological context.

Currently, two classic signal pathways are believed to mediate apoptotic signals: the caspase-8-mediated type- I apoptotic pathway and caspase-9-mediated type- II pathway. Caspase-3 ultimately executes the apoptotic signal. In a study on hepatic ischemia-reperfusion injury, the researchers demonstrated that TNF-α, but not Fas, medi-

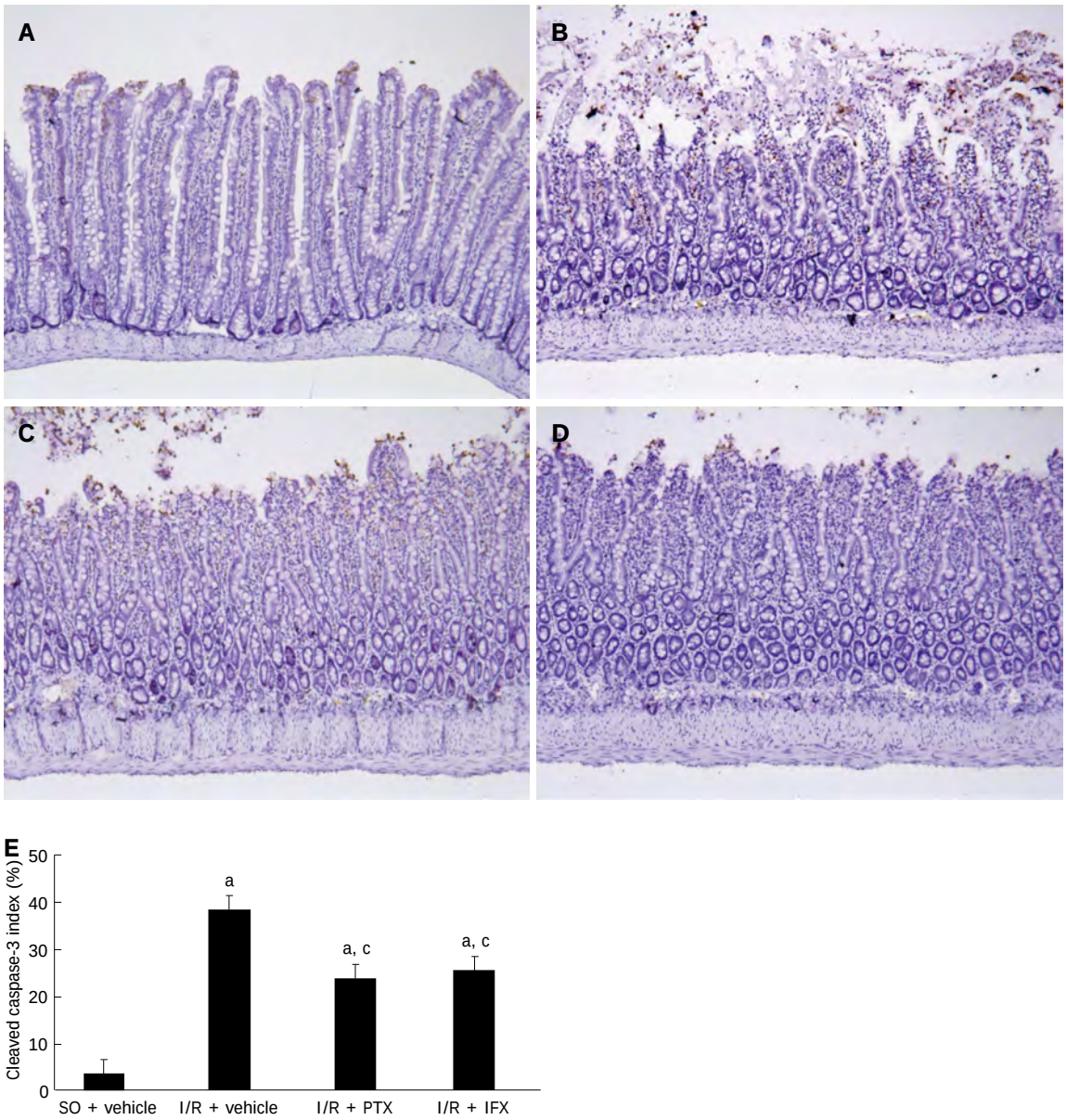

Figure 4 Suppression of tumor necrosis factor-α repressed caspase-3 activity in ischemia-reperfusion intestinal mucosa. A: Sham-operation (SO) pretreated with vehicle; B: Ischemia-reperfusion (I/R) pretreated with vehicle; C: I/R pretreated with a tumor necrosis factor-α (TNF-α) inhibitor pentoxifylline (PTX); D: I/R pretreated with a TNF-α antibody infliximab (IFX); Activated caspase-3 was assessed by immunohistochemical staining. Magnifications: × 100; E: The active caspase-3 index was calculated by counting a minimum of 20 randomly selected villi and crypts in the sections following cleaved caspase-3 staining. The index was obtained by dividing the cleaved caspase-3 positive cells by the total number of cells. Values are mean ± SE. Six rats were tested in each group. $^{a}P < 0.05$ *vs* SO rats pretreated with vehicle (SO + vehicle); $^{c}P < 0.05$ *vs* I/R rats pretreated with vehicle (I/R + vehicle). Six rats were studied in each group, and a similar pattern was seen in six different rats in each group.

ated apoptosis in hepatocytes[18]. Our previous study indicated that TNF-α mediated mucosal cell apoptosis in rat intestines that suffered venous congestion[19]. However, the effect of TNF-α is poorly understood in I/R-induced intestinal cell apoptosis.

TNF-α can activate both the stress-activated protein kinase cascade, including JNK[20,21], and the NF-κB signaling pathway[22,23]. Therefore, the stimulation of cells with TNF-α may lead to both pro-apoptotic and anti-apoptotic consequences. JNK is a member of the mitogen-activated protein kinases (MAPKs). It has been shown that JNK mediates inflammatory processes by inducing the expression of adhesion molecules and inflammatory chemokines. The activation of JNK is closely related to cell apoptosis in I/R-induced injury, and TNF-α is a strong stimulating factor[24,25]. Under I/R conditions, JNK is activated by the dual phosphorylation of threonine (Thr) and tyrosine (Tyr), and the phosphorylated-JNK translocates to the nucleus, where it phosphorylates and activates different transcription factors and transactivates target genes, including c-Jun[26]. Phosphorylation of c-Jun leads to the formation of AP-1, which is involved in the transcription of a wide variety of proteins, and some of them are known to be pro-apoptotic proteins[27].

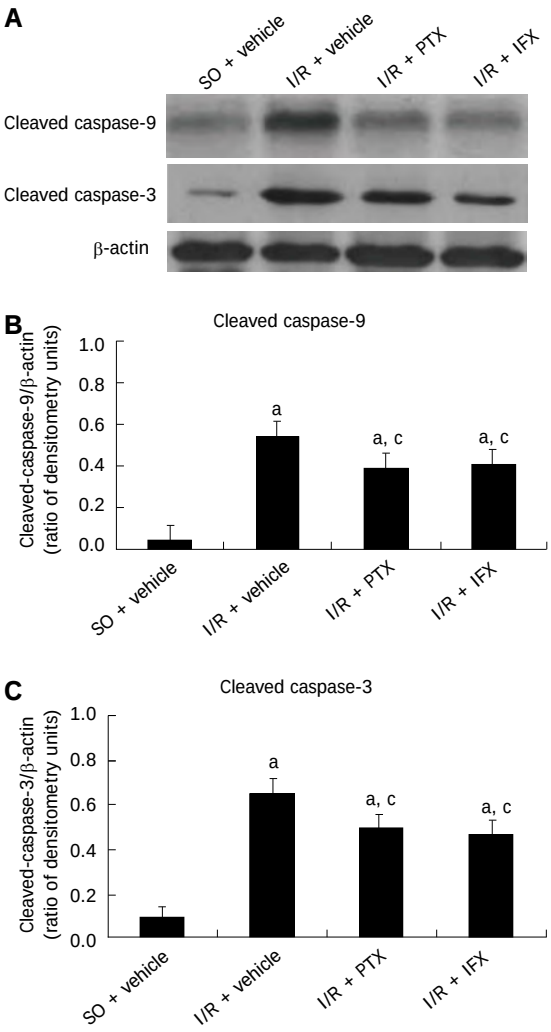

Figure 5 Tumor necrosis factor-α mediated ischemia-reperfusion-induced mucosal apoptosis *via* caspase activation in intestinal mucosa. Total protein was extracted from intestinal mucosa, and subjected to SDS-PAGE and Western blotting analysis. β-actin was used as the control for loading. The results were expressed as a ratio to β-actin densitometry units. A: Western blotting analysis; B: The ratio of cleaved caspase-9 and β-actin; C: The ratio of cleaved caspase-3 and β-actin. Values are mean ± SE. Six rats were tested in each group. [a]*P* < 0.05 *vs* sham-operation (SO) rats pretreated with vehicle (SO + vehicle); [c]*P* < 0.05 *vs* ischemia-reperfusion(I/R) rats pretreated with vehicle (ischemia-reperfusion + vehicle). PTX: Pentoxifylline; IFX: Infliximab.

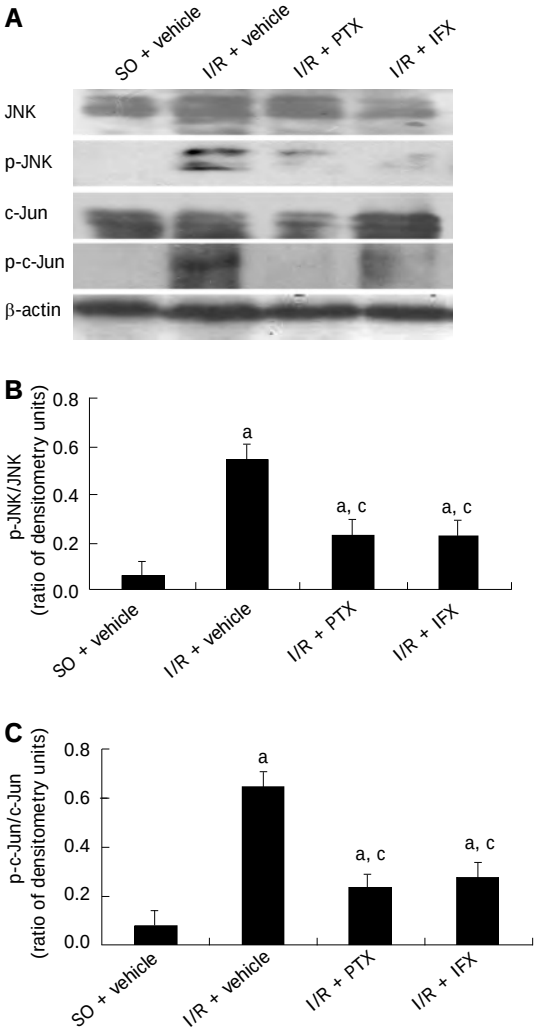

Figure 6 Tumor necrosis factor-α mediated c-Jun N-terminal kinase activation response to mucosal injury in ischemia-reperfusion-induced intestine. Equal quantities of protein were subjected to Western blotting analysis, and β-actin was used as the control for loading. The results were expressed as a ratio of densitometry units. A: Western blotting analysis; B: The ratio of p-c-Jun N-terminal kinase (JNK) and JNK; C: The ratio of p-c-Jun and c-Jun. Values are mean ± SE. Six rats were tested in each group. [a]*P* < 0.05 *vs* sham-operation(SO) rats pretreated with vehicle (SO + vehicle); [c]*P* < 0.05 *vs* ischemia-reperfusion (I/R) rats pretreated with vehicle (I/R + vehicle). PTX: Pentoxifylline; IFX: Infliximab.

Pentoxifylline has been proven to be a potent inhibitor of TNF-α production[28]. Recently, several studies have suggested that pretreatment with pentoxifylline in intestinal I/R not only attenuates the local intestinal injury but also improves the tolerance of the remote organ to I/R[29,30]. However, the specific effects of pentoxifylline on the cell apoptosis and apoptotic signal pathways in intestinal I/R are not clear. Infliximab, a chimeric TNF-α monoclonal antibody, has been shown to inhibit the function of TNF-α in a variety of studies[31]. Because infliximab is a potent antibody against TNF-α, capable of neutralizing all forms (extracellular, transmembrane, and receptor-bound) of TNF-α[32], it may also attenuate TNF-α-mediated injury in I/R. Few studies have been conducted that examine the effect of infliximab on I/R injury. Guven *et al*[33] suggested that infliximab exerted neuroprotective effects in a study involving an experimental spinal cord ischemic injury. A recent study indicated that infliximab might have protective effects in intestinal I/R injury because of its anti-inflammatory and antioxidant properties[7].

In this study, our data identified that I/R dramatically induced TNF-α expression at both the mRNA and protein levels using real time-PCR, ELISA and Western bloting analysis, and pretreatment with pentoxifylline significantly downregulated TNF-α expression. Morphological analysis indicated that I/R induced apparent intestinal mucosal injury, which was characterized by the appearance of hemorrhage, extensive epithelial disruption and disintegration of lamina propria. Pretreatment with pentoxifylline or infliximab significantly alleviated the injury, which was confirmed by mucosal injury scor-

ing. TUNEL assay and caspase-3 immunohistochemical staining demonstrated that I/R remarkably induced mucosal apoptosis, and pretreatment with pentoxifylline or infliximab suppressed cell apoptosis, thus contributing to the alleviation of injury. The observed apoptotic index values were also consistent with these results.

The Western blotting results showed that apoptotic proteins, including caspase-9 and caspase-3, were significantly activated after intestinal I/R, and the activation of caspases was suppressed by pretreatment with pentoxifylline or infliximab. The present data suggest that TNF-α mediates caspase-dependent mitochondrial apoptotic signaling. In addition, our data showed that the phosphorylation of JNK and c-Jun increased after intestinal I/R. When TNF-α was suppressed by pretreatment with pentoxifylline or infliximab, the phosphorylation of JNK and c-Jun, as well as intestinal apoptosis, were also significantly inhibited. The data suggest that the activation of the JNK signaling pathway is involved in TNF-α-mediated caspase-dependent apoptosis.

In summary, intestinal I/R dramatically induced TNF-α expression, and TNF-α activated the JNK signaling response to caspase-dependent apoptosis, resulting in intestinal injury. Clinically, a treatment involving inhibition of TNF-α by pentoxifylline or infliximab might ameliorate intestinal injury in I/R patients, and pentoxifylline has potential as a low-cost and efficacious anti-TNF-α compound.

COMMENTS

Background

Intestinal ischemia-reperfusion (I/R) injury may occur in numerous situations, such as small bowel transplantation, strangulated hernias, neonatal necrotizing enterocolitis, cardiopulmonary bypass surgery and hypovolemic/septic shock. A series of factors, including reactive oxygen species production, calcium overload, neutrophil infiltration and cytokine release, are involved in I/R injury. Among these mediators, tumor necrosis factor-α (TNF-α) acts as an initial inflammation factor in I/R injury and is believed to play a pivotal role. However, the exact mechanism of TNF-α in intestinal I/R is still not clearly understood.

Research frontiers

TNF-α is believed to initiate three signaling pathways: the apoptotic signaling pathway, c-Jun N-terminal kinase (JNK) signaling pathway and nuclear factor kappa-B signaling pathway. These pathways are all involved in cell injury and apoptosis. However, the role of TNF-α in I/R-induced injury and its mechanisms remain to be elucidated. The effect of TNF-α-mediated JNK signaling pathways on cell apoptosis requires further exploration.

Innovations and breakthroughs

The study demonstrated that I/R significantly enhanced expression of mucosal TNF-α at both the mRNA and protein levels, induced severe mucosal injury and cell apoptosis, and activated JNK. Pretreatment with the TNF-α inhibitor pentoxifylline or the TNF-α antibody infliximab dramatically suppressed I/R-induced mucosal injury and cell apoptosis and significantly inhibited the activation of caspase-9/3 and JNK. The results of this study indicate that TNF-α played a pivotal role in intestinal I/R injury, and TNF-α mediated a JNK activation response to intestinal I/R injury.

Applications

In the present study, the authors investigated the mechanism of TNF-α in I/R-induced intestinal injury, which will provide new insight into the pathogenesis of I/R injury. Moreover, the study indicated that treatment *via* inhibiting TNF-α by pentoxifylline or infliximab might ameliorate intestinal injury in I/R patients.

Terminology

TNF-α is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. It is produced chiefly by activated macrophages (M1), although it can be produced by many other cell types such as CD4+ lymphocytes, NK cells and neurons. JNK were originally identified as kinases that bind and phosphorylate c-Jun on Ser-63 and Ser-73 within its transcriptional activation domain. They belong to the mitogen-activated protein kinase family and are responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock. They also play a role in T-cell differentiation and the cellular apoptosis pathway.

Peer review

The authors have done some intriguing studies in rats in TNF-α mediated JNK activation response to intestinal ischemic reperfusion injury. This is very interesting and well written paper.

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BRIEF ARTICLE

Analysis of single nucleotide polymorphisms in the region of *CLDN2-MORC4* in relation to inflammatory bowel disease

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Abstract

AIM: To investigate a possible genetic influence of claudin (*CLDN*)*1*, *CLDN2* and *CLDN4* in the etiology of inflammatory bowel disease.

METHODS: Allelic association between genetic regions of *CLDN1*, *CLDN2* or *CLDN4* and patients with inflammatory bowel disease, Crohn's disease (CD) or ulcerative colitis were investigated using both a case-control study approach (one case randomly selected from each of 191 Swedish inflammatory bowel disease families and 333 controls) and a family-based study (463 non-Swedish European inflammatory bowel disease -families). A nonsynonymous coding single nucleotide polymorphism in *MORC4*, located on the same linkage block as *CLDN2*, was investigated for association, as were two novel *CLDN2* single nucleotide polymorphism markers, identified by resequencing.

RESULTS: A single nucleotide polymorphism marker (rs12014762) located in the genetic region of *CLDN2*

was significantly associated to CD (case-control allelic OR = 1.98, 95%CI: 1.17-3.35, *P* = 0.007). *MORC4* was present on the same linkage block as this CD marker. Using the case-control approach, a significant association (case control allelic OR = 1.61, 95%CI: 1.08-2.41, *P* = 0.018) was found between CD and a nonsynonymous coding single nucleotide polymorphism (rs6622126) in *MORC4*. The association between the *CLDN2* marker and CD was not replicated in the family-based study. Ulcerative colitis was not associated to any of the single nucleotide polymorphism markers.

CONCLUSION: These findings suggest that a variant of the *CLDN2-MORC4* region predisposes to CD in a Swedish population.

Key words: Crohn's disease; Genetic predisposition; Inflammatory bowel disease; Single nucleotide polymorphism; Tight junctions

Core tip: Tight junction proteins are key components in the regulation of paracellular permeability and therefore we considered claudin genes as candidate genes in the study. Association was identified between a single nucleotide polymorphism marker (rs12014762) in the *CLDN2-MORC4* region and the occurrence of Crohn's disease (CD) in a Swedish population. Additionally, a nonsynonymous coding single nucleotide polymorphism (rs6622126) in *MORC4* was associated to CD. Our findings add further support for a genetically impaired intestinal epithelial barrier as one predisposing factor in the etiology of CD.

Söderman J, Norén E, Christiansson M, Bragde H, Thiébaut R, Hugot JP, Tysk C, O'Morain CA, Gassull M, Finkel Y, Colombel JF, Lémann M, Almer S. Analysis of single nucleotide polymorphisms in the region of *CLDN2-MORC4* in relation to inflammatory bowel disease. *World J Gastroenterol* 2013; 19(30): 4935-4943 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/4935.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.4935

INTRODUCTION

Chronic inflammatory bowel disease (IBD) encompasses Crohn's disease (CD), ulcerative colitis (UC) and, in the absence of a confident diagnosis, unclassified colitis (IBD-U). Susceptibility to IBD and the broad spectrum of phenotypic expressions depends on contributions from environmental factors and a genetic predisposition. Several etiological factors have been suggested, including the presence of specific strains of commensal enteric bacteria, defective bacterial killing, aberrant regulation of innate and adaptive immune responses and an impaired intestinal barrier[1]. Consistent with this, several genetic associations in IBD have been described[2-8].

Both CD and UC have been associated with an increase in intestinal permeability[9-11]. Based on findings of an increased intestinal permeability among healthy first-degree relatives to CD patients, a role for a tight junction (TJ) based genetic contribution to permeability changes has been suggested as a predisposing susceptibility factor for CD[12]. This suggestion has been contradicted by other studies[13-15]. However, by defining a normal range of intestinal permeability in healthy controls, a subgroup of healthy first-degree relatives to CD patients with increased intestinal permeability has been identified[16]. An increased permeability response to acetylsalicylic acid has been observed in CD patients and their relatives, indicating hereditary factors underlying this responsiveness[14]. An increased permeability may be primary or a consequence of subclinical intestinal inflammation present as an inherited abnormality in relatives of CD-patients[17]. It is still unknown whether this disturbed permeability is caused by genetic or environmental factors, but several studies provide support for a genetic rather than environmental induced increase[18,19].

The barrier of epithelial cells, with their apical TJ-structure, is critical for the permeability properties of the intestine. The TJ-structure is a multicomponent protein complex that serves to seal and regulate permeability across the paracellular space between adjacent epithelial cells, with the family of membrane-spanning claudin-proteins as the major determinants[20,21]. Claudin-1 and claudin-4 have been associated with a tight TJ-structure whereas claudin-2 expression results in a more leaky epithelial layer[22-24]. Expressions of claudins, and other TJ-proteins, are subject to regulation by different cytokines[20]. Claudin-4 seems to be preferentially expressed in M-cells[25] and the dome area of the follicle-associated epithelium[26], which has been suggested to be the site of initial inflammation in ileal CD[27].

Our aim was to elucidate a possible genetic influence of tight junction-components to IBD-susceptibility and therefore we conducted genetic association studies using single nucleotide polymorphism (SNP) markers of the genetic regions of claudin 1 (*CLDN1*, chromosome 3q28-q29), claudin 2 (*CLDN2*, chromosome Xq22.3-q23) and claudin 4 (*CLDN4*, chromosome 7q11.23). Furthermore, in order to identify putative functional sequence variants of *CLDN2*, the promoter region, exon-intron boundaries and exons harbouring the 5' untranslated region and protein coding region were amplified and resequenced.

MATERIALS AND METHODS

Study subjects

The IBD-families in this study originated from the large European collaboration that lead to the discovery of *NOD2/CARD15* as a CD susceptibility gene[5,28]. Swedish IBD-patients were selected for inclusion in a case-control study, whereas the remaining non-Swedish families were used in a family based genetic association study (Table 1). Samples from an anonymized regional DNA bank con-

Table 1 General study outline

Study design[1]	Cohort and disease	Number of families	Number of individuals	Women
Case control study	Healthy unrelated controls		333	162
	Swedish IBD families	191		
	IBD		347	157
	CD		150	69
	UC		166	71
	IBD-U		31	17
Family based approach	Non-Swedish families	463		
	IBD		715	398
	CD		528	297
	UC		151	83
	IBD-U		36	18

[1]Including Claudin (*CLDN*)*1, CLDN2, CLDN4*. IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; IBD-U: Unclassified colitis.

sisting of randomly selected individuals (n = 800) living in the southeastern part of Sweden were used as controls in the case-control studies. The study was conducted under approval by the ethics committees of Linköping University (DNR 97271) and Karolinska Institutet (DNR 97-327).

SNP selection for genetic association studies

Linkage blocks were defined using SNP data from the HapMap CEPH collection and the SNPbrowser software version 3.5 (Applied Biosystems, Foster City, CA, United States) and a default value of 0.3 linkage disequilibrium units (LDU)[29]. SNP markers were selected for *CLDN1*, *CLDN2* and *CLDN4* (Table 2). A distance less than 1 LDU has been considered useful for allelic association[30]. SNP markers were also chosen from adjacent linkage blocks.

Genotyping

Allele discrimination was carried out using TaqMan SNP genotyping assays (Table 2) and TaqMan Genotyping Master Mix or TaqMan Fast Universal polymerase chain reaction (PCR) Master Mix without AmpErase UNG (Applied Biosystems), using either a 7300 Real-Time PCR System or a 7500 Fast Real-Time PCR System (Applied Biosystems). All genotype data were analyzed using the 7500 Fast System SDS Software version 1.3.1.21 (Applied Biosystems).

In addition, a nonsynonymous coding SNP in *MORC4* (rs6622126; Applied Biosystems assay ID C_22273025_10) and two novel *CLDN2* SNP markers (this study; rs62605981 and rs72466477) were genotyped. The two novel *CLDN2* sequence variants were ordered as custom assays from Applied Biosystems. Primer and probe sequences are available from the authors upon request.

Resequencing of CLDN2

The promoter region, exon-intron boundaries and exons harbouring the 5' untranslated region and protein coding region of *CLDN2* were amplified by PCR and resequenced (Table 3). PCR amplifications were in accordance with the manufacturer's recommendations, using HotStarTaq DNA polymerase (Qiagen, Hilden, Germany), 2.0 mmol/L $MgCl_2$, and 0.2 μmol/L per PCR primer (Operon Biotechnologies GmbH, Cologne, Germany, and Scandinavian Gene Synthesis AB, Köping, Sweden). The following PCR cycle was repeated 45 times: 94 ℃ for 30 s, 60 ℃ for 30 s, and 72 ℃ for 60 s. A total of 93 individuals (21 CD, 21 UC, 5 IBD-U, and 46 healthy; 60 females) were resequenced.

All PCR products were purified in accordance with the QIAquick PCR purification kit protocol (Qiagen), and analyzed using the DNA 1000 assay on the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, United States). Cycle sequencing was carried out using the CEQ8800 system and accompanying sequencing reagents from Beckman Coulter (Fullerton, CA, United States) and conducted using half concentrated CEQ DTCS quick start kit, purification with an ethanol-precipitation protocol. Sequence data were analyzed with the heterozygote detection option activated in the software (version 8.0.52) and relative a reference sequence. New sequence variants were deposited in the NCBI SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) and provided with rs-numbers.

Prediction of transcription factor binding sites

Transcription factor binding sites were predicted using the Alibaba 2.1 software[31] available through Biobase (www.gene-regulation.com), in conjunction with the Transfac database public release version 7.0[32] at Biobase.

Statistical analysis

Allelic OR with accompanying 95%CI, and P values based on χ^2 statistics were calculated using a likelihood-based analysis for genetic association with the Unphased software version 3.0.13[33], both in the case-control approach and in the family-based study. In order to avoid bias due to genetic relatedness in the case-control study, one case per family was randomly selected from 191 Swedish IBD families. These random samplings of cases were repeated 15 times and the median OR was used as a representative measure of association. The case-control studies with respect to IBD, CD and UC were based on 191, 103 and 102 cases of IBD, CD, and UC, respectively, and 333 controls. No deviations from Hardy-Weinberg equilibrium were observed.

Since *CLDN2-MORC4* are located in a non-pseudo-autosomal region of the X-chromosome, males contribute one allele and females two alleles. Because the analysis did not identify sex as a confounder, males and females were analyzed jointly. Association to clinical features was tested for using a chi-square test for qualitative variables and one way ANOVA for quantitative variables, and carried out using the GraphPad Prism 4 software (GraphPad Software, La Jolla, CA, United States). For all statistical

Table 2 Single nucleotide polymorphism markers in the genetic regions of *Claudin-1*, *Claudin-2* and *Claudin-4*

Candidate gene	SNP rs number[1]	MAF[2]	Assay ID[3]	Position in kbp relative candidate gene and location[4]	Coverage[5]
CLDN1	rs1491991	0.25	C_7550365_10	-66.3 (5′-flanking region)	*CLDN16*
	rs3732923	0.41	C_27509271_10	5.5 (intron 1)	*CLDN1* (from promoter until first two thirds of intron 1)
	rs3732924	0.29	C_8528578_10	5.6 (intron 1)	*CLDN1* (from promoter until first two thirds of intron 1)
	rs9848283	0.49	C_2057729_10	6.4 (intron 1)	*CLDN1* (from promoter until first two thirds of intron 1)
	rs12629166	0.47	C_2057718_10	13.8 (intron 3)	*CLDN1* (from second intron until 3′-flanking region)
	rs7620166	0.41	C_8528273_10	45.9 (3′-flanking region)	*CLDN1* (from second intron until 3′-flanking region)
	rs567408	0.42	C_1587588_10	94.5 (3′-flanking region)	intergenic block
	rs536435	0.42	C_1587674_10	155.3 (3′-flanking region)	*LOC391603*
CLDN2	rs4409525	0.34	C_382795_10	-23.3 (5′-flanking region)	*RIPPLY1*, *TBC1D8B*
	rs5917027	0.48	C_11771710_10	-1.0 (5′-flanking region)	*CLDN2*, *MORC4*
	rs12014762	0.21	C_2013132_20	20.0 (3′-flanking region)	*CLDN2*, *MORC4*
CLDN4	rs4131376	0.43	C_26657639_10	-56.9 (5′-flanking region)	*ABHD11*, *CLDN3*, *CLDN4*, *WBSCR27*, *WBSCR28*
	rs8629	0.18	C_7493975_10	0.3 (exon 1)	*ABHD11*, *CLDN3*, *CLDN4*, *WBSCR27*, *WBSCR28*

[1]Initially all of the selected single nucleotide polymorphism (SNP) markers were evaluated, using the Hapolview software 4.0[52], for linkage disequilibrium (LD) and association to inflammatory bowel disease (IBD) in a case-control study using a subset of Swedish IBD patients (73, 39 and 42 individuals with IBD, Crohn's disease (CD) or ulcerative colitis, respectively). Because of significant associations to CD, SNP markers for claudin (*CLDN)1* (rs7620166) and *CLDN2* (rs12014762) were chosen for further evaluation on the complete case-control. Even though non of the *CLDN4* SNP markers showed evidence of association to any of the disease categories rs8629 was included for further evaluation; [2]Minor allele frequency (MAF) according to SNP data from the HapMap CEPH collection; [3]TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA, United States); [4]With respect to each candidate gene, SNP positions are defined relative a genomic reference sequence. The first nucleotide of a reference cDNA sequence has been designated +1, with the preceeding genomic position being -1. The following reference sequences have been used for *CLDN1* NT_005612.15 (genomic) and NM_021101.3 (mRNA), *CLDN2* NT_011651.16 (genomic) and NM_020384.2 (mRNA) and *CLDN4* NT_007758.11 (genomic) and NM_001305.3 (mRNA); [5]Linkage blocks have been defined using SNP data from the HapMap CEPH collection and the SNP browser software with 0.3 LDU (linkage disequilibrium units) as threshold for linkage block computation.

Table 3 Primers for polymerase chain reaction amplification and sequencing of *Claudin-2*

Candidate gene	Exon	Forward primer[1]	Reverse primer[2]	Size of PCR-product
CLDN2	1	GTAGGACCTGCTCTTTGAACTC[2,3]	TGAATTTAAAAGGCAGCAACTA[2,3]	708 bp
	1	TCAATCTTTCCCAGCCTCCA[3]	TTTCTGTCAAAAACTCCACTCC[3]	
	2	TTGTAGAGAATGGGGAGGTGTGC[2,3]	TCAATTGCAGACTGAGGCCAAA[2]	599 bp
	2		TGCAGACTGAGGCCAAACTG[3]	
	2	CTAGGCCCCTGGAGATTCAAGA[2,3]	TGTGCCCAAAAGCCCCAGAA[2,3]	682 bp
	2	TTCCTTTCTCATGTGTTATTTCTAA[3]	GAGAAAGGAAAAAAAAAACAAC[3]	
	2	TGAGGGATTAGAGGTGTTCAA[2,3]	GCAGCACCTTCTGACACGA[2,3]	892 bp
	2	ATCCTACGGGACTTCTACTCA[3]	ACTCCACCTGCTACCGCCACT[3]	

[1]q, Q-solution for polymerase chain reaction (PCR)-amplification; [2]Primer used for PCR; [3]Primer used for sequencing. CLDN: Claudin.

tests, two-sided *P* values < 0.05 were considered significant. Correction for multiple testing was not performed.

RESULTS

Genetic association - case-control approach

Based on a subset of Swedish IBD patients (73, 39 and 42 individuals with IBD, CD or UC, respectively) one marker per gene was selected for analysis in the full study material (Table 2). Allelic association between any of the three markers for the genetic regions of *CLDN1*, *CLDN2* or *CLDN4* and patients with IBD, CD or UC were investigated using a case-control study approach (Table 4). Significant associations were observed between the *CLDN2* marker (rs12014762; associated C allele frequency of 0.776 among controls) and CD (*P* = 0.007), and the *CLDN1* marker (rs7620166; associated T allele frequency of 0.470 among controls) and IBD (*P* = 0.025). No associations were observed for the *CLDN4* marker (rs8629; C allele frequency of 0.772 among controls), neither to IBD, CD nor UC.

Genetic association - family-based approach

The same three SNP markers were further investigated using a family-based approach in non-Swedish families (Table 4). The significant associations identified in the case-control study were not confirmed (*P* = 0.126 and *P* = 0.177 for the *CLDN2* and *CLDN1* marker, respectively). The *CLDN2* marker rs12014762 was not related to any demographic or disease characteristics in CD-patients (Table 5).

Resequencing of CLDN2

Based on the most significant association (CD and the *CLDN2* marker), *CLDN2* was chosen for resequencing in an approach to identify novel sequence variants of putative importance for the risk of developing CD. Two novel non-coding sequence variants were identified for

Table 4 Inflammatory bowel disease-phenotypes were investigated by performing family-based and case-control approach in genetic association studies

		rs7620166 (*CLDN1*)		rs12014762 (*CLDN2*)		rs8629 (*CLDN4*)	
		allelic OR (95%CI)	*P* value	allelic OR (95%CI)	*P* value	allelic OR (95%CI)	*P* value
IBD	Swedish case-control	1.33 (1.04-1.72)	0.025	1.39 (0.95-2.01)	0.083	1.21 (0.89-1.65)	0.225
	Non-Swedish families	0.87 (0.72-1.06)	0.177	1.25 (0.89-1.77)	0.195	1.09 (0.88-1.33)	0.432
CD	Swedish case-control	1.17 (0.86-1.60)	0.319	1.98 (1.17-3.35)	0.007	1.25 (0.84-1.85)	0.258
	Non-Swedish families	0.80 (0.64-1.00)	0.052	1.37 (0.91-2.07)	0.126	1.14 (0.89-1.46)	0.287
UC	Swedish case-control	1.35 (0.98-1.84)	0.064	1.27 (0.80-2.02)	0.304	1.18 (0.80-1.73)	0.409
	Non-Swedish families	1.19 (0.77-1.84)	0.436	0.91 (0.39-2.14)	0.827	1.15 (0.75-1.77)	0.512

The family-based association studies included a total of 463 families. For the single nucleotide polymorphism -markers rs7620166 [claudin (*CLDN*)*1*], rs12014762 (*CLDN2*) and rs8629 (*CLDN4*) genotyping failed for 5 [including 2 Crohn's disease (CD)], 9 (including 3 CD) and 7 (including 2 CD) samples, respectively. Results were based on 191, 103 and 102 cases of inflammatory bowel disease (IBD), CD and ulcerative colitis (UC), respectively and 333 controls. OR (and its associated 95%CI) and *P* values (based on log likelihood ratio χ^2 statistics) were calculated for the T allele of rs7620166, the C allele of rs12014762, and the C allele of rs8629.

Table 5 Genotype-phenotype correlation between *Claudin-2* marker (rs12014762) polymorphism and clinical characteristics of 677 patients with Crohn's disease (all families)

rs12014762 Crohn's disease	At least one C	T	*P* value
Sex			
Male	265	47	
Female	354	11	
Age at diagnosis (yr)			
Mean (SD)	24.48 (12.11)	24.36 (11.46)	0.90
Median (range)	22 (3-70)	22 (8-63)	
Location at onset:	*n* = 583	*n* = 54	
Pure colonic disease	78	6	0.64
Pure ileal disease	117	14	0.31
Ileocolonic disease	274	309	0.87
Any colonic disease	414	37	0.70
Any ileal disease	424	42	0.42
Upper digestive tract	118	12	0.73
Perineal disease	147	11	0.53
Granuloma	237/478	26/43	0.12
Penetrating disease	218/478	22/43	0.48
Strictures	221/478	15/43	0.15
Extra-digestive symptoms	190/619	15/58	0.44
Smoking habits	*n* = 505	*n* = 51	
Non-smoker	272	21	0.08
Smoker	157	21	0.14
Ex-smoker	76	9	0.62

CLDN2 (Table 6).

Prediction of transcription factor binding sites in the CLDN2 promoter and analysis of genetic association to the newly identified sequence variants

The two novel *CLDN2* polymorphisms were located in a region corresponding to the *CLDN2* promoter, as described by Sakaguchi *et al*[34] Analyzing the promoter region of *CLDN2* for the presence of possible transcription factor binding sites, revealed that these new variants were located in a putative specificity protein 1 (Sp1) binding site/GC-box (rs62605981) and a putative upstream stimulatory factor (USF) binding site/E-box (rs72466477). The genotyping results of these two novel SNPs were based on 166, 89 and 89 cases of IBD, CD and UC, respectively, from the Swedish families and their 333 non-related controls (Table 1). Neither rs62605981 (C allele frequency of 0.860 among controls) nor rs72466477 (AT allele frequency of 0.842 among controls) showed any significant associations to IBD overall or to any sub-entities (Table 7).

MORC4 and genetic association

In addition to *CLDN2*, *MORC4* is located on the same linkage block as the CD associated marker (rs12014762). A nonsynonymous coding SNP (rs6622126) was identified in the *MORC4* gene using the NCBI SNP database, for which a high minor allele frequency (A allele = 0.440) was present in the non-related Swedish control samples. This SNP was investigated for genetic association to IBD overall and to sub-entities, using the same individuals as used for the two novel *CLDN2* variants. A significant association was observed between the G allele (frequency of 0.560 among controls) and CD ($P = 0.018$), but not to UC or IBD (Table 7).

DISCUSSION

TJ proteins are considered key components in the regulation of paracellular permeability of both epithelial and endothelial cell linings[20,21], and due to their barrier-forming properties we considered claudin genes as candidate genes affecting a leaky gut phenotype of IBD.

Using the case-control approach, an association was identified between the *CLDN2-MORC4* region (rs12014762) and the occurrence of CD. This SNP marker was associated with an overall increased risk of having CD, but not to any distinctive phenotypic pattern. This association was not confirmed in the family-based approach, where non-Swedish families were included. Such a regional heterogeneity may in part be explained by different genetic backgrounds. Substantial genetic heterogeneity due to geographic stratification has been demonstrated in genome-wide association studies[6]. A geographically homogenous population (this study) should be advantageous for unveiling regionally restricted genetic risk factors. The case of underrepresented *NOD2* mutations in CD patients from the Nordic countries[35-37] together with our data on the presence of *CLDN2* vari-

Table 6 Sequence variants identified by resequencing of selected gene regions

Gene[1]	Position of SNP	rs-number	Part of gene/predicted consequence	VAF/control[2]	VAF/patients[2]
CLDN2	c.-178-678C>G (g.29466911)	rs62605981	Intron/5′ gene flanking region[3]	0.175	0.139
	c.-178-104_-103delAT (g.29467485_29467486delAT)	rs72466477	Intron/5′ gene flanking region[3]	0.175	0.083

[1]The following reference sequences have been used for claudin (*CLDN*)2 NT_011651.16 (genomic) and NM_020384.2 (mRNA); [2]Variant allele frequencies (VAF) are defined with respect to the corresponding reference sequence; [3]The intronic region of *CLDN2*, according to the *CLDN2* reference mRNA (NM_020384), correspond to the promoter region described by Sakaguchi *et al*[34]. SNP: Single nucleotide polymorphism.

Table 7 Genetic association between inflammatory bowel disease-phenotype and either newly discovered *Claudin-2* promoter polymorphisms or a nonsynonymous coding polymorphisms in *MORC4* was analyzed by performing case-control studies

	rs62605981 (*CLDN2*)		rs72466477 (*CLDN2*)		rs6622126 (*MORC4*)	
	allelic OR (95%CI)	*P* value	allelic OR (95%CI)	*P* value	allelic OR(95%CI)	*P* value
IBD	1.11 (0.71-1.73)	0.659	1.23 (0.79-1.91)	0.350	1.24 (0.91-1.70)	0.179
CD	0.83 (0.49-1.41)	0.501	1.16 (0.68-2.00)	0.584	1.61 (1.08-2.41)	0.018
UC	1.24 (0.69-2.26)	0.463	1.19 (0.68-2.06)	0.543	0.903 (0.61-1.33)	0.606

Results were based on 166, 89 and 89 cases of inflammatory bowel disease (IBD), Crohn's disease (CD) and Ulcerative colitis (UC), respectively, available from the Swedish families and 333 controls (Table 1). OR (and its associated 95%CI) and *P* values (based on log likelihood ratio chi-square statistics) were calculated for the C allele of rs62605981, the AT allele of rs72466477, and the G allele of rs6622126. CLDN: Claudin.

ants in Swedish patients add support for such an assumption taking into account that IBD, and especially CD, are polygenic disorders[2,3]. When we investigated a possible interaction between *NOD2* and the *CLDN2-MORC4* region (rs12014762) with individuals stratified on the basis of carrying none or at least one *NOD2* mutant allele, no significant association was identified between these two genetic regions (data not shown).

Both CD and UC have been associated with an increase in intestinal permeability[9-11]. Experimental data reveal an altered expression of claudin-2, and other claudin proteins, in the intestinal epithelium of IBD patients, and such alterations affect the permeability characteristics[38]. Claudin-2 expression was increased in a cell culture of human colonic epithelial cells (HT-29/B6) in response to tumor necrosis factor-α (TNF-α)[38], a finding consistent with an increased expression of claudin-2 in the inflamed epithelium of CD patients[38,39]. Using another human colonic epithelial cell line (T84), Prasad *et al*[39] demonstrated an increase both in paracellular permeability and claudin-2 expression in response to interleukin-13, but not in response to interferon-γ/TNF-α treatment.

Within the resequenced parts of *CLDN2* we identified two novel polymorphisms in the promoter region. These two sequence variants were located in different putative transcription factor binding sites, a Sp1 binding site/GC-box (rs62605981) and an USF binding site/E-box (rs72466477). It has been shown that the *CLDN2* transcription is affected by several transcription factors[34], and both E-box binding[40-42] and Sp1 binding site[43,44] transcription factors have been identified as important determinants of claudin gene expression. However, neither of the two novel promoter variants were associated to CD.

MORC4 is present on the same linkage block as *CLDN2* and the CD associated marker (rs12014762). It is therefore possible that the C allele of rs12014762 is a marker for a functional variant of *MORC4* that results in an increased overall risk of developing CD. A significant association was observed between CD and the G allele of the nonsynonymous coding SNP in the *MORC4* gene (rs6622126), resulting in a substitution of the more hydrophobic amino acid isoleucine at position 473, located immediately outside the region predicted to be a zinc finger (420-472), with threonine.

MORC4 encodes a member of the MORC family of CW-type zinc finger proteins, which contain a number of predicted domains and motifs suggestive of being transcription factors[45]. *MORC4* exhibits a low-level mRNA expression in a variety of normal tissues, including the intestine. Using the SKI-like protein as bait in a two-hybrid screen, MORC4 has been identified as a putative interacting protein, linking MORC4 to the transforming growth factor-β (TGF-β) signaling pathway and the SMAD family of signal transduction proteins[46]. Increased intestinal expression of TGF-β has been observed in patients with CD[47] and, in monolayers of intestinal epithelial cells, TGF-β has been shown to preserve or enhance the paracellular barrier[48,49]. These two findings seem to contradict the increased intestinal permeability that has been associated to both CD and UC[9-11]. Possibly a genetic variant in the *CLDN2-MORC4* region could disturb a TGF-β mediated signal that preserves or enhances the paracellular barrier, or exert an effect on *CLDN2* expression that dominates a TGF-β mediated effect on paracellular permeability. In addition, a SMAD4-dependent, but TGF-β-independent, repression of *CLDN1* transcription[50] and a ZEB2-mediated repression of *CLDN4*[42] support a role for the SMAD signal transduction pathway in the regulation of claudin genes.

An increased intestinal permeability has been associated with the CD susceptibility allele CARD15 3020insC[18], and TJ associated genes have been suggested as suscepti-

bility genes for UC (*e.g.*, *GNA12*[2]) and for both UC and CD (*MAGI2*[51]).

In conclusion, our findings add further support for a genetically impaired intestinal epithelial barrier as one predisposing factor in the etiology of CD, either directly through *CLDN2* or indirect *via* a tentative link between *MORC4*, TGF-β/SMAD signalling and an effect on paracellular permeability. This putative genetic link between the *CLDN2-MORC4* region, intestinal epithelial integrity and the risk of developing CD needs to be further explored.

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COMMENTS

Background

For chronic inflammatory bowel disease (IBD) - Crohn's disease (CD), ulcerative colitis - a number of studies have demonstrated a substantial genetic predisposition. These inflammatory conditions have been associated with an increased intestinal permeability. Consistent with the phenotype of these diseases, several genetic association studies have implicated mainly components of the immune response, but also factors implicated in intestinal permeability. In order to further investigate intestinal permeability as a predisposing genetic risk factor for IBD, the authors have conducted genetic association studies on claudin genes (*CLDN1*, *CLDN2* and *CLDN4*), key components in the regulation of permeability.

Research frontiers

Although large-scale genome-wide association studies have uncovered a large number of genetic susceptibility loci in relation to IBD these factors still explain only a minority of the total genetic risk for IBD. The genetic background regulating intestinal barrier functions largely remains unknown.

Innovations and breakthroughs

The barrier of epithelial cells is critical for the permeability properties of the intestine. The tight junction structure is a multicomponent protein complex that serves to seal and regulate permeability across the space between adjacent epithelial cells, with significant contribution from members of the claudin family. This is the first study to report genetic link between claudin gene (*CLDN2*) and the risk of developing CD.

Applications

In this study the authors have identified a genetic link between the *CLDN2-MORC4* region and the risk of developing CD, and thereby highlighted claudins as therapeutic targets.

Terminology

The tight junction structure is critical for the permeability properties of the intestine. The structure is a multicomponent protein complex that serves to seal and regulate permeability across the paracellular space between adjacent epithelial cells.

Peer review

This is a small study on the importance of claudins (*CLDN1*, *CLDN2* and *CLDN4*) in Swedish and non-Swedish case-control and family-based approach. A weak suggestive association was reported in the case-control setting, while this was not replicated in the non-Swedish sample. The paper is interesting and well written: its main limitation lies in the study design, comparing Swedish and non-Swedish individuals using two different approaches.

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BRIEF ARTICLE

Treatment of hemorrhagic radiation-induced proctopathy with a 4% formalin application under perianal anesthetic infiltration

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Abstract

AIM: To evaluate the results of hemorrhagic radiation proctopathy treatment with a 4% formalin application.

METHODS: A prospective study was performed. Over a three-year period, 38 patients underwent 4% formalin application under perianal anesthetic infiltration for hemorrhagic radiation proctopathy. All patients included in the study were irradiated for prostate cancer. The patients ranged in age from 56-77 years (average 70 ± 5 years). All of the patients were referred for formalin therapy after noninvasive management had failed. Twenty-four (63.2%) patients underwent a single application, 10 (26.3%) patients underwent 2 applications, and 4 (10.5%) patients underwent 3 applications.

RESULTS: Two to 36 mo (average 12 ± 3 mo) following treatment, 34 patients were interviewed (four were lost to follow-up). Twenty (58.8%) subjects reported complete cure, 8 (23.5%) subjects reported significant improvement, and 6 (17.7%) subjects reported no change. One patient (who underwent a colostomy at a regional hospital with no specialized services available for previous bleeding episodes from radiation proctopathy) was cured, and the colostomy was closed. One patient (2.6%) developed rectal mucosal damage after the second application.

CONCLUSION: A 4-min application of 4% formalin for hemorrhagic radiation-induced proctopathy under perianal anesthetic infiltration in patients who have received external radial radiation therapy for prostate cancer is simple, reasonably safe, inexpensive, generally well tolerated, and effective.

Key words: Formalin application; Radiation proctopathy; Rectal bleeding; Prostate cancer

Core tip: In a prospective study conducted from 2006 to 2009, 38 patients underwent 4% formalin application under perianal anesthetic infiltration for hemorrhagic radiation proctopathy. Based on the rectal-telangiectasia density classification, eight (21.1%) patients had grade Ⅰ proctitis, 23 (60.5%) patients had grade Ⅱ proctitis, and seven (18.4%) patients had grade Ⅲ proctitis. A piece of gauze soaked with 4% formalin was applied to the entire diseased rectal mucosa and remained for 4 min under perianal anesthetic infiltration. Twenty patients (58.8%) reported complete cure, eight patients (23.5%) reported significant improvement, and

six patients (17.7%) reported no change. Application of 4% formalin under perianal anesthetic infiltration in patients who received external radial radiation therapy for prostate cancer was simple, safe, and effective.

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INTRODUCTION

Radiotherapy is a common treatment modality for carcinoma of the female genital tract, prostate, and urinary bladder[1]. Because of its fixed position in the pelvis and because of its proximity to the treated organs, the anorectal area is the most common site of bowel injury following pelvic radiotherapy. Chronic hemorrhagic radiation proctopathy occurs in 1%-5% of patients following radiotherapy for pelvic malignancy[2].

The gross pathologic changes can be acute, subacute, or chronic. Acute changes occur during and immediately after radiotherapy in the form of hyperemia, edema, and extensive inflammatory cell infiltration of the mucosa. To a variable extent, subacute and chronic changes begin after 2 to 12 mo of regeneration. In the vessels, there may be endothelial swelling leading to fibrosis of the connective tissues (intima) and endarteritis. Damage of the vessels increases the formation of arteriovenous shunts, *i.e.*, telangiectatic neovasculature that is fragile and prone to bleeding. Ulcers, strictures, and fistulae may also develop[3]. More often, patients will experience functional symptoms of proctopathy, such as urgency, tenesmus, mucoid rectal discharge, abdominal pain, and sphincter irritability[4].

No standard treatment exists for this condition. The primary treatment of radiation proctopathy is medical (or non-invasive). If non-invasive treatment is ineffective, then invasive treatment is considered. One such treatment is formalin application. Formalin is a solution of formaldehyde mixed with methanol that is commonly used to fix tissue samples for histological examination. Applied topically, formalin acts as a chemical cautery of telangiectatic mucosal vessels, and its main action is the sclerosing and sealing of fragile neovasculature[4]. In 1969, Brown *et al*[5] were the first to use formalin to treat radiation-induced hemorrhagic cystitis. Inspired by this experience, in 1986, Rubinstein *et al*[6] were the first to apply formalin to treat hemorrhagic radiation proctopathy. However, this treatment modality did not become popular until 1993, when Seow-Choen *et al*[4] reported their data, which indicated a high success rate. Rectal instillation of various concentrations of formalin solution has been used by several groups to control severe or refractory bleeding from radiation proctopathy, with encouraging results[1-16]. The local application of 4% formalin is safe and highly effective in both radiation cystitis and radiation proctopathy. We evaluated the use of a 4% formalin gauze (surgical swab) in patients with radiation proctopathy as an outpatient procedure under perianal anesthetic infiltration[17].

MATERIALS AND METHODS

This study was approved by the Lithuania Bioethical Committee in 2006.

We conducted a prospective study from July 2006 to July 2009 (3 years). Thirty-eight patients were included. The following inclusion criteria were applied: male patients older than 18 years who had undergone external beam radiotherapy for prostate cancer and developed rectal bleeding as the main symptom of proctopathy. The rectal bleeding occurred not more than two years post-radiotherapy. Proctopathy (classified using the rectal telangiectasia density score) was diagnosed with colonoscopy. Patients were excluded from the study if they met any of the following criteria: the bleeding occurred more than two years after radiotherapy; other symptoms dominated, such as tenesmus, pain, ulceration, or impaired defecation; the patient had impaired coagulation; or the patient was using anticoagulants. All patients provided written informed consent prior to the formalin application.

All patients were referred for formalin therapy after the failure of noninvasive management (peroral sucralfate and topical cortisone were used). The subjects received radical treatment for prostate cancer, including 3D conformal external radiotherapy to the prostate and the base of the seminal vesicles, up to a total dose of 74 Gy (70-74 Gy) over a 7.5-wk period. The patients ranged in age from 56-77 years, with an average of 70 ± 5 years.

Bleeding occurred for all patients during the first two years following treatment. The mean timepoint for the onset of symptoms was 9 ± 4 mo (range one week to 24 mo). In one case, hemorrhage occurred one week after treatment; in two cases, during treatment; and in the remainder of cases, three to 24 mo after treatment. Nineteen patients reported daily blood in their stools, and 19 patients reported bleeding two or three times per week. Two patients received blood transfusions for severe anemia, and one patient was treated with a colostomy for severe rectal bleeding at a regional hospital before coming to our institution.

A total colonoscopy was performed in all of the patients to exclude other synchronous causes of hemorrhage and to determine the extent of the radiation-induced damage. We used the rectal telangiectasia density score[18], in which the radiation proctopathy was graded into the following four grades: normal mucosa (Grade 0), fewer than 10 discrete telangiectasias within a luminal view (Grade I), a single coalescing patch of telangiectasias and/or greater than or equal to 10 discrete telangiec-

tasias (Grade Ⅱ), and the presence of two or more coalescing telangiectatic patches (Grade Ⅲ). Based on this classification, eight (21.1%) patients had grade I proctitis, 23 (60.5%) patients had grade Ⅱ proctitis, and seven (18.4%) patients had grade Ⅲ proctitis.

The formalin application was performed on an outpatient basis in an operating theater. All of the procedures were conducted with the patient in the prone, jack-knife position under perianal anesthetic infiltration, which was performed by injecting a mixture of lidocaine and bupivacaine solution. Vaseline (petroleum jelly) was applied to the perineum and upper anal canal up to the level of the dentate line, both to serve as a lubricant and to protect the skin from unnecessary exposure to formalin. A piece of gauze (surgical swab) soaked with 4% formalin was applied to the entire diseased rectal mucosa and left in place for four minutes. A Fansler proctoscope was used to visualize the radiation-induced rectal lesions and to avoid formalin application to the healthy rectum. At the end of the procedure, the anal canal and the rectum were abundantly rinsed with water.

A complete response was recorded if there were no further episodes of bleeding. Significant improvement was recorded if there was less than one bleeding episode per month. No response was recorded if the bleeding continued as prior to treatment. The patients were treated repeatedly if they exhibited no improvement after four weeks. All of the patients were interviewed using a questionnaire administered by mail or by telephone at 1, 2, 3, 6, 9, 12, 18, 24 and 36 mo after the treatment. Colonoscopy was not repeated after the treatment.

All of the statistical analyses were performed using SPSS version 17 was used (*SPSS* Inc., Chicago, IL, United States) for Windows.

RESULTS

Two to 36 mo after treatment (average 12 ± 3 mo), 34 patients were interviewed (four were lost to follow-up). Twenty-four (63.2%) patients were treated with only one formalin application, and 10 (26.3%) patients required a second application because of persistent bleeding. Four patients (10.5%) required three applications. The treatment was effective in 28 cases (82.3%); of these cases, 20 (58.8%) patients reported complete cessation of the bleeding, and eight (23.5%) patients reported significant improvement. Six patients (17.7%) reported no change in the bleeding. One patient, who underwent a colostomy for previous episodes of bleeding due to radiation proctopathy at another hospital, was cured, and the colostomy was closed. One patient (2.6%) developed rectal mucosal damage after the second application and underwent prolonged conservative management (*i.e.*, topical sucralfate, sucralfate enemas, cortisone enemas, analgesics, and mesalazine suppositories); in this case, the bleeding was controlled completely. No other complications occurred.

DISCUSSION

Currently, no "best" treatment exists for hemorrhagic radiation proctopathy. Non-invasive therapy includes a low-residue diet, laxatives and retention enemas with steroids, rebamipide[19] or hyperbaric oxygen therapy[20], oral antibiotics with colonic irrigation[21,22], short-chain fatty acids, pentoxifylline[23], hormonal therapy[24], antioxidants[25], and retinol palmitate[26]. However, these treatment modalities have not been proven effective in all cases of chronic hemorrhagic radiation proctopathy.

Studies of hyperbaric oxygen therapy suggest a clear benefit of this modality in the control of bleeding. Unfortunately, this procedure is expensive and requires additional prospective randomized studies to determine its efficacy in cases of rectal bleeding[16,20]. In a randomized placebo-controlled trial, retinol palmitate was proven effective in significantly reducing rectal functional symptoms[26]. One comparative study recently demonstrated that oral antibiotics combined with colonic irrigation was superior to 4% formalin application in reducing rectal functional symptoms but yielded the same results in controlling bleeding[22].

If non-invasive treatment is ineffective, then invasive procedures may be used. Successful results using endoscopic therapy have been reportedly achieved in controlling bleeding and providing symptomatic relief by reducing the frequency of hematochezia and the necessity for transfusion[16,27-31]. Initially, endoscopists used cryoablation[27] and heater and bipolar probes[30], followed by neodymium/yttrium aluminum garnet and potassium titanyl phosphate lasers[29,32], which were beneficial. Argon plasma coagulation (APC) is an innovative, no-touch electrocoagulation technique that is used to treat hemorrhagic digestive malformations. Studies have demonstrated the superior efficacy and safety of APC in treating hemorrhagic radiation proctopathy[28,33].

The treatment of hemorrhagic radiation proctopathy with formalin was first reported by Rubinstein *et al*[6] in 1986. The concentration of the formalin solution used, the treatment method (application *vs* instillation[12]), and the mucosal contact time vary largely, as reported by different authors. Diverse techniques have been used by different investigators with varying success rates; examples include irrigation of the rectum with a large volume of formalin for 15 min[33], insertion of a formalin-soaked gauze for 2 to 3 min[4] or up to cessation of the symptoms[7,14], and repeated instillation of 50 mL of formalin for 30 s[8]. Cullen *et al*[2] used 20 mL of a 5% formalin instillation for two or three minutes, with success rates of up to 85%. We used a piece of gauze (surgical swab) soaked with 4% formalin solution, which was applied to the entire diseased rectal mucosa for 4 min.

Several studies have indicated that systemic toxicity arises after more prolonged contact with formalin[31]. Systemic toxicity also increases when formalin instillation is used. The optimal concentration of formalin for the

procedure is unknown. Varying concentrations of formalin solution, ranging from 2% to 10%, have been used[1-4]. However, a 4% formalin solution has been used most widely. A lower concentration may be safer but is associated with a lower response rate[1]. In a study in which 2% formalin was used, the overall response rate was 78.2%, while the complete success rate was only 47.5%[1]. The use of 10% formalin has resulted in an overall success rate of 93%[3], which is comparable to 4% formalin, for which the success rates ranges from 70% to 100%. A higher concentration of formalin may result in a higher incidence of complications[4].

Formalin usually causes cessation of bleeding within a short period by acting as a local chemical cautery. It stops the bleeding by sealing the sites of leakage from the neovascularized telangiectatic spots and ulcers. Multiple sessions of formalin application were required in some of the nonresponsive or relapsed patients. In the study by Seow-Choen *et al*[4], 17 of 29 patients experienced the complete cessation of bleeding one month after a single application; 11 patients experienced only minor bleeding, and one patient continued to experience major rectal bleeding.

Repeated formalin applications resulted in further success in this study. In the investigation by Parikh *et al*[14], the number of formalin treatments ranged from 1 to 13, with a mean of 3.4. The response rates in other studies have been similar, ranging from 81% to 100%[1-4]. We used 4% formalin with a success rate of 82.3%. This response rate is comparable to that of previous studies. Twenty-four of 38 patients in our study were treated with only one formalin application, and 10 patients required a second application because of persistent bleeding. Four patients needed three applications. Decreased cost is a major advantage of formalin over APC and the other treatment modalities. However, APC poses the advantage of reaching lesions beyond the rectum[33].

One patient, who underwent a colostomy at another institution for previous episodes of bleeding from radiation proctopathy, was cured, and the colostomy was closed. One patient (2.6%) developed rectal mucosal damage after the second application. No other complications were observed. Several published reports have also shown no serious complications of local formalin therapy[7,8,13,14]. However, a higher incidence of local complications (*e.g.*, anorectal strictures, incontinence, anal ulcers and/or stenosis) has been reported[10]. These events may not be entirely caused by formalin, as a higher proportion (36%) of patients in the latter case series had anorectal malignancies. Other complications that have been reported in certain studies include the proximal migration of formalin, which is caused when a rigid sigmoidoscope is used for instillation. Overdistension of the distal rectum with subsequent proximal migration of the formalin should be avoided. In the present study, we used a Fansler proctoscope to visualize the damaged rectal mucosa.

One recently published randomized trial has compared formalin dab treatment with a sucralfate-steroid retention enema; in that investigation, Nelamangala Ramakrishnaiah *et al*[15] concluded that a 4% formalin dab was superior to a sucralfate-steroid retention enema for treating hemorrhagic proctopathy caused by radiotherapy. Surgery should be reserved for patients who have intractable symptoms, such as strictures and/or fistulas. However, surgery may be technically demanding because of adhesions and other radiation damage in the pelvis. Another surgical concern is that anastomoses involving radiated tissue may break down. Abdominoperineal resection may be the only reliable option in some patients.

The present study had the following limitations: colonoscopy was not repeated after the treatment, we could not relate our results to possible endoscopic changes in the rectum, and the follow-up time was markedly different for our patients in our study.

In conclusion, radiation-induced hemorrhagic proctopathy is a frequent complication following pelvic radiation. In our experience, formalin application therapy was an inexpensive, simple and highly effective therapy for radiation-induced hemorrhagic proctopathy and yielded few complications. We reported a clinical response rate of 82.3%. Therefore, we recommend 4% formalin application as a low-cost treatment for chronic hemorrhagic radiation proctopathy.

COMMENTS

Background

Radiation-induced hemorrhagic proctopathy is a common late complication that manifests after irradiation treatment for pelvic malignancies. The condition may present with signs and symptoms ranging from clinically insignificant bleeding during defecation to a debilitating disorder requiring blood transfusions, repeated admissions to the hospital and even surgery, thereby reducing the patient's quality of life. Preventing radiation-induced hemorrhagic proctopathy *via* different agents during the radiotherapy course has not been successful, and newer external radiation techniques or brachytherapy have not precluded this complication in all cases. Other types of radiotherapy-related damage to the rectum, such as ulcers, strictures or fistulas, are reported much more rarely. This article focuses on formalin applications for hemorrhagic radiation-induced proctopathy in patients undergoing radical external radiotherapy for prostate cancer.

Research frontiers

Conservative treatment (peroral drugs, suppositories or enemas) may be useful in certain patients suffering from radiation-induced hemorrhagic proctopathy. However, non-responders require other options to treat this condition. Formalin application, argon plasma coagulation, and hyperbaric oxygen therapy appear to be the most effective modalities after failed conservative treatment. Argon plasma coagulation requires specific equipment, hyperbaric oxygen (which is not accessible everywhere), multiple sessions, and a long treatment period, whereas formalin application is a simple and low-cost method that is available in most hospitals.

Innovations and breakthroughs

Despite the many published articles on formalin therapy for radiation-induced hemorrhagic proctopathy, several questions remain unanswered. What percentage of formalin solution should be used? Which method (application or instillation) is safer and more effective? How long should the mucosal contact period last? How should the application be performed? What type of anesthesia should be used? In this article, the authors attempt to standardize formalin application to address the above-mentioned considerations.

Applications

The perspectives regarding the future use of the method described in this article are as follows. After failed conservative treatment, other treatment modalities are not universally available. Because of the simplicity of this method, formalin

application for radiation-induced hemorrhagic proctopathy under perianal anesthetic infiltration may be performed in most hospitals by general surgeons who do not necessarily have extensive colorectal experience. Therefore, the standard technique described by their group may be useful for achieving the best-possible results with an existing approach.

Terminology

The authors used the term "radiation proctopathy" rather than "radiation proctitis," both of which describe the same condition. "Perianal anesthetic infiltration" was a better definition of the anesthesia method employed by their group, although it exhibits certain similarities with the "pudendal block" technique described a few decades ago.

Peer review

There is bot much new things, but it is ok to report these clinic data.

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BRIEF ARTICLE

Validation of the chronic liver disease questionnaire in Serbian patients

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Abstract

AIM: To translate into Serbian and to investigate the validity of the cross-culturally adapted the chronic liver disease questionnaire (CLDQ).

METHODS: The questionnaire was validated in 103 consecutive CLD patients treated between October 2009 and October 2010 at the Clinic for Gastroenterology, Clinical Centre of Serbia, Belgrade (Serbia). Exclusion criteria were: age < 18 years, psychiatric disorders, acute complications of CLD (acute liver failure, variceal bleeding, and spontaneous bacterial peritonitis), hepatic encephalopathy (grade > 2) and liver transplantation. Evaluation of the CLDQ was done based on the following parameters: (1) acceptance is shown by the proportion of missing items; (2) internal reliabilities were assessed for multiple item scales by using Cronbach alpha coefficient; and (3) in order to assess whether the allocation of items in the domain corresponds to their distribution in the original questionnaire (construction validity), an exploratory factor analysis was conducted. Discriminatory validity was determined by comparing the corresponding CLDQ score/sub-score in patients with different severity of the diseases.

RESULTS: The Serbian version of CLDQ questionnaire completed 98% patients. Proportion of missing items was 0.06%. The total time needed to fill the questionnaire was ranged from 8 to 15 min. Assistance in completing the questionnaire required 4.8% patients, while 2.9% needed help in reading, and 1.9% involved writing assistance. The mean age of the selected patients was 53.8 ± 12.9 years and 54.4% were men. Average CLDQ score was 4.62 ± 1.11. Cronbach's alpha for the whole scale was 0.93. Reliability for all domains was above 0.70, except for the domain "Activity" (0.49). The exploratory factor analysis model revealed 6 factors with eigenvalue of greater than 1, explaining 69.7% of cumulative variance. The majority of the items (66%) in the Serbian version of the CLDQ presented the highest loading weight in the domain assigned by the CLDQ developers: "Fatigue" (5/5), "Emotional function" (6/8), "Worry" (5/5), "Abdominal symptoms" (0/3), "Activity"

(0/3), "Systemic symptoms" (3/5). The scales "Fatigue" and "Worry" fully corresponded to the original. The factor analysis also revealed that the factors "Activity" and "Abdominal symptoms" could not be replicated, and two new domains "Sleep" and "Nutrition" were established. Analysis of the CLDQ score/sub-score distribution according to disease severity demonstrated that patients without cirrhosis had lower total CLDQ score (4.86 ± 1.05) than those with cirrhosis Child's C (4.31 ± 0.97). Statistically significant difference was detected for the domains "Abdominal symptoms" [F (3) = 5.818, P = 0.001] and "Fatigue" [F (3) = 3.39, P = 0.021]. *Post hoc* analysis revealed that patients with liver cirrhosis Child's C had significantly lower sub-score "Abdominal symptoms" than patients without cirrhosis or liver cirrhosis Child's A or B. For domain "Fatigue", patients with cirrhosis Child's C had significantly lower score, than non-cirrhotic patients.

CONCLUSION: The Serbian version of CLDQ is well accepted and represents a valid and reliable instrument in Serbian sample of CLD patients.

Key words: Chronic liver disease; Quality of life; Questionnaire; Validation; Factor analysis

Core tip: The Serbian validation of the chronic liver disease questionnaire (CLDQ) confirmed the 6-domain structure of the original United States version. However, in our investigation the original structure was only partially reproduced. The most prominent changes are related to the fact that the factors "Activity" and "Abdominal symptoms" could not be replicated, and two new domains "Sleep" and "Nutrition" were established Moreover, the domain "Nutrition" has been introduced for the first time. Our results of factors analysis gave the evidence that at list some items from the original version of CLDQ should be allocated or eliminated from the questionnaire because of the multiple loadings.

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INTRODUCTION

The concept of health-related quality of life (HRQoL) incorporates many aspects of an individual's experience, the general well-being, satisfaction, social and physical functioning[1]. Chronic liver disease (CLD) includes a wide range of disorders that are characterized by chronic inflammation and often progress to the cirrhosis. This group of diseases has a significant impact on HRQoL, and therefore its assessment is widely used as important outcome in clinical trials[2,3]. The most widely used general questionnaire is the short form health survey-36[4]. Furthermore, the liver disease-specific instruments comprise items that are specific for patients with CLD, and therefore they are more sensitive for capturing all relevant disease-burdened quality of life domains than a generic measure. These disease-specific questionnaires such as the CLD questionnaire (CLDQ)[5], liver disease quality of life instruments[6] and hepatitis quality of life questionnaire[7] are more sensitive and responsive to changes in HRQoL.

The CLDQ is a specific quality of life instrument designed for patients with liver disease, regardless of the underlining severity and etiology of CLD[5]. Its original version was developed by Younossi *et al*[5] and has demonstrated appropriate validity and reliability. The CLDQ has already been cross-culturally adapted and validated into different languages in previously published studies[8-18].

Up to now, there is no CLD-specific quality of life instruments adapted for Serbian patients. Therefore, the aim of this study was to investigate the validation of the translated and culturally adapted CLDQ questionnaire on a group of Serbian CLD patients.

MATERIALS AND METHODS

A cross-sectional study has been performed at the Clinic for Gastroenterology, Clinical Centre of Serbia, Belgrade. Between October 2009 and October 2010, consecutive inpatients and outpatients with CLD were considered for inclusion. Inclusion criteria were chronic hepatitis or liver cirrhosis. Diagnosis of liver disease was made by medical doctor-specialist in hepatology. Chronic hepatitis was defined as elevation of aminotransferases for 1.5 times greater than the upper limit of the reference interval, for more than 6 mo duration, and/or presence of histopathologic criteria for chronic hepatitis. The diagnosis of cirrhosis was based on clinical, laboratory, echo sonographic, endoscopic and histopathological criteria[9,19]. Ascites was diagnosed by ultrasound. Hepatic encephalopathy was assessed clinically, and patients were graded on a scale from 1 to 4. The presence of hypersomnia indicated grade 1, somnolentia grade 2, severe somnolence or stupor grade 3 and severe stupor or coma grade 4[8]. Exclusion criteria were: age < 18 years, psychiatric disorders (psychosis or dementia), acute complications of CLD (acute liver failure, variceal bleeding, and spontaneous bacterial peritonitis), hepatic encephalopathy (grade > 2) and liver transplantation. We also excluded the patients undergoing antiretroviral therapy because of a very small number of these subjects.

Severity of liver cirrhosis was determined by the Child-Pugh classification[20,21]. According to the severity of the diseases, patients were categorized into the following groups: non-cirrhotic, cirrhotic Child's A, cirrhotic Child's B and cirrhotic Child's C. According to the etiol-

ogy of the diseases, patients were categorized into the following categories: alcoholic, viral (viral hepatitis B and viral hepatitis C), autoimmune (autoimmune hepatitis, primary biliary cirrhosis and primary sclerosing hepatitis) and other (non-alcoholic steatohepatitis, Wilson's disease, hereditary hemochromatosis and cryptogenic).

This study was approved by the Ethics Committee of the Faculty of Medicine, University of Belgrade. All subjects gave written consent to participate in the study. Permission to use and validate CLDQ questionnaire was obtained by author of the original version (Younossi ZM).

The demographic data (age, gender, education, occupation, employment, marital status), clinical information (duration of the liver disease, haematemesis, ascites, hepatic encephalopathy), as well as the results of hematological, biochemical, virological and immunological analyses, were obtained from medical records.

The CLDQ was developed in 1999, by Younossi *et al*[5]. The questionnaire consists of 29 questions, which are divided into 6 domains as follows: "Fatigue", "Activity", "Emotional function", "Abdominal symptoms", "Systemic symptoms" and "Worry". Scores for each question were ranked from 1 (the worst quality of life - "All of the time") to 7 (the best quality of life - "None of the time"), for to the period of 2 wk ago. These scores were created using the Likert method. Domain scores are the means of the items contained. A summary score is calculated by the mean value of all subscale scores. The scores range from 1 to 7, whit higher values indicating better quality of life[5]. The CLDQ questionnaire was self-administered for all types of patients and filled in by the patients. In case of help in understanding and/or writing, the physician provided assistance when necessary.

The CLDQ adaptation was based on internationally accepted methodology for cultural adaptation of HRQoL questionnaires[22,23]. We used a standard methodology for the production of the Serbian version and it's included: (1) "Foward translation" - translation of the original version from English to Serbian language, so that the Serbian's version, semantically and conceptually corresponds to the original questionnaire. Translation was conducted by two independent, professional translators. Following review and editing by translators and experts, one single translation was formed; (2) "Backward translation" implied translation of the Serbian's version of CLDQ into English. Conducted by two translators, one an expert in quality of life and another one a clinician, with discussion on controversial items, it resulted in the final version of CLDQ culturally corresponding with Serbian's patients with CLD chronic disease liver; (3) Serbian version CLDQ questionnaire was tested on five patients with CLD who have had the opportunity to present their comments and suggestions. Test results are discussed by the group of experts, who created the final Serbian's version of the CLDQ (CLDQ-S); and (4) the final version was tested in 15 patients with CLD. During adaptation and pretesting of the CLDQ, there were no disputed items and any change from the original questionnaire items. Patients had no difficulty in understanding and completing the questionnaire.

Statistical analysis

In the data analysis, descriptive and analytical statistics were used. Continuous variables were described as mean ± SD, while the categorical variables were presented as proportions (percentages). For comparison of continuous variables between groups one-way Analysis of variance was used, including Bonferroni post hoc test for multiple comparisons.

Evaluation of the CLDQ was done through the following parameters: (1) acceptance is shown by the proportion of missing items; (2) internal reliabilities of Serbian version CLDQ were assessed for multiple item scales by using Cronbach alpha coefficient, ranges from 0-1, latter meaning perfect reliability; (3) in order to assess whether the allocation of items in the domain corresponds to their distribution in the original questionnaire (construction validity), an exploratory factor analysis (principal component analysis with varimax rotation) was conducted. A factor was considered as important if its eigenvalue exceeded 1.0; and (4) discriminatory validity was determined by comparing the corresponding CLDQ score/sub-score in patients with different severity of the diseases.

RESULTS

Out of 107 patients who met the inclusion criteria, 96.2% (n = 103) patients agreed to participate in the study. The reason for not accepting participation was a lack of interest or time. The mean age of the selected patients was 53.8 ± 12.9 years (range 21-79 years) and 54.4% were men (Table 1). According to the etiology of CLD the largest proportion was alcoholic liver disease (35%), and then autoimmune liver disease (28.2%). CLD in the stage of cirrhosis had 77.6% (n = 80) patients (Table 1).

The Serbian version of CLDQ questionnaire was completed by 98% (n = 101) patients. Proportion of missing items was 0.06% (2/2987). Two patients filled the questionnaire, but did not answered to all questions, for the "Systemic symptoms" domain (one for Question No.6 and one for No.27). The total time needed to fill the questionnaire ranged from 8 to 15 min. Assistance in completing the questionnaire was required by 4.8% (n = 5) patients, while 2.9% (n = 3) needed help in reading, and 1.9% (n = 2) involved writing assistance.

Analysis of distribution characteristics and reliability of the Serbian version of CLDQ showed that the average CLDQ score was 4.62 ± 1.11 and varied from 1.90 to 6.78. Cronbach's alpha for the whole scale (items 1-29) was 0.93. Reliability for all domains was above 0.70, except for the domain "Activity" (0.49) (Table 2).

In our validation study the exploratory factor analysis model revealed 6 factors with eigenvalue of greater than 1, explaining 69.7% of cumulative variance (Table 3). The majority of the items (66%) in the Serbian version of the

Table 1 Demographic and clinical characteristics of patients with chronic liver disease *n* (%)

Characteristics	Statistics
Age[1] (yr)	53.8 ± 12.9
Gender	
Male	56 (54.4)
Female	47 (45.6)
Education	
Unqualified[2]	6 (5.8)
Primary school	18 (17.5)
Secondary school	43 (41.7)
High school	17 (16.5)
University	18 (17.5)
Missing data	1 (1.0)
Current employment status	
Employed	29 (28.2)
Unemployed	25 (24.3)
Retired	49 (47.5)
Profession	
Housewife	13 (12.6)
Peasant	3 (2.9)
Worker	40 (38.8)
Official	21 (20.4)
Expert	21 (20.4)
Missing data	5 (4.9)
Marital status	
Single	15 (14.6)
Married/cohabiting	68 (66.0)
Separated/divorced	13 (12.6)
Widowed	7 (6.8)
Alcohol consumption	56 (54.4)
Smoker	32 (31.1)
Disease severity	
Non cirrhotic	23 (22.3)
Cirrhotic Child's A	25 (24.3)
Cirrhotic Child's B	30 (29.1)
Cirrhotic Child's C	25 (24.3)
Etiology	
Alcoholic	36 (35.0)
Viral	16 (15.5)[3]
Autoimmune/cholestatic	29 (28.2)
Other	22 (21.3)

[1]mean ± SD; [2]Without primary school; [3]Four patients with hepatitis B surface antigen positive chronic liver disease (CLD) and 12 patients with anti-hepatitis C virus positive CLD.

Table 2 Distribution and reliability of the chronic liver disease guestionnaire

Scale	*n*	mean ± SD	Min value	Max value	Missing items[1]	Cronbach alpha
Abdominal symptoms	103	4.75 ± 1.63	1.33	7	0%	0.82
Fatigue	103	4.20 ± 1.60	1.60	7	0%	0.90
Systemic symptoms	101	5.27 ± 1.60	1.60	7	1.94%	0.74
Activity	103	4.47 ± 1.33	1.33	7	0%	0.49
Emotional function	103	4.61 ± 1.62	1.62	7	0%	0.89
Worry	103	4.24 ± 1.61	1.00	7	0%	0.85
CLDQ total	101	4.62 ± 1.11	1.90	6.78	1.94%	0.93

[1]Proportion of patients with missing any item on the subscale. CLDQ: Chronic liver disease questionnaire.

CLDQ presented the highest loading weight in the domain assigned by the CLDQ developers: "Fatigue" (5/5), "Emotional function" (6/8), "Worry" (5/5), "Abdominal symptoms" (0/3), "Activity" (0/3), "Systemic symptoms" (3/5). The scales "Fatigue" and "Worry" corresponded fully to the original. An important difference compared to the original version was inclusion of two new factors, "Sleep" and "Nutrition". A new factor named "Sleep" was derived from the two items, No. 16 ("difficulty sleeping") and No. 20 ("incapable to fall asleep"), of the original subscale "Emotional function". An additional new factor "Nutrition" consisted of two items, No. 7 ("not able as much as would like") and 14 ("restriction of diet"), belonging to the "Activity" domains in original version of CLDQ. Furthermore, the factor "Activity", which consists of three items (No. 7, 9 and 14), could not be reproduced at all. Items No. 7 and 14 constructed the new factor "Nutrition", and item No. 9 had highest loading on "Fatigue". Also, the factor "Abdominal symptoms", which consists of three items (No. 1, 5 and 17) was not be replicated in the form like in the original version. Namely, in the Serbian version of CLDQ all of these three items had the highest loading in the same group and jointly with questions 3, 21 and 23 constituted a factor called "Systemic symptoms". In the original version of the questionnaire items No. 3, 21 and 23 are also part of the domain "Systemic symptoms", with the difference that in Serbian CLDQ questionnaire the two issues (No. 6 and 27) from the original version showing higher loadings on more than one other factors rather than the factor "Systemic symptoms". Explicitly, the item No. 6 ("shortness of breath in daily activities") showed higher loadings on "Fatigue", and "Nutrition", while the question No. 27 ("itching") revealed a higher degree of belonging to the domains of "Nutrition", "Worry" and "Sleep" (Table 3).

The analysis of etiology-specific scores of CLDQ have shown that the lowest total quality of life score (4.45 ± 1.11) was registered in the group of autoimmune/cholestatic origin of CLD, while the highest total score (4.84 ± 0.91) was observed in the CLD subcohort with the causes different from alcoholic, viral and autoimmune/cholestatic. However, there were no statistically significant differences between etiology-specific total quality of life scores, as well, as, among etiology-specific domain scores of CLDQ (data was not shown).

Analysis of the CLDQ scores distribution according to disease severity demonstrated that patients without cirrhosis had lower the total CLDQ score than those with cirrhosis Child's C, but without statistic significance [F (3) = 0.97, P = 0.402]. Statistically significant difference was detected for the domains "Abdominal symptoms" [F (3) = 5.818, P = 0.001] and "Fatigue" [F (3) = 3.39, P = 0.021]. *Post hoc* analysis revealed that patients with liver cirrhosis Child's C had significantly lower sub-score "Abdominal symptoms" than patients without cirrhosis or liver cirrhosis Child's A or B. For domain "Fatigue", patients with cirrhosis Child's C had significantly lower

Table 3 Exploratory factor analysis of the serbian version of the chronic liver disease questionnaire

Original CLDQ items	Factor 1 Systemic symptoms	Factor 2 Emotional function	Factor 3 Fatigue	Factor 4 Worry	Factor 5 Sleep	Factor 6 Nutrition
Fatigue						
(2) tired or fatigued	0.542	0.173	0.636[1,2]	0.271	0.018	0.140
(4) sleepy during the day	0.105	0.200	0.868[1,2]	0.004	0.156	-0.012
(8) reduced strength	0.489	0.119	0.595[1,2]	0.310	-0.019	0.220
(11) decreased level of energy	0.374	0.159	0.600[1,2]	0.385	0.055	0.282
(13) drowsy	0.168	0.287	0.824[1,2]	0.114	0.172	-0.085
Emotional function						
(10) anxious	0.313	0.521[1,2]	0.354	0.358	0.022	0.267
(12) unhappy	0.034	0.677[1,2]	0.283	0.284	0.096	0.264
(15) irritable	0.118	0.823[1,2]	0.157	0.082	0.036	0.091
(16) difficulty sleeping	0.256	0.184	0.175	0.229	0.745[1,3]	0.096
(19) mood fluctuations	0.127	0.794[1,2]	0.178	0.248	0.139	-0.042
(20) incapable to fall asleeP	0.252	0.183	0.123	0.155	0.822[1,3]	0.046
(24) felt depressed	0.162	0.815[1,2]	-0.003	0.288	0.203	0.092
(26) problem concentrating	0.161	0.697[1,2]	0.078	0.240	0.089	0.224
Worry						
(18) impact on family	0.237	0.269	0.037	0.760[1,2]	0.081	0.036
(22) symptoms developing into major problems	0.153	0.361	0.170	0.696[1,2]	0.229	0.015
(25) condition getting worse	-0.028	0.269	0.188	0.791[1,2]	0.240	0.152
(28) never feeling any better	0.005	0.293	0.162	0.659[1,2]	0.151	0.232
(29) availability of a liver	-0.142	-0.018	0.103	0.599[1,2]	0.460	0.127
Abdominal symptoms						
(1) abdominal bloating	0.763[1,3]	0.098	0.242	0.063	-0.041	0.074
(5) abdominal pain	0.785[1,3]	0.050	0.136	-0.054	0.207	0.110
(17) abdominal discomfort	0.811[1,3]	0.228	0.138	0.205	0.072	-0.049
Activity						
(7) not able to eat as much as would like	0.186	0.406	0.175	0.066	-0.079	0.543[1,3]
(9) trouble lifting or carrying heavy objects	0.389	-0.105	0.481[1,3]	0.162	0.116	0.236
(14) restriction of diet	-0.073	0.182	0.100	0.212	0.199	0.703[1,3]
Systemic symptoms						
(3) bodily pain	0.798[1,2]	0.103	0.116	0.097	0.121	-0.053
(6) shortness of breath in daily activities	0.367	0.296	0.433[1,3]	-0.047	0.083	0.389
(21) muscle cramps	0.470[1,2]	0.223	0.102	0.046	0.422	0.350
(23) dry mouth	0.556[1,2]	0.305	0.261	-0.065	0.316	0.172
(27) itching	0.252	0.011	-0.193	0.370	0.358	0.481[1,3]

[1]Highest factor loadings for each factor; [2]Factor loadings corresponding to the factors in the original version; [3]Factor loadings indicate highest loadings on other factors than the original ones. The factors "activity" and "abdominal symptoms" could not be reproduced; a new factors "sleep" and "nutrition" were found. CLDQ: Chronic liver disease questionnaire.

score, than non-cirrhotic patients. Significant difference was not detected for the following domains: "Systemic symptoms", "Activity", "Emotional function" and "Worry" (Table 4).

DISCUSSION

The CLDQ is a disease-specific instrument for assessment HRQoL in patients with CLD. It is reliable, reproducible, valid, short, easy to administer and economic questionnaire, which is validated and cross-culturally adapted into many different languages[8-18].

According to internationally accepted methodology for the validation of HRQoL questionnaires, we developed a Serbian version of CLDQ. Patients had no difficulty in understanding and completing the questionnaire. Only 4.8% of the patients required assistance in filling the questionnaire. The frequency of missing items is 0.06%, although this parameter seen in other studies varied from 0.4% to 23.5%[8,24].

In all validation studies CLDQ questionnaire shows outstanding reliability which ranged up to 0.96[9]. In our study, Cronbach's alpha is 0.93, for the overall scale which is the same as in Lithuanian[14], Greece[11], and the Spanish[10] versions. For all domains, internal reliability is acceptable, except for "Activity" where it is 0.49. However, this finding is in accordance with those obtained in Spanish[10] and Germany[8] validation study, where the Cronbach's alpha is 0.57 and 0.69, respectively. High reliability for this domain was found in Thais[9] and Pakistani[15] study.

The domain "Activity" includes three questions: No. 7 ("Not able to eat as much as you would like"), No. 9 ("Trouble lifting or carrying heavy objects") and No. 14 ("Limitation of diet"). The reason for the low internal reliability of this domain could be a cultural relationship between diet and disease in our population.

Exploratory factor analysis was carried out to establish whether the changes introduced in the Serbian version of CLDQ affected the structure of the questionnaire. The Serbian validated version confirmed the 6-domain structure of the original United States ver-

Table 4 Distribution of chronic liver disease questionnaire-S score/sub-score according disease severity

	n	Abdominal symptoms	Fatigue	Systemic symptoms	Activity	Emotional function	Worry	Total score
Non cirrhotic	23	5.37 ± 1.39[1]	4.81 ± 1.38[1]	5.54 ± 1.14	4.80 ± 1.32	4.40 ± 1.52	4.23 ± 1.54	4.86 ± 1.05
Child's class A	25	5.08 ± 1.65[1]	4.35 ± 1.30	5.34 ± 1.38	4.40 ± 1.39	4.62 ± 1.31	4.36 ± 1.70	4.69 ± 1.22
Child's class B	30	4.88 ± 1.58[1]	4.18 ± 1.51	5.25 ± 1.34	4.37 ± 1.59	4.77 ± 1.30	4.27 ± 1.61	4.63 ± 1.17
Child's class C	25	3.68 ± 1.46	3.52 ± 1.45	4.95 ± 0.99[2]	4.40 ± 1.37	4.60 ± 1.24	4.09 ± 1.65	4.31 ± 0.97
P value[3]		0.001	0.021	0.442	0.68	0.808	0.952	0.402

[1]Significantly better score compared with "cirrhosis Child's C" score (*post hoc* analysis); [2]For domain "systemic symptoms" (n = 23); [3]*P* value for Analysis of variance.

sion[5]. Such composition of the questionnaire has also been supported by the Hamburg[20] and Chinese (Hong Kong)[17]. The Italian version has five factors versions[13], while Spanish[10] and Greek[11] validated CLDQ revealed 7 factors. However, in our investigation the original structure was only partially reproduced. The most prominent changes are related to the fact that the factors "Activity" and "Abdominal symptoms" could not be replicated, and two new domains "Sleep" and "Nutrition" were established. In the validation studies of the CLDQ carried out in Italy[13], Spain[10], Germany[24] and Chinese (Hong Kong)[17] a new factor described as "Sleep" has already been found and composed of the same two items as in our analysis. Ferrer *et al*[10] pointed out that sleeping habits could vary among cultures (napping habits and bed-times) and therefore influenced cluster potential of sleeping-related items. Moreover, in Serbian version of CLDQ, the domain "Nutrition" has been introduced for the first time. This domain consisted of two items ("Not able to eat as much as would like" and "Restriction in diet") belonging to the "Activity" in original version of CLDQ. Keeping in mind the fact that the original factor "Activity" contains one additional question ("Trouble lifting or carrying heavy objects") that is not strictly related to the previous two, special allocation of the domain of nutrition makes the assessment of quality of life more sensitive. In accordance with our findings, the factor "Activity" could not also be reproduced in investigations conducted in Germany[24] and Italy[13]. Furthermore, majority of the studies dealing with the validation of this questionnaire have shown that the factor "Systemic symptoms" was difficult to be fully reproduced[10-13,24]. In our exploratory analysis the factor "Systemic symptoms" was also partially confirmed (3/5). In Serbian version two of five items in this original domain revealed a higher degree of belonging to the other factors. Namely, the item No. 6 ("Shortness of breath in daily activities") showed higher loadings on "Fatigue", and "Nutrition", while the question No. 27 ("Itching") revealed a higher degree of belonging to the domains of "Nutrition", "Worry" and "Sleep". Ferrer *et al*[10] found that three questions (No. 3, 6 and 23) derived from original "Systemic symptoms" had considerably higher loadings on more than one other factor. Additionally, in Spanish validated version, two items (No. 3 and 6) showed multiple loading on different factors. The other studies also confirmed the hypothesis that the original domain of "Systemic symptoms" consisted of items that could not be assigned clearly and strictly to any particular dimension[11,13].

Our results of factors analysis gave the evidence that at least some items from the original version of CLDQ should be allocated or eliminated from the questionnaire because of the multiple loadings. However, we do not yet want to recommend a change of domains because direct comparisons between the validated versions of CLDQ in different populations would no longer be possible.

The decreasing in total CLDQ score with increasing disease severity was shown in several studies[5,9-11,14,16,25-28]. Reduction of the total CLDQ-S score, between patients without cirrhosis and those with cirrhosis Child's C is 0.55 points, but without statistically significance. However, Younossi *et al*[5] described that a change of 0.5 points on the 1 to 7 point scale approximates the important difference in questionnaire score. Significant reduction of the CLDQ-S sub-score, with severe CLD is detected for the subscales "Abdominal symptoms" and "Fatigue".

In Germany validation study[8] a significant reduction of the CLDQ sub-score was detected for the domains "Abdominal symptoms", "Systemic symptoms", "Activity" and "Worry", while in Spanish validation[10] this finding was obtained for "Fatigue", "Activity" and "Worry". The US validation study reported a significant reduction of the CLDQ score and sub- score for domains: "Fatigue", "Systemic symptoms" and "Activity"[5]. Additionally, Sobhonslidsuk *et al*[9] has shown that severity of CLD affecting the quality of life in all domains of CLDQ, while Ray *et al*[16] confirmed these results for all subscales except for "Worry".

In our research, the etiology of CLD did not significantly affect the HRQoL, which is consistent with previously published results[14,28-30]. In patients with early stages of CLD, etiology does not affect HRQoL, while in patients with cirrhosis, cholestatic etiology is associated with better HRQoL, than hepatocellular CLD[25]. Ray *et al*[16] described that the etiology of CLD did not affect the overall score and most CLDQ sub-score, but had effect on sub-score "Abdominal symptoms" as well as the average scores for some questions. Etiology associated with a worse HRQoL are: chronic viral hepatitis C[16,31], nonalcoholic etiology[27] and non-alcoholic fatty liver disease[32]. In our validation sample, these results could not be reproduced, probably due to the small number of patients with chronic viral hepatitis C included in our study. Besides the impact of chronic hepatitis C and interferon therapy

has an impact on HRQoL[33]. However, data on its effects are controversial[7,33,34].

In conclusion, our results provide considerable support to the appropriate metric properties of the Serbian version of CLDQ. Therefore, it could be emphasized that the questionnaire might be reliable and valid instrument for indentifying HRQoL among liver disease patients and it can be used by health professionals in their clinical practices to improve assessment of patients, especially those with low scores of quality of life. Furthermore, the results reconfirmed psychometric characteristics of the questionnaire observed in other CLD patients populations.

COMMENTS

Background

Chronic liver disease (CLD) has a significant impact on health-related quality of life (HRQoL), and therefore its assessment is widely used as important outcome in clinical trials. The CLD questionnaire (CLDQ) is a specific quality of life instrument designed for patients with liver disease, regardless of the underlining severity and etiology of CLD. Its original version was developed by Younossi *et al* and has demonstrated appropriate validity and reliability.

Research frontiers

The CLDQ has already been cross-culturally adapted and validated into different languages in previously published studies. Up to now, there is no CLD-specific quality of life instruments adapted for Serbian patients. Therefore, the aim of this study was to investigate the validation of the translated and culturally adapted CLDQ questionnaire on a group of Serbian CLD patients.

Innovations and breakthroughs

The Serbian validation of the CLDQ confirmed the 6-domain structure of the original United States version. However, in our investigation the original structure was only partially reproduced. The most prominent changes are related to the fact that the factors "Activity" and "Abdominal symptoms" could not be replicated, and two new domains "Sleep" and "Nutrition" were established Moreover, the domain "Nutrition" has been introduced for the first time. Their results of factors analysis gave the evidence that at list some items from the original version of CLDQ should be allocated or eliminated from the questionnaire because of the multiple loadings.

Applications

The authors' results provide considerable support to the appropriate metric properties of the Serbian version of CLDQ. Therefore, it could be emphasized that the questionnaire might be reliable and valid instrument for indentifying HRQoL among liver disease patients and it can be used by health professionals in their clinical practices to improve assessment of patients, especially those with low scores of quality of life.

Terminology

Cross-cultural adaptation and validation procedures create a version of the original questionnaire in a target language that is conceptually equivalent to the origin instrument and psychometrically valid to allow for data pooling and cross-national comparisons.

Peer review

The manuscript investigated the validation of a CLDQ in Serbian CLD patients. The study is of clinical significance. The manuscript from Popovic *et al* reports the validation of a CLDQ in Serbian patients. Evaluation of health related quality of life is a very relevant issue as life expectancy for several chronic diseases has increased significantly. Adaptation of a preexisting questionnaire instead of proposing an alternative procedure allows comparison with results from other patient communities allowing cross-cultural validations and future refinements. Congratulations on this article which is well written and is a good initiative to better understnd the repercussions of CLD on the quality of life of these patients.

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BRIEF ARTICLE

Low-dose amitriptyline combined with proton pump inhibitor for functional chest pain

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Abstract

AIM: To investigate the efficacy of amitriptyline with proton pump inhibitor (PPI) for the treatment of functional chest pain (FCP).

METHODS: This was a randomized, open-label trial investigating the addition of low dose amitriptyline (10 mg at bedtime) to a conventional dose of rabeprazole (20 mg/d) (group A, *n* = 20) *vs* a double-dose of rabeprazole (20 mg twice daily) (group B, *n* = 20) for patients with FCP whose symptoms were refractory to PPI. The primary efficacy endpoints were assessed by global symptom score assessment and the total number of individuals with > 50% improvement in their symptom score.

RESULTS: The between-group difference in global symptom scores was statistically significant during the last week of treatment (overall mean difference; 3.75 ± 0.31 *vs* 4.35 ± 0.29, the between-group difference; *P* < 0.001). Furthermore, 70.6% of patients in group A had their symptoms improve by > 50%, whereas only 26.3% of patients in group B had a similar treatment response (70.6% *vs* 26.3%, *P* = 0.008). Specifically, patients in group A had a significantly greater improvement in the domains of body pain and general health perception than did patients in group B (52.37 ± 17.00 *vs* 41.32 ± 12.34, *P* = 0.031 and 47.95 ± 18.58 *vs* 31.84 ± 16.84, *P* = 0.01, respectively).

CONCLUSION: Adding amitriptyline to a PPI was more effective than a double-dose of PPI in patients with FCP refractory to a conventional dose of PPI.

Key words: Functional chest pain; Proton pump inhibitor; Amitriptyline

Core tip: Hypersensitivity and psychological problems have an important role in the pathogenesis of functional chest pain (FCP). In this regard, the principal treatment of FCP has moved towards hypersensitivity modulation and antidepressant agents on the basis that the underlying mechanisms were increased pain perception or visceral hyperalgesia in addition to psychologic causes. This is the first study to report that adding low-dose amitriptyline to a conventional dose of proton pump inhibitor (PPI) is more effective than a double-dose of PPI in patients with FCP resistant to a conventional dose of PPI treatment.

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INTRODUCTION

Noncardiac chest pain (NCCP) is a common condition that affects up to one third of the general population. Moreover, the effect of NCCP on an individual's quality of life and use of health care resources is considerable because evaluation of new patients with NCCP may require a variety of costly tests. Gastroesophageal reflux disease (GERD) is the most common cause of NCCP and is present in up to 60% of patients with NCCP in Western countries[1]. In addition, some patients with NCCP are regarded as having functional chest pain (FCP) by Rome Ⅲ criteria[2-4].

Despite extensive evidence indicating that the causes of FCP are visceral hypersensitivity and psychiatric pathology[5], the underlying mechanism for FCP has not been fully understood. This problem makes the treatment of FCP quite difficult. Indeed, therapeutic gains with a conventional dose of empirical PPI treatment may be obtained in only 9%-39% of patients with FCP[6,7]. A Cochrane review suggests that doubling the PPI dose is associated with greater relief of symptoms for those with NCCP. However, there is no clear PPI dose-response relationship for symptom resolution[8].

Reflecting recent interest, several authors have confirmed an important role for hypersensitivity in the pathogenesis of FCP[9,10]. Furthermore, psychological evaluation of patients with FCP has been suggested because a significant proportion may meet the criteria for panic disorder and depressive symptoms[11,12]. In this regard, several studies have assessed the psychological treatment of FCP with tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors, and serotonin-norepinephine reuptake inhibitors[11,13,14]. Hence, the principal treatment of FCP has moved towards hypersensitivity modulation and antidepressant agents on the basis that the underlying mechanisms are increased pain perception or visceral hyperalgesia in addition to psychologic causes[15,16]. Consequently, it would be reasonable to assume a beneficial effect of amitriptyline (a TCA) on the symptoms of FCP. As mentioned earlier, these drugs could reduce the severity of psychological manifestations which are thought to exacerbate the symptoms of FCP. In addition, amitriptyline has central analgesic actions, in addition to local pharmacological actions on the upper gut which specifically alter transit and gastric accommodation[16,17]. Although widely used for FCP, the combined therapy of PPI and antidepressant agents is not evidence-based. The purpose of this study is to investigate whether adding low-dose amitriptyline to a conventional dose of PPI is more effective than a double-dose of PPI in patients with FCP resistant to conventional dose of PPI treatment.

MATERIALS AND METHODS

Patients and study protocol

This was a single-center, prospective, randomized, open-label trial. Over 8 wk we investigated the addition of a subtherapeutic dose of amitriptyline to a conventional dose of PPI (group A) *vs* a double-dose of PPI (group B) for the treatment of refractory FCP. Consecutive patients were recruited for the study who presented to the Yonsei University Medical Center with persistent unexplained midline chest pain for a minimum of 3 mo, and who had a normal upper endoscopy, 24 h impedance esophageal pH monitoring, and esophageal manometry. Patients were only considered eligible for enrollment if they were free from cardiac, musculoskeletal, and pulmonary diseases, and if they had < 50% improvement of their global symptom scores after treatment with a conventional dose of PPI (rabeprazole 20 mg/d) for at least 1 mo. Patients were excluded if they had erosive esophagitis, Barrett's esophagus, other GERD-related disorders, or peptic ulcer disease during upper endoscopy. In addition, patients were excluded if they were unable to complete 24 h impedance esophageal pH monitoring or esophageal manometry, and if the results of these tests indicated GERD or a definite motility disorder. Finally, patients were excluded if they had a depressive disorder [Beck Depression Inventory (BDI) score > 19] or if they refused all procedures of this study. After signing a written informed consent patients were asked to complete a baseline Short-Form (SF-36) to generate quality of life (QOL) data, a global symptom score, and a BDI score. Enrollees were randomized by an independent investigator using a computer-generated random number table to one of two groups, whereby those in group A were treated with the combination of amitriptyline (10 mg at bedtime) and rabeprazole (20 mg/d), and those in group B were treated with a double-dose of rabeprazole (20 mg twice daily).

Efficacy was assessed by patient evaluation of global symptom relief scores using a daily symptom diary each week. At each visit the symptom diary was checked, side effects were reported, and compliance was assessed. At the end of the 8 wk study patients were asked to complete a final QOL questionnaire, global symptom and BDI scores were generated, and any side effects were reported. Primary efficacy endpoints were assessed by the subjective global symptom relief score and the total number of individuals with > 50% improvement in their symptom score. Secondary endpoints were related to QOL indices and the BDI score. The study described in this report was approved by the ethics committee of Yonsei University School of Medicine, Seoul, South Korea.

Demographics

All subjects completed a demographic questionnaire including age, gender, residence area, smoking and alcohol history, and body mass index (BMI).

Symptom assessment

The overall clinical assessment (global symptom score) was made using an analogue scale ranging from 0 (no symptoms) to 10 (intolerable), carried out immediately before treatment[18]. All patients were also questioned regarding symptoms possibly related to complications of the treatment. Subsequently, patients were contacted every week and re-evaluated for the presence of clinical symptoms. In addition, patients were asked to report to the center if any additional symptoms occurred during the study period. In both study groups the treatment of FCP was considered effective if the global symptom score improvement was > 50%[19].

SF-36

Health-related QOL was assessed with the SF-36, which contains 36 items that, when scored, yield eight domains. This approach was chosen because of its reliability and validity among both diseased and general populations, and given its usefulness in comparing the health burden of different conditions and the benefits of treatment. Specifically, the physical functioning domain (10 items) assesses limitations of physical activities, such as walking and climbing stairs. The physical role (4 items) and emotional role (3 items) domains measure problems with work or other daily activities as a result of physical health or emotional problems. The body pain domain (2 items) assesses limitations due to pain, and the vitality domain (4 items) measures energy and tiredness. The social functioning domain (2 items) examines the effect of physical and emotional health on normal social activities, and the mental health domain (5 items) assesses happiness, nervousness, and depression. Finally, the general health perception domain (5 items) evaluates personal health and the expectation of changes in health. All domains were scored on a scale from 0 to 100, with 100 representing the best possible health state.

Depression

We assessed depression using the BDI instrument. The BDI is a self-administered 21-item self-reported scale measuring supposed manifestations of depression. In particular, a BDI score between 9 and 18 implies mild-to-moderate depression, a score between 19 and 30 signifies moderate-to-severe depression, and a score > 30 implies severe depression. This score was measured before and after treatment.

Statistical analysis

On the basis of a previous meta-analysis with antidepressants[20], a two-sided comparison of the primary outcome variable with 17 patients per group, at the end of the treatment period, had the required 90% power and 5% type I error rate to detect a difference of 40% between the groups receiving additional amitriptyline and high dose PPI in the number of patients reporting > 50% improvement in symptoms (MedCalc® Version 12.3; MedCalc Software: Mariarkerke, Belgium). To allow for possible dropouts, defined as patients who failed to present or failed to follow the medication instructions, 20 subjects were required for each group.

To account for missing data, analysis of the primary and secondary endpoints was performed according to intention to treat (ITT) and per-protocol (PP) analyses. Descriptive statistics were provided for the binary and continuous variables using the incidence frequency (%) and the mean (standard distribution). The χ^2 test or Fisher's exact test was used to compare binary variables, and the two sample *t* test was used to compare continuous variables. Between-group differences of global symptom scores over time were analyzed using a linear mixed model with an unstructured residual covariance matrix. There were also two fixed effects that were assessed, including a between-subjects treatment effect (group A: amitriptyline + rabeprazole, group B: double dose of rabeprazole) and a within-subject time effect (time: week 0 to week 8). A possible group difference across time was analyzed by the group and time interaction effect. We also evaluated treatment effects of the drug on each SF-36 domain measured at baseline and during follow-up using a two sample *t* test. Two-sided *P* values were calculated with significance accepted at the 5% level. When necessary, the *P* value was adjusted by Bonferroni correction for multiple pair-wise comparisons. We primarily report the outcomes evaluated by ITT analysis since there were no differences for test results between ITT and PP analyses. All of the statistical analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC, United States).

RESULTS

Baseline characteristics

Figure 1 shows the progress of patients throughout the study. A total of 73 patients were enrolled in the study, all of whom had persistent unexplained midline chest pain for a minimum of 3 mo, a normal upper endoscopy, 24 h impedance esophageal pH monitoring, and esophageal manometry, and an improvement of their global symptom score by < 0% after treatment with a conventional dose of PPI (rabeprazole 20 mg/d) for at least 1 mo. The random assignment of patients into two arms resulted in 20 patients in group A designated to receive the addition of amitriptyline 10 mg once daily to rabeprazole 20 mg/d, and 20 patients in group B designated to receive a rabeprazole dose of 20 mg twice daily. Overall, 4 patients dropped out of the study, including three patients because of mild medication side effects (all of whom belonged to group A) and one who was lost to follow-up (this patient belonged to group B). Of the 36 patients who completed the 8-wk trial 17 were assigned to group A and 19 to group B. The ITT population consisted of 40 patients.

Baseline characteristics for each treatment group in the ITT population are summarized in Table 1. The mean age of individuals in group A was 51.1 ± 8.5 years *vs* 49.7 ± 9.59 years for those in group B. There was a slight

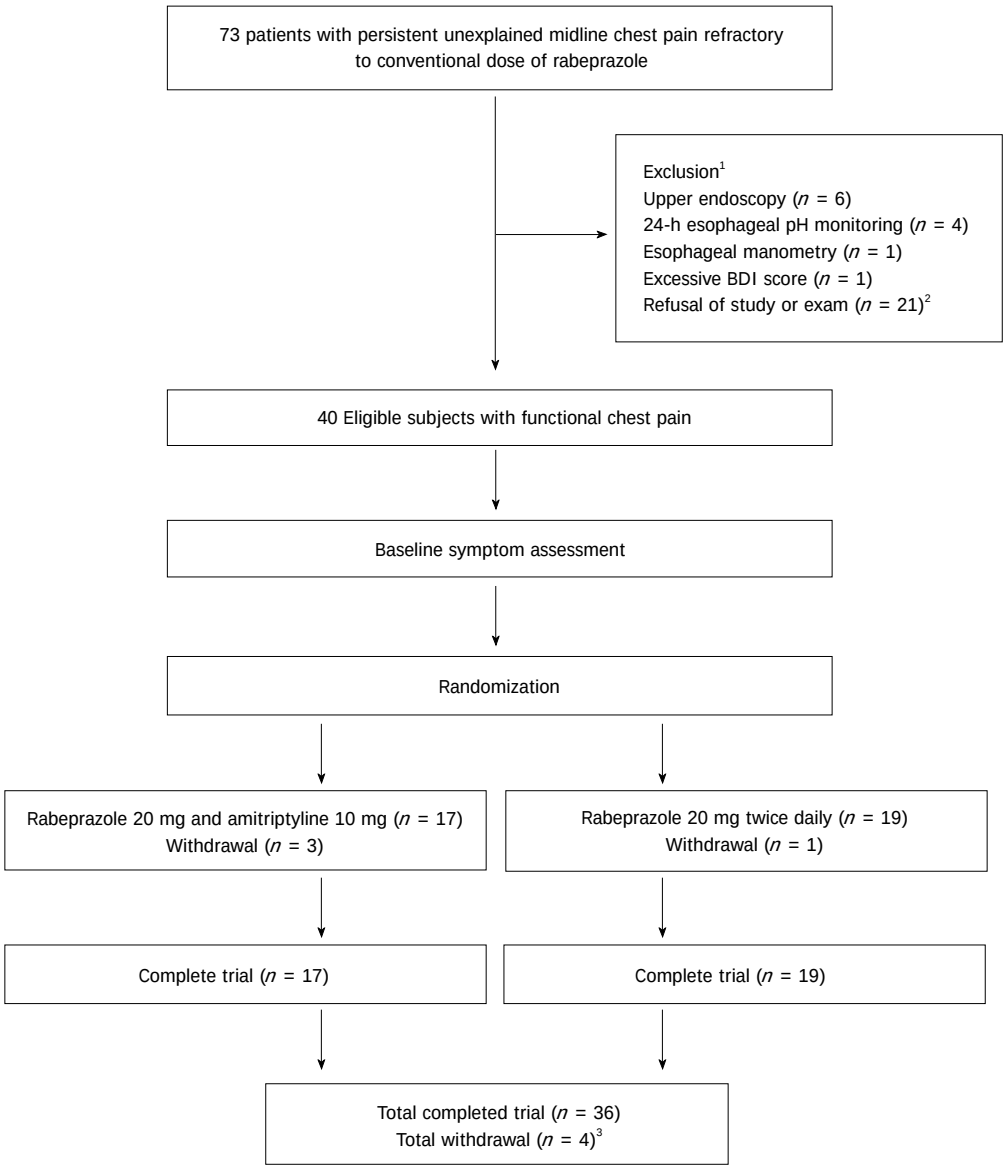

Figure 1 Flow of patients throughout the trial. [1]Upper gastrointestinal endoscopy showed erosive gastroesophageal reflux disease (n = 5), and peptic ulcer disease (n = 1). Pathological acid exposure was found in four patients by ambulatory 24 h esophageal pH monitoring. An esophageal motility disorder was found in one patient by esophageal manometric examination. The Beck Depression Index score of one patient exceeded 19 points; [2]Sixteen patients refused to take part in this study, and five patients refused examination by esophageal manometry or ambulatory 24 h esophageal pH monitoring; [3]Out of 4 patients, three in group A withdrew because of an amitriptyline-associated adverse event. One patient in group B dropped out of the trial because of loss to follow-up. BDI: Beck Depression Inventory.

female predominance in both groups: 55% in group A and 60% in group B. Of those in groups A and B respectively, 15% *vs* 20% used alcohol and 20% *vs* 45% smoked cigarettes. The mean BMI was 21.56 ± 1.74 for subjects in group A and 21.79 ± 2.2 in group B. There were no significant differences between group A and group B regarding symptom index, acid exposure time, and baseline BDI, indicating adequate randomization. Furthermore, subjects in both treatment groups of the ITT population showed generally similar values for most laboratory test results.

Global symptom score assessment

The global symptom scores over time associated with each treatment are shown in Figure 2. The overall mean difference between the two groups was not significantly different (3.75 ± 0.31 *vs* 4.35 ± 0.29, P = 0.172). However, we found that the time effect and time × group interaction effect were significant ($P < 0.001$ and $P = 0.006$, respectively). For instance, the global symptom scores significantly declined in group A. For group B, however, the global symptom scores decreased until week 2 and then somewhat increased after week 5 until the end of study follow-up (Table 2). Consequently, the between-group difference in global symptom scores was statistically significant at the last week ($P < 0.001$). Figure 3A shows the response rates in patients with FCP treated with amitriptyline and rabeprazole *vs* the double-dose of rabeprazole on the PP analysis. We found that 70.6% of amitriptyline and rabeprazole-treated patients showed im-

Table 1 Baseline characteristics of the intention to treat population

Variables	Group		*P* value
	A (*n* = 20)	B (*n* = 20)	
Age (yr)	51.1 ± 8.5	49.7 ± 9.59	0.628
Gender (female)	11 (55)	12 (60)	0.749
Alcohol	3 (15)	4 (20)	0.677
Smoking	4 (20)	9 (45)	0.091
BMI (kg/m^2)	21.56 ± 1.74	21.79 ± 2.2	0.716
Region (rural)	9 (52.94)	7 (36.84)	0.332
Symptom index[1]	5 (25)	5 (25)	-
Acid exposure time	0.52% ± 0.68%	0.44% ± 0.8%	0.719
Baseline BDI	6.9 ± 2.22	7.35 ± 1.93	0.498

Data are expressed as absolute numbers (percentage) or mean ± SD. [1]Symptom index indicated number of patients who has score of symptom index by > 50% on 24-h esophageal pH monitoring. BMI: Body mass index; BDI: Beck Depression Inventory.

Table 2 Global symptom scores by follow-up time

Time	Group A	Group B	*P* value[1]
Week 0	5.85 (5.09-6.61)	6.10 (5.34-6.86)	0.642
Week 1	4.90 (4.14-5.66)	5.10 (4.34-5.86)	0.709
Week 2	3.94 (3.08-4.80)	3.90 (3.06-4.74)	0.948
Week 3	3.76 (2.86-4.66)	3.95 (3.10-4.80)	0.755
Week 4	3.82 (2.96-4.68)	3.90 (3.10-4.70)	0.889
Week 5	3.18 (2.38-3.98)	3.80 (3.05-4.55)	0.259
Week 6	3.00 (2.21-3.79)	3.95 (3.21-4.69)	0.084
Week 7	2.89 (2.12-3.65)	4.05 (3.33-4.77)	0.031
Week 8	2.47 (1.68-3.27)	4.37 (3.63-5.11)	0.001

Data are least square means (95%CI). Group A: rabeprazole + amitriptyline group *vs* Group B: double dose of proton pump inhibitor group. [1]*P* value < 0.006 is considered statistically significant after adjusting the significance level of 0.05 using Bonferroni correction method for multiple comparisons. The significant difference between two groups was found at week 8 only (*P* < 0.006).

provement by > 50%, and that 29.4% failed to respond, given a response of < 50%. On the contrary, only 26.3% of patients showed a response to a double-dose of rabeprazole. This difference was significant (χ^2 = 7.06, *df* = 1, *P* = 0.008).

Health-related QOL assessment

Table 3 shows the health-related QOL as assessed by the SF-36 before and after treatment. There were no statistically significant differences between the two patient groups at baseline in any of the eight SF-36 domains. Moreover, the treatment effect at the end of the study was not significantly different between most domains of the SF-36, except for the body pain and general health perception factors. Patients who received amitriptyline and rabeprazole treatment had a significantly greater improvement in the domains of body pain and general health perception than those who received a double-dose of rabeprazole treatment (*P* = 0.031 and 0.01, respectively). The majority of the other domains of the SF-36 did not reach statistical significance.

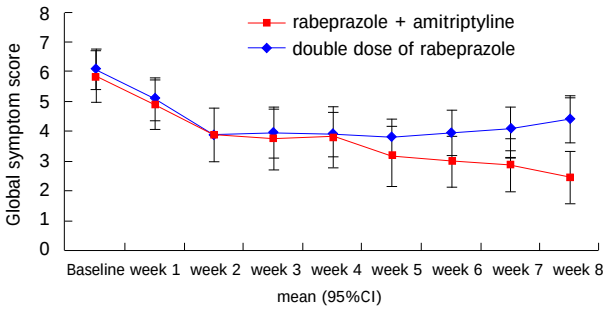

Figure 2 Efficacy of both treatment groups on the global symptom score. The global symptom scores over time was associated with each treatment. The overall mean difference between the two groups was not significantly different (*P* = 0.172). However, we found that the time effect and time × group interaction effect were significant (*P* < 0.001 and *P* = 0.006, respectively). Consequently, the between-group difference in global symptom scores was statistically significant at the last week (*P* < 0.001).

Depression

The overall mean difference of BDI at baseline and after the 8-wk treatment period was not significantly different between group A and group B (6.9 ± 2.22 *vs* 7.35 ± 1.93, *P* = 0.498, and 6.71 ± 1.99 *vs* 7.16 ± 1.89, *P* = 0.49, respectively). There was no significant difference in the depression score from baseline to the end of treatment in the double dose of PPI treatment group (*P* = 0.3). In the group receiving rabeprazole and amitriptyline, this result was marginally significant (*P* = 0.06). The change in value of the BDI scores associated with treatment is shown in Figure 3B.

Tolerability and safety assessment

Three patients withdrew from the study because of non life-threatening adverse events while receiving the combination of amitriptyline and rabeprazole (excessive sleeping, dizziness and general weakness).

DISCUSSION

The primary aim of this study was to determine the efficacy of low-dose amitriptyline with a conventional dose of PPI for the treatment of FCP with refractory symptoms to a conventional dose of PPI. We found favorable evidence for the efficacy of antidepressants in improving global symptom scores and health related QOL in patients with FCP refractory to conventional doses of PPIs. In other words, we found that adding low-dose amitriptyline to a conventional dose of PPI resulted in significantly decreased symptoms compared with a double-dose of PPI, with minimal side effects. Interestingly, this outcome is very similar to the open-label response to antidepressants seen with irritable bowel syndrome[21].

Anti-reflux therapy with PPIs plays an important role in the diagnosis and treatment of patients with NCCP because the major cause of NCCP is GERD, and since the management of NCCP is largely empirical[22,23]. However, in patients with non GERD-related NCCP (espe-

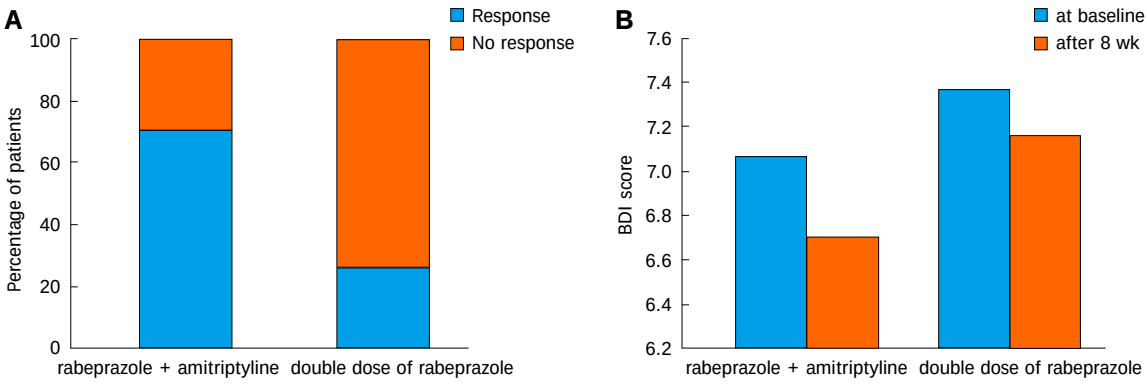

Figure 3 Symptom response rate (A) and Beck Depression Inventory scores (B) after treatment. A: The response rates in patients after treatment with rabeprazole + amitriptyline *vs* double-dose of rabeprazole on the per-protocol analysis are demonstrated. In this analysis, the difference of the response rate between both groups was statistically significant; B: The Beck Depression Inventory (BDI) scores after treatment with amitriptyline and rabeprazole *vs* double-dose of rabeprazole are represented. The overall mean difference of BDI scores after 8 wk of treatment was not significantly different between group A and group B (6.71 ± 1.99 *vs* 7.16 ± 1.89, respectively; $P = 0.49$).

Table 3 Treatment effect on the health-related quality of life assessed with Short-Form 36

SF-36 score	Group		*P* value
	Group A (n = 20)	Group B (n = 20)	
Physical functioning			
Baseline	42.01 ± 13.47	32.83 ± 17.06	0.211
End of treatment	37.28 ± 12.76	38.42 ± 7.83	0.753
Role -physical			
Baseline	31.51 ± 14.9	29.45 ± 15.51	0.670
End of treatment	37.88 ± 10.16	39.02 ± 12.49	0.768
Role-emotional			
Baseline	32.01 ± 12.12	38.39 ± 12.68	0.112
End of treatment	36.99 ± 12.62	30.14 ± 16.95	0.182
Social functioning			
Baseline	37.95 ± 14.06	33.82 ± 13.90	0.356
End of treatment	36.41 ± 13.03	30.07 ± 12.83	0.151
Body pain			
Baseline	34.94 ± 14.39	30.13 ± 12.09	0.260
End of treatment	52.37 ± 17.00	41.32 ± 12.34	0.031
General health perceptions			
Baseline	38.63 ± 11.66	31.82 ± 12.94	0.088
End of treatment	47.95 ± 18.58	31.84 ± 16.84	0.010
Mental health			
Baseline	38.82 ± 14.72	39.20 ± 10.67	0.927
End of treatment	44.69 ± 10.79	38.88 ± 10.50	0.111
Energy/vitality			
Baseline	38.08 ± 12.50	34.39 ± 12.64	0.360
End of treatment	42.95 ± 15.32	35.14 ± 10.87	0.084

All results are expressed as mean ± SD. Group A received amitriptyline and rabeprazole and group B received double dose of rabeprazole. *P* values are for the comparison of amitriptyline and rabeprazole *vs* double dose of rabeprazole at baseline and end of the treatment. Patients who received amitriptyline and rabeprazole treatment had a significantly greater improvement in the domains of body pain and general health perception than those who received a double dose of rabeprazole treatment ($P = 0.031$ and $P = 0.01$, respectively). The majority of the other domains of the Short-Form 36 (SF-36) did not reach statistical significance.

cially those refractory to conventional doses of PPI), treatment should be targeted to alternative drugs, such as pain modulating agents[24]. Indeed, therapeutic gains with PPI treatment have been obtained in only 9%-39% of non GERD-related NCCP patients[6,22,25]. Recent studies have focused on modulating nociception and visceral pain sensation pathways for decreasing chest pain using antidepressants[11,26]. For these reasons, it would be reasonable to assume a beneficial effect of antidepressant drugs, such as TCAs, on the symptoms of FCP. In fact, to investigate the efficacy of antidepressant treatments for FCP, one meta-analysis of seven studies and 319 participants indicated that there was strong evidence for an association of antidepressants with a reduction in pain and psychological symptoms. However, the drugs assessed in this analysis were varied and included a TCA, selective serotonin reuptake inhibitors, and a serotonin-norepinephine reuptake inhibitor[14]. Therefore, the possible effect of amitriptyline for FCP is unclear. Moreover, until now there has been no randomized controlled study to investigate the effects of combining TCAs with PPIs for the treatment of FCP refractory to conventional therapy.

The results of our clinical study suggest that FCP may respond favorably to low-dose amitriptyline in combination with a PPI. Eventually the symptomatic overlap with functional gastrointestinal disorders, the recognized association of functional dyspepsia with visceral hypersensitivity, and the response of several other functional gut disorders to TCAs may all hold clues to the seeming success in our patients. In our study, we used doses of amitriptyline far below those necessary for an antidepressant benefit. This likely explains the positive association of amitriptyline for reducing pain in the absence of a benefit for depressive symptoms. The synergistic effects of amitriptyline in combination with a conventional dose of PPI could have an important role in improving QOL of those with FCP. Because the therapeutic effects of amitriptyline are usually achieved within 4-6 wk, the duration of our trial was considered adequate for evaluating the efficacy of this drug. Our results show that an 8-wk treatment regimen with amitriptyline and rabeprazole significantly improved the global symptom score given the response rate of 70.6%, which was in comparison to the

response rate for a double-dose of rabeprazole of only 26.3%. However, analysis of the SF-36 as a QOL measurement showed satisfactory efficacy in only two domains. This might have been caused by the small sample size, which may have been inadequate for detecting differences in secondary outcomes, although it was adequate for detecting the required difference in the primary outcome variable.

Our study has a few limitations. The open-label nature of this study could lead to some biases with generalization of the results. The sample size was also relatively small and further investigation based on a larger number of patients is necessary to corroborate our data. Finally, our study duration was relatively short. Indeed, the short duration of most studies and the lack of follow-up after treatment cessation leave the question unanswered whether antidepressants have long-term beneficial effects on FCP symptoms, as well as the optimal treatment duration. Nevertheless, this study is of value because it is the first study examining the efficacy of amitriptyline on patients with FCP with symptoms refractory to a conventional dose of PPI. We did not encounter major or unexpected side effects related to amitriptyline.

In conclusion, the combination of low-dose amitriptyline with a standard PPI regimen was more effective than a double-dose of PPIs in patients with FCP refractory to conventional PPI therapy, without serious adverse events. The safety profile and efficacy in the subjects using low-dose amitriptyline as well as the significant improvement in global symptom scores may justify the addition of amitriptyline for the treatment of refractory FCP.

COMMENTS

Background

Despite extensive evidence indicating that the causes of functional chest pain (FCP) are visceral hypersensitivity and psychiatric pathology, the underlying mechanism for FCP is largely unknown. This problem makes the treatment of FCP quite difficult.

Research frontiers

The principal treatment of FCP has moved towards hypersensitivity modulation and antidepressant agents on the basis that the underlying mechanisms were increased pain perception or visceral hyperalgesia in addition to psychologic causes. In this study, the authors demonstrate that amitriptyline, as a tricyclic antidepressant, would appear to have a beneficial effect on the symptoms of FCP.

Innovations and breakthroughs

Recent reports have highlighted the psychological treatment of FCP with tricyclic antidepressants, selective serotonin reuptake inhibitors, and serotonin-norepinephine reuptake inhibitors. This is the first study to report that adding low-dose amitriptyline to a conventional dose of proton pump inhibitor (PPI) is more effective than a double-dose of PPI in patients with FCP resistant to a conventional dose of PPI treatment.

Applications

The authors' result demonstrates that the safety profile and efficacy in the subjects using low-dose amitriptyline as well as the significant improvement in global symptom scores may justify the addition of amitriptyline for the treatment of refractory FCP.

Terminology

FCP was defined as recurrent angina-like retrosternal chest pain with normal coronary anatomy and no detectable gastroenterological and respiratory causes after an adequate evaluation by Rome Ⅲ criteria. The SF-36 as health-related quality of life contains 36 items that, when scored, yield eight domains. The BDI instrument as assessment for anxiety and depression is a self-administered 21-item self-reported scale measuring supposed manifestations of depression.

Peer review

The authors have focused on modulating nociception and visceral pain sensation pathways for decreasing chest pain using antidepressants. It revealed that the combination of low-dose amitriptyline with a standard PPI regimen was more effective than a double-dose of PPIs in patients with FCP refractory to conventional PPI therapy, without serious adverse events.

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BRIEF ARTICLE

Residual common bile duct stones on direct peroral cholangioscopy using ultraslim endoscope

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Abstract

AIM: To detect and manage residual common bile duct (CBD) stones using ultraslim endoscopic peroral cholangioscopy (POC) after a negative balloon-occluded cholangiography.

METHODS: From March 2011 to December 2011, a cohort of 22 patients with CBD stones who underwent both endoscopic retrograde cholangiography (ERC) and direct POC were prospectively enrolled in this study. Those patients who were younger than 20 years of age, pregnant, critically ill, or unable to provide informed consent for direct POC, as well as those with concomitant gallbladder stones or CBD with diameters less than 10 mm were excluded. Direct POC using an ultraslim endoscope with an overtube balloon-assisted technique was carried out immediately after a negative balloon-occluded cholangiography was obtained.

RESULTS: The ultraslim endoscope was able to be advanced to the hepatic hilum or the intrahepatic bile duct (IHD) in 8 patients (36.4%), to the extrahepatic bile duct where the hilum could be visualized in 10 patients (45.5%), and to the distal CBD where the hilum could not be visualized in 4 patients (18.2%). The procedure time of the diagnostic POC was 8.2 ± 2.9 min (range, 5-18 min). Residual CBD stones were found in 5 (22.7%) of the patients. There was one residual stone each in 3 of the patients, three in 1 patient, and more than five in 1 patient. The diameter of the residual stones ranged from 2-5 mm. In 2 of the patients, the residual stones were successfully extracted using either a retrieval balloon catheter (n = 1) or a basket catheter (n = 1) under direct endoscopic control. In the remaining 3 patients, the residual stones were removed using an irrigation and suction method under direct endoscopic visualization. There were no serious procedure-related complications, such as bleeding, pancreatitis, biliary tract infection, or perforation, in this study.

CONCLUSION: Direct POC using an ultraslim endoscope appears to be a useful tool for both detecting and treating residual CBD stones after conventional ERC.

Key words: Balloon-occluded cholangiography; Common bile duct stones; Endoscopic retrograde cholangiography; Peroral cholangioscopy; Residual stones

Core tip: Balloon-occluded cholangiography is generally performed to confirm bile duct clearance after performing endoscopic retrograde cholangiography (ERC) for stone retrieval. However, balloon-occluded cholangiography may be an imperfect tool for this diagnostic purpose. In this case series, we demonstrated that 22.7%

of patients still had residual stones detected on peroral cholangioscopy after a negative balloon-occluded cholangiography was obtained. All of the residual stones were retracted on the cholangioscopy. Our results reveal that peroral cholangioscopy appears to be a useful tool for both detecting and treating residual common bile duct stones after conventional ERC.

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INTRODUCTION

Endoscopic sphincterotomy (ES) has become the cornerstone of therapeutic endoscopic retrograde cholangiography (ERC). It is most commonly performed to remove common bile duct (CBD) stones[1-3]. Endoscopic papillary balloon dilatation (EPBD) has been used as an alternative approach to ES[4,5]. After ES/EPBD for stone retrieval, balloon-occluded cholangiography is generally performed to confirm bile duct clearance. However, small stones may be left undetected by the balloon-occluded cholangiography[6-8]. These small stone fragments run the risk of acting as nidi for future stone formation, leading to the recurrence of CBD stones[9-11]. Therefore, it is crucial to achieve a level of stone clearance that is as complete as possible to prevent stone recurrence. Intraductal ultrasound (IDUS) has been applied to confirm the clearance of CBD stones after stone retrieval by ES[6,9,12]. Tsuchiya *et al*[9] reported that performing IDUS after stone extraction decreased the recurrence rate of CBD stones to 3.4% from the 13.2% recurrence rate of the control group. However, IDUS poses problems, such as probe fragility and a high cost. In addition, it is a highly operator-dependent technology. Poor images and the consequent oversight of residual stones are possible, especially in patients with extensive pneumobilia[9]. Ohashi *et al*[6] reported that IDUS examination failed to detect residual stones after bile duct clearance in 14.6% (6/41) of the patients.

Cholangioscopy offers a crucial advantage over IDUS in that it permits direct visualization of the bile duct and further management of any bile duct stones[13-15]. Direct peroral cholangioscopy (POC) may play a role in the detection of bile duct stones, but there is a lack of studies on this subject[16]. Although conventional POC using a mother-baby endoscopic system has been available for more than three decades, its role remains limited because of its many disadvantages[17,18]. Recently, direct POC using an ultraslim endoscope has been reported to be feasible and superior to the conventional mother-baby endoscopic system because it provides superior endoscopic images and a larger working channel[19-22]. Furthermore, it can be performed by a single endoscopist. The aim of this study is to evaluate the utility of ultraslim endoscopic POC in the diagnosis and management of residual CBD stones after performing bile duct clearance and confirming the procedure using balloon-occluded cholangiography.

MATERIALS AND METHODS

Patients

From March 2011 to December 2011, the patients who underwent ERC performed by the two endoscopists (Tsou YK and Lin CH) in Chang Gung Memorial Hospital who met the following criteria were prospectively enrolled in this study: CBD stones were diagnosed based on imaging studies, such as abdominal ultrasonography, computed tomography scans, and/or magnetic resonance cholangiopancreatography before the index ERC (n = 92); The exclusion criteria were as follows: (1) patients who were younger than 20 years of age, pregnant, or critically ill (n = 5); (2) patients with concomitant gallbladder stones (n = 42); (3) patients with CBD diameters of less than 10 mm (n = 9); and (4) patients who were unable to provide informed consent for POC (n = 14).

ERC

ERC was performed using a duodenoscope (JF or TJF 260-V, Olympus, Tokyo, Japan) under conscious sedation with the patients in a prone position. ES was performed using a standard pull-type sphincterotome (Ultratome; Boston Scientific Co., Spencer, IN, United States). EPBD was carried out using a controlled radial expansion (CRE) balloon. During the index ERC, EPBD was carried out in 13 patients (including the 8 patients who had previously undergone ES or EPBD); ES then EPBD were performed in 6 patients (including 1 patient who had received a previous ES); and extended ES was performed in 1 patient to facilitate the stone extraction and/or the performance of the POC. Two other patients did not undergo ES or EPBD during the index ERC because the papillary orifice created by EPBD during the previous procedure(s) was large enough to permit stone extraction and performing the POC.

A contrast medium at a 1:1 dilution was used for the cholangiography. The instruments used to extract the CBD stones included a retrieval balloon catheter alone (n = 16) and a combination of a balloon and a basket catheter (n = 6). Two patients underwent lithotripsy because the CBD stones were difficult to extract using a balloon catheter. After the stone extraction, balloon-occluded cholangiography was performed to confirm the complete clearance of the CBD stones. If any residual stones were observed during the balloon-occluded cholangiography, additional endoscopic treatments were performed until the balloon-occluded cholangiography was negative (Figure 1).

Direct peroral cholangioscopy

An ultraslim endoscope (GIF-N260, Olympus) and an

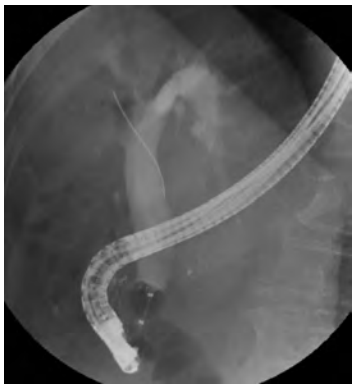

Figure 1 Balloon-occluded cholangiography failed to reveal any filling defects of stones in the biliary tree after endoscopic retrograde cholangiography with bile duct clearance.

overtube (ST-SB1, Olympus) were used for the POC procedures. All of the POC procedures were performed by two endoscopists (Tsou YK and Lin CH) who are experienced in this endoscopy and were carried out immediately after a negative balloon-occluded cholangiography was obtained during a single endoscopic session. The details of the POC procedures are described in our previous study[20]. Briefly, the overtube containing the endoscope is advanced into the distal gastric antrum (or into the afferent loop for patients with a post-operative stomach); the overtube balloon is then inflated to anchor the overtube. The endoscope is further advanced into the orifice of the major papilla either directly or after performing a J-turn of the endoscopic tip. Then, the endoscope is advanced into the bile duct as far as possible. The POC time is defined as the interval between the ultraslim endoscope entering the mouth of the patient and reaching the farthest site of the biliary tree.

In the text and tables, the continuous variables are expressed in the form mean ± SD. The study protocol was approved by the ethical committee at Chang Gung Memorial Hospital (IRB No: 99-2585C).

RESULTS

A cohort of 22 patients with CBD stones undergoing both ERC and direct POC were prospectively enrolled in this study (Table 1). The patient age was 73.4 ± 11.8 years (range, 40-89 years), and 15 (68%) of the patients were men. The patients were categorized into the following gallbladder status groups: intact without stones (n = 3), post laparoscopic cholecystectomy (n = 2), and post open cholecystectomy (n = 17). Eight patients (36.4%) had juxtapapillary diverticulum. Three patients (13.6%) had a medical history of subtotal gastrectomy with Billroth-Ⅱ anastomosis (n = 2) or total gastrectomy with Roux-en-Y anastomosis (n = 1).

The ERC results are listed in Table 2. The maximum diameter of the CBD was 17.9 ± 5.1 mm (range, 10-30 mm). Twelve of the patients (54.5%) had recurrent CBD stones. The number of CBD stones was one each in 10 patients, two in 2 patients, three in 4 patients, and more than three in 6 patients. The maximum stone diameter was 13.4 ± 5.6 mm (range, 5-25.4 mm). Sixteen patients had brown stones, 2 patients had black stones, and 4 patients had mixed brown and black stones. During the index ERC, the CBD stones were removed intact in 11 patients (50%).

The results of POC are given in Table 3. The POC time was 8.2 ± 2.9 min (range, 5-18 min). The ultraslim endoscope was able to be advanced to the hepatic hilum or the intrahepatic bile duct (IHD) in 8 patients (36.4%) (Figure 2); to the extrahepatic bile duct where the hilum could be visualized in 10 patients (45.5%); and to the distal CBD where the hilum could not be visualized in 4 patients (18.2%). During POC, residual CBD stones were found in 5 patients (22.7%) (Figure 3). One residual stone was found in each of 3 patients, three in 1 patient, and multiple (more than five) in 1 patient. The diameter of the residual stones ranged from 2-5 mm. In 2 patients, the residual stones were extracted successfully using a retrieval balloon catheter (n = 1) or a basket catheter (n =

Table 1 Patient characteristics *n* (%)

Characteristics	*n* = 22
Age (yr)	73.4 ± 11.8 (range, 40-89)
Gender (male)	15 (68)
Prior cholecystectomies[1]	19 (86.4)
Acalculous gallbladder	3 (13.6)
Juxtapapillary diverticulum	8 (36.4)
Subtotal gastrectomy with Billroth-Ⅱ anastomosis	2 (9.1)
Total gastrectomy with Roux-en-Y anastomosis	1 (4.5)
Patients with recurrent CBD stones	12 (54.5)

[1]Including 17 cases of open cholecystectomy and 2 cases of laparoscopic cholecystectomy. CBD: Common bile duct.

Table 2 Results of endoscopic retrograde cholangiography *n* (%)

Characteristics	*n* = 22
CBD stones	
No. of stones (one/two/three/more than three)	10/2/4/6
Mean maximum diameter (mm)	13.4 ± 5.6 (range, 5-25.4)
Mean maximum diameter of CBD (mm)	17.9 (range, 10-30)
ES and/or EPBD	
ES	1 (4.5)
EPBD	13 (59.1)
ES + EPBD	6 (27.3)
None[1]	2 (9.1)
Mean maximum inflated diameter during EPBD[2] (mm)	13.6 (range, 12-15)
Stone extraction methods	
Balloon and/or basket	20 (90.9)
Mechanical lithotripter	2 (9.1)
Intact stone extraction	11 (50)

[1]Endoscopic papillary balloon dilatation (EPBD) was performed during the previous endoscopic session, and the papillary orifice was adequate; [2]A total of 15 cases underwent EPBD. CBD: Common bile duct; ES: Endoscopic sphincterotomy.

Table 3 Results of peroral cholangioscopy *n* (%)

Characteristics	*n* = 22
Mean procedure time (min)	8.2 (range, 5-18)
The endoscope reached	
Hilum or IHD	8 (36.4)
CBD and the hilum was seen	10 (45.5)
Distal CBD and the hilum was not seen	4 (18.2)
Residual stones on the POC	
No. of patients	5 (22.7)
No. of residual stones (one/three/multiple)	3/1/1
Maximum diameter of stones (range, mm)	2-5

IHD: Intrahepatic duct; CBD: Common bile duct; POC: Peroral cholangioscopy

Table 4 Clinical features between the patients with and without residual bile duct stones *n* (%)

	With residual stones (*n* = 5)	Without residual stones (*n* = 17)
Age (yr)	69 ± 18.6	74.7 ± 9.5
Sex (male)	4 (80)	11 (64.7)
Recurrent CBD stones	4 (80)	7 (41.2)
Prior choledocholithotomy	3 (60)	6 (35.3)
Mean maximum CBD diameter (mm)	19 ± 7.6	17.6 ± 4.5
Stones number (single)	2 (40)	8 (47)
Mean maximum stone diameter (mm)	14.1 ± 7.2	13.2 ± 5.2
Lithotripsy	0 (0)	2 (11.8)
Intact stone extraction	1 (20)	10 (58.9)
Diameter of EPBD[1] (mm)	13.5 ± 1.7 (*n* = 4)	13.5 ± 1.4

[1]Mean maximum inflated diameter of the balloon during endoscopic papillary balloon dilatation (EPBD). CBD: Common bile duct.

1) under direct endoscopic visualization (Figure 4). In the remaining 3 patients, the residual stones were removed using direct endoscopic suction after normal saline irrigation. For the patient with multiple residual stones, a balloon catheter was inserted proximally to the stones through the endoscope and the endoscopic tip was placed distally to the stones. Using synchronic normal saline irrigation *via* the balloon catheter and endoscopic suction, the stones and the endoscope were slowly pulled down to the distal bile duct and finally to the duodenum. No serious procedure-related complications, such as bleeding, pancreatitis, biliary tract infection, or perforation, were observed in this study. The ultraslim endoscope used as the cholangioscope did not sustain any obvious damage during the study period.

The clinical data on the patients with (*n* = 5) and without (*n* = 17) residual stones on POC are listed in Table 4. Because the patient sample size was small, we did not perform any statistical analysis. However, recurrent CBD stones and prior choledocholithotomy were more frequently observed in the patients with residual stones than in the patients without (80% *vs* 41.2% and 60% *vs* 35.3%, respectively). Intact stone extraction during the index ERC was less common in patients with residual stones than in patients without (20% *vs* 58.9%, respectively). These patients were followed up for 17.5 ± 4.9 mo (range, 12-24 mo) after the POC. Four patients (18.2%) had recurrent CBD stones documented on ERC during a follow-up; two of these four had residual stones on POC.

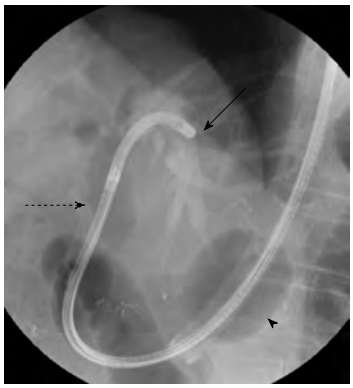

Figure 2 Ultraslim endoscope was advanced to the common bile duct (dotted arrow) and up to the left intrahepatic duct (arrow) using an over-tube balloon (arrow head)-assisted technique.

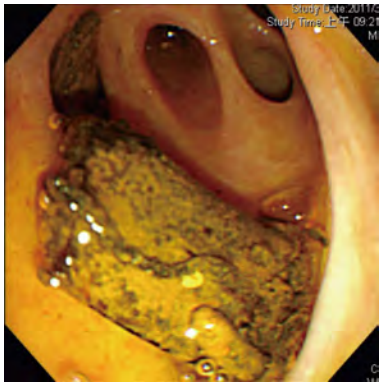

Figure 3 Residual stones in the bile duct visualized using direct peroral cholangioscopy.

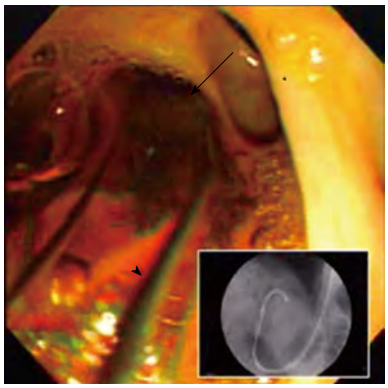

Figure 4 A basket catheter (arrow head) was used to retrieve the residual bile duct stones (arrow) under direct endoscopic control.

DISCUSSION

Balloon-occluded cholangiography is an imperfect tool to confirm complete bile duct clearance after ES/EPBD for

stone retrieval. In this study, we observed that balloon-occluded cholangiography failed to detect residual CBD stones in 22.7% of the patients. Tsuchiya *et al*[9] used IDUS and reported that balloon-occluded cholangiography did not detect any residual CBD stones in 23.7% (14/59) of the patients[9]. Itoi *et al*[7] performed POC using a mother-baby system 0-20 d (median, 6.2 d) after ERC with CBD stone retrieval. In all of these patients, the bile duct was confirmed to be free of stones using balloon-occluded cholangiography. It was later found that 24% (26/108) of these patients had residual CBD stones on the POC, although in some of the patients, the CBD stones might have migrated from the gallbladder after the stone retrieval. Our results are consistent with those of other studies, revealing that balloon-occluded cholangiography fails to detect residual CBD stones in nearly one quarter of patients.

The residual CBD stones not detected by balloon-occluded cholangiography in the previous reports using IDUS were usually small[23-25]. The present study confirmed (using POC) that the undetected residual CBD stones are small and no more than 5 mm in diameter. It is unclear whether these small residual stones have clinical significance[26]. Because the orifice of the major papilla after ES/EPBD is large enough, spontaneous passage of the stones is possible. However, Itoi *et al*[7] performed POC an average of 6 d after stone retrieval by ERC and found that 24% of the patients still had residual stones. Their result suggests that residual stones might not be excreted for a long time and may eventually cause stone recurrence. Several studies that have aimed to analyze the risk factors for recurrent CBD stones suggest that residual stones are a possible cause of recurrent CBD stones[9,10,27]. Therefore, it may be important to detect and remove residual CBD stones after ES/EPBD for stone retrieval. Further studies are needed to clarify whether the residual stones have clinical significance and whether the removal of residual stones minimizes the risk of stone recurrence.

The ultraslim endoscopic POC has several advantages over the mother-baby endoscopic system[20,21]. One is that it enables the extraction of residual CBD stones under direct endoscopic visualization, as demonstrated in the present study. In contrast, the residual stones in the 26 patients reported by Itoi *et al*[7] were not directly removable using the mother-baby system. A 5-Fr balloon or basket catheter can pass through the 2-mm working channel of the ultraslim endoscope to grasp the residual stones. However, to remove the grasped stones, the ultraslim endoscope with the balloon/basket must be withdrawn to the duodenum. This maneuver can be complicated when there are multiple residual stones. In this case, the irrigation and suction method described above offers an effective way to remove the stones, especially for small soft stones. Placing the tip of a balloon catheter proximally to the stones can avoid flushing them upstream.

There are two major limitations to this study. One is that the hilum or IHD could be reached by the POC in only 36.4% of the patients. For the purpose of this study, seeing the hilum may be enough to verify bile duct clearance, and in 71.8% of the patients, the hilum could be seen. However, in 18.2% of the patients, the hilum could not be seen by the POC. One possible reason for this limitation was that many (41%) of our patients had undergone choledocholithotomy with T-tube drainage, resulting in a tortuous CBD. Direct POC using an intraductal anchoring balloon method may be able to improve the rate of seeing the hilum, but the anchoring device was withdrawn from the market due to an increased risk of air embolism[28]. Therefore, the rate of residual CBD stones may be higher if some of the residual stones were not visualized during the POC in our patients.

The other limitation is that we enrolled only patients without GB stones or without GB to avoid the risk of "migrated GB stones" being confused with the residual stones. As a result, 19 of the 22 patients (86.4%) had a previous cholecystectomy. Therefore, the study results may be only applied to this subgroup of patients. In this study, we found that recurrent CBD stones as an indication for index ERC, prior choledocholithotomy, and fragmented stones during stones retrieval on the index ERC were more frequently observed in patients with residual stones. It might be worthwhile to perform POC for patients with these characteristics.

In conclusion, conventional ERC with balloon-occluded cholangiography is not a reliable method for confirming the complete extraction of CBD stones. Direct POC using an ultraslim endoscope appears to be a useful tool to confirm the clearance of CBD stones and to extract the residual CBD stones in selected patients.

COMMENTS

Background

Balloon-occluded cholangiography is generally performed to confirm bile duct clearance after bile duct stone retrieval. However, small stones may remain undetected on the balloon-occluded cholangiography.

Research frontiers

Cholangiography is a direct image which is at least theoretically better than the indirect image of balloon-occluded cholangiography. Therefore, the authors perform direct peroral cholangiography (POC) to examine if there are residual bile duct stones after obtaining a negative balloon-occluded cholangiography. The method of POC is using an ultraslim endoscope with overtube balloon-assisted technique.

Innovations and breakthroughs

The authors demonstrate that 22.7% of the patients still have residual stones detected on the direct POC after a negative balloon-occluded cholangiography is obtained. All of the residual stones are small (range 2-5 mm) and extracted successfully during the POC.

Applications

The results indicate that direct POC using an ultraslim endoscope appears to be a useful tool for both detecting and treating residual bile duct stones after conventional endoscopic retrograde cholangiography.

Terminology

Direct POC is to insert an endoscope perorally into the bile ducts. Direct POC using a mother-baby endoscope system is not widely used because of its many disadvantages. Recently, direct POC using an ultraslim endoscope has been

reported to be feasible and superior to the conventional mother-baby endoscopic system because it provides superior endoscopic images and a larger working channel. Furthermore, it can be performed by a single endoscopist.

Peer review

In this study, the value of peroral cholangioscopy for detecting remaining bile duct stones after balloon-occluded cholangiography was evaluated in 22 patients. Despite a negative balloon-occluded cholangiography, additional bile duct stones were detected in 5 patients (23%). The stone diameter was generally small (2-5 mm). Unfortunately the authors did not perform intraductal ultrasound in their 22 patients simultaneously, that would have allowed comparison of the two methods.

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BRIEF ARTICLE

A systematic analysis of pneumatosis cystoids intestinalis

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Abstract

AIM: To increase the understanding, diagnosis and treatment of pneumatosis cystoides intestinalis (PCI) and to find the characteristics and potential cause of the disease in China.

METHODS: We report here one case of PCI in a 70-year-old male patient who received a variety of treatment methods. Then, we systematically searched the PCI eligible literature published from an available Chinese database from May 2002 to May 2012, including CBM, CBMDisc, CMCC, VIP, Wanfang, and CNKI. The key words were pneumatosis cystoides intestinalis, pneumatosis, pneumatosis intestinalis, pneumatosis coli and mucosal gas. The patients' information, histories, therapies, courses, and outcomes were reviewed.

RESULTS: The study group consisted of 239 PCI cases (male:female = 2.4:1) from 77 reported incidents. The mean age was 45.3 ± 15.6 years, and the median illness course was 6 mo. One hundred and sixty patients (66.9%) were in high altitude areas. In addition, 43.5% (104/239) of the patients had potential PCI-related disease, and 16.3% had complications with intestinal obstruction and perforation. The most common symptom was abdominal pain (53.9%), followed by diarrhea (53.0%), distention (42.4%), nausea and vomiting (14.3%), bloody stool (12.9%), mucous stool (12.0%) and constipation (7.8%). Most multiple pneumocysts developed in the submucosa of the colon (69.9%). The efficacy of the treatments by combined modalities, surgery, endoscopic treatment, conservative approach, oxygen, and antibiotics were 100%, 100%, 100%, 93.3%, 68.3% and 26.3%, respectively.

CONCLUSION: PCI can be safely managed by conservative treatments, presents more frequently in males, in the large bowel and submucosa, than in females, in the small intestine and subserosa. High altitude residence maybe associated with the PCI etiology.

Key words: Pneumatosis cystoides intestinalis; Pneumatosis; Cyst; Intestinal; Colon

Core tip: Pneumatosis cystoids intestinalis (PCI) is a rare disease characterized by the presence of multiple gas-filled cysts in the submucosa and/or subserosa of the intestinal wall. PCI is still a poorly understood entity, and nearly all of the studies for PCI are case reports. In this work, we systematically evaluated and demonstrated for the first time the characteristics of PCI patients in China.

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INTRODUCTION

Pneumatosis cystoids intestinalis (PCI) is an uncommon disease with an unknown etiology, characterized by the

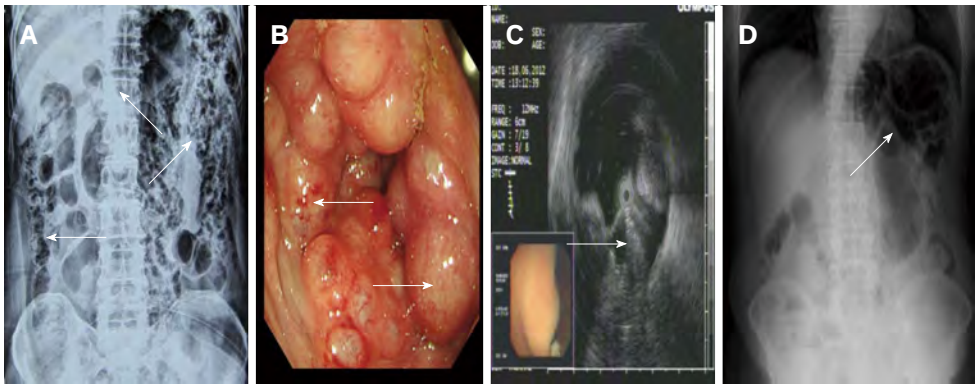

Figure 1 Imaging features of pneumatosis cystoides intestinalis. A: Barium enema study revealing multiple polypoid lesions with air shadows (arrow) and grape-like intramural gas in the whole colon; B: Colonoscopy revealing multiple round and smooth-surfaced elevated lesions (arrow) similar to submucosal tumors in the colon; C: Endoscopic ultrasonography revealing hyperechoic lesions and acoustic shadows in the submucosal layer (arrow); D: Plain radiography of the left upper quadrant abdomen revealing dilatation of the intestine and small linear, round radiolucent areas (arrow) on the clusters in the wall of the colon.

presence of gas within the submucosa or subserosa of the intestine[1,2]. Since it was first described by Du Vernoy[1] in autopsy specimens in 1730 and subsequently named by Mayer as PCI in 1825, it has been reported in various publications. However, after reviewing the literature, we found no epidemiologic studies, no randomized clinical trials, very few case series, and a large number of case reports. Many of the patients underwent misdiagnosis, mistreatment or even surgical exploration[3-5].

Our case report and systematic analysis are based on the Chinese publications to increase the understanding, diagnosis and treatment of PCI and to find the potential cause of the disease in China.

MATERIALS AND METHODS

Materials

A 70-year-old male was admitted for intermittent diarrhea accompanied by abdominal pain and bloody purulent stool for almost 2 years. The abdomen showed no relevant physical findings. Routine biochemical tests, inflammation indices and tumor markers were within normal values. He was diagnosed with hypertension and diabetes 10 years ago. Barium enema (Figure 1A) and colonoscopy (Figure 1B) disclosed multiple submucosal cysts protruding into the lumen of the whole colon. When a cyst was biopsied, it disappeared immediately. Endoscopic ultrasonography revealed gas in the cysts (Figure 1C). PCI was diagnosed. The patient received antibiotics and became asymptomatic with normal bowel movements. However, the diarrhea recurred after 4 mo. The patient then started hyperbaric oxygenation therapy. Unfortunately, he suffered from a hearing disorder and could not tolerate the hyperbaric therapy. Thus, the conservative approach was employed (observation only). Regular follow-up visits half a year later revealed improved clinical and radiological signs of PCI (Figure 1D).

Search strategy

The literature search used the available Chinese databases from May 2002 to May 2012, including CBM, CBMDisc, CMCC, VIP, Wanfang, and CNKI. The key words were pneumatosis cystoides intestinalis, pneumatosis, pneumatosis intestinalis, pneumatosis coli and mucosal gas. The patients' information, histories, therapies, courses, and outcomes were reviewed. Moreover, extended information was collected with regard to the nature and pathophysiology of PCI, and the incomplete reports were removed.

Statistical analysis

All data are presented as the mean ± SE. The demographic characteristics are presented as number (%).

RESULTS

The study group included 77 reports that contained an adequate amount of clinical information on 239 PCI cases (168 male:71 female = 2.4:1). The number of case reports was 62 (80.5%). The mean age was 45.3 ± 15.6 years (range: 2-81 years). The group was nationwide and particularly included high altitude areas and poor areas, including Qinghai, Sinkiang and Gansu (Table 1).

One hundred and four cases (43.5%) had comorbidities that may be related to PCI, with peptic ulcer being the most common concomitant disorder. In addition, 16.3% of the patients had complications including intestinal obstruction and perforation (Table 2).

The illness course from onset to identification ranged from 0 to 20 years, with a median of 6 mo. Overall, 217 cases (90.8%) had symptoms, and the most common symptom was abdominal pain (n = 117, 53.9%), followed by diarrhea (n = 115, 53.0%), distention (n = 92, 42.4%), nausea and vomiting (n = 31, 14.3%), bloody stool (n = 28, 12.9%), mucous stool (n = 26, 12.0%) and constipation (n = 17, 7.8%) (Figure 2A). PCI was most frequently diagnosed by colonoscopy (51.9%, 124/239), followed by surgery (40.6%, 97/239) and X-ray (10.9%, 26/239) (Figure 2B). The primary involved site was the colon, followed by the small intestine, especially the descending

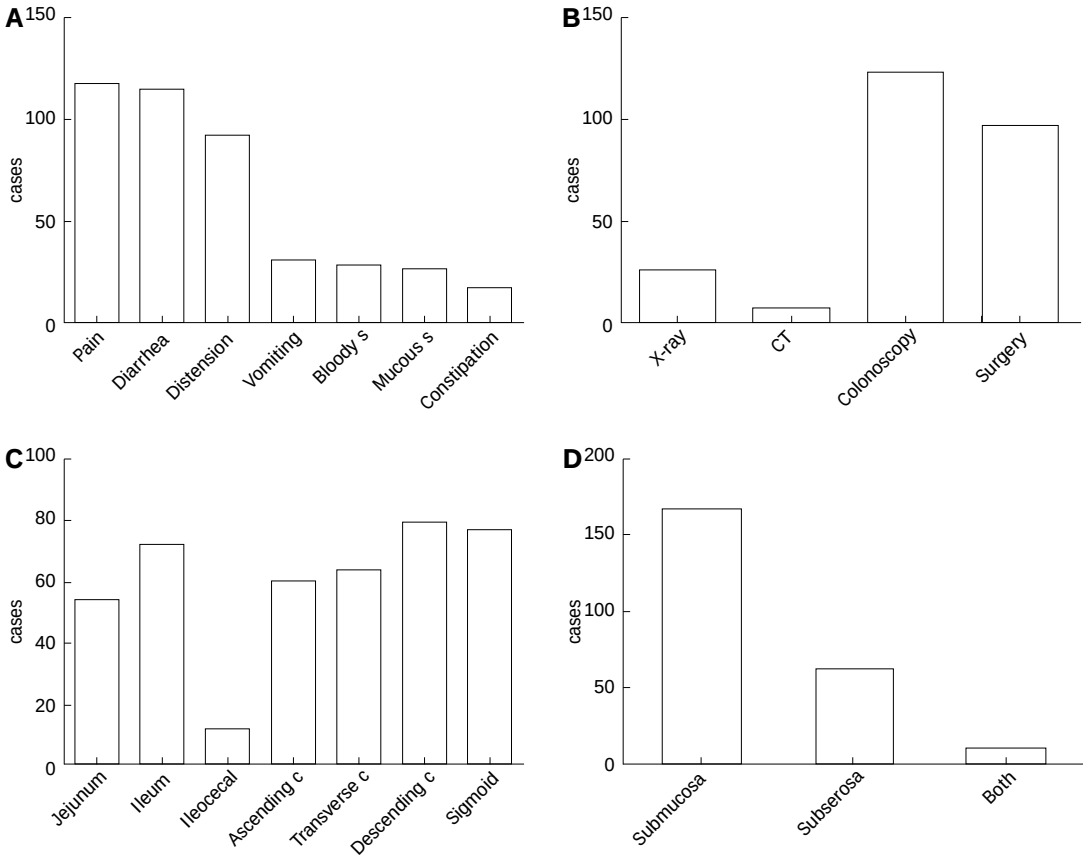

Figure 2 Clinical information of all 217 cases. A: The chief complaints; B: The methods of diagnosis; C: The primary involved site; D: The localization of gas in the intestinal wall. S: Stool; C: Colon; CT: Computed tomography.

colon (*n* = 79, 33.1%), sigmoid (*n* = 77, 32.2%) and ileum (*n* = 72, 30.1%) (Figure 2C). The majority of the cysts were found in the submucosa (69.9%, 167/239). Only 11 (4.6%) patients had both submucosa and subserosa involvement (Figure 2D).

The management of PCI included antibiotics, oxygen therapy, endoscopic therapy, surgery and the conservative approach. The efficiency of the conservative treatment reached up to 93.3% (Table 3). During the follow-up, which ranged from 1 mo to 20 years (median, 1 year), no symptoms recurred.

DISCUSSION

PCI is a rare disease and is still poorly understood. In a retrospective review of PCI, Koss[6] found a 3.5:1 male-to-female ratio of the occurrence of PCI in an age group of 30-50 years, and Jamart[7] found a 3:1 male-to-female ratio (aged from 41-50 years old) of the occurrence of PCI. However, both of these old reports contained few patients. A prospective study by Knechtle *et al*[8] showed equal incidence among males and females. PCI was previously thought to occur most frequently in the small intestine, but in recent barium enemas and colonoscopies studies, PCI has been reported to more commonly affect the colon. Most older studies showed PCI to occur more commonly in the small intestine. However, Horiuchi *et al*[9] showed that PCI appeared more commonly in the colon (61.8%) of females (mean age 55.4 years), followed by the small intestine (15.4%). Recently, Morris *et al*[10] showed the incidence of PCI was 46% in the colon; 27% in the small intestine, only 7% in the colon and small intestine combined. In contrast to previous reports using different ethnic cohorts, PCI in the patients in this study (Chinese cohort) showed a male-to-female ratio of 2.4:1 and a mean age of 45.3 ± 15.6 years. The most frequent location of PCI was the colon instead of the small bowel (rate of 1.3:1), with only 2.9% (7/239) of the cases being combined colon and small intestine. The most common localization of gas was in the submucosa (69.9%) (Figure 1).

PCI has been associated with a wide variety of underlining etiologies to explain the abnormal accumulation of gas[11-23]. There are five major theories: (1) The mechanical theory: Intestinal obstruction, inflammatory bowel disease, ischemic bowel disease, gastroenteric tumor, anorectal surgery, bowel preparation or colonoscopy resulting in intestinal wall injury or increased intraluminal pressure serve as the driving force in PCI that causes the intramural gas[14,15]. However, this theory cannot explain how the cysts are maintained once they have formed; (2) The pulmonary theory: Pulmonary diseases, such as chronic obstructive pulmonary disease, asthma, and interstitial pneumonia, may result in pulmonary alveolar rupture

Table 1 Geographical distribution of pneumatosis cystoides intestinalis in China *n* (%)

Province	Mean altitude (m)	Cases
Qinghai	4000	92 (38.5)
Sinkiang	2000	28 (11.7)
Beijing	50	21 (8.8)
Gansu	3000	17 (7.1)
Shanghai	4	10 (4.2)
Yunnan	1500	8 (3.3)
Shanxi	1000	8 (3.3)
Henan	1500	7 (2.9)
Tianjin	5	7 (2.9)
Sichuan	500	7 (2.9)
Tibet	4000	7 (2.9)
Shandong	1500	4 (1.7)
Shaanxi	1000	4 (1.7)
Zhejiang	50	3 (1.3)
Jiangxi	50	2 (0.8)
Guangdong	100	2 (0.8)
Kiangsu	50	2 (0.8)
Hainan	200	2 (0.8)
Heilongjiang	200	2 (0.8)
Jilin	800	2 (0.8)
Liaoning	500	2 (0.8)
Inner mongolia	1000	1 (0.4)
Hebei	400	1 (0.4)

Table 2 Pneumatosis cystoids intestinalis concomitant diseases and pneumatosis cystoids intestinalis complications *n* (%)

Total	*n* = 239
PCI concomitant diseases	104 (43.5)
Pyloric obstruction	31 (29.8)
Duodenal ulcer	18 (17.3)
Gastric ulcer	17 (16.3)
Pulmonary diseases	15 (14.4)
Intestinal diseases	17 (16.3)
Abdominal external injury or surgery	10 (9.6)
Malnutrition	27 (26.0)
Connective tissue disease	3 (2.9)
Diabetes mellitus	5 (4.8)
Hypertension	3 (2.9)
PCI complications	39 (16.3)
Intestinal obstruction	20 (51.3)
Intestinal perforation	14 (35.9)
Atypical hyperplasia and canceration	4 (10.2)
Intussusception and intestinal necrosis	1 (2.6)

PCI: Pneumatosis cystoids intestinalis.

and then produce a pneumomediastinum that dissects along the aorta and then along the mesenteric vessels to the bowel wall. However, this theory alone also fails to account for the finding that hydrogen, a gas never produced by mammalian cells, may comprise up to 50% of the gas content of the cysts[16]; (3) The bacterial theory: The gas is produced by gas-forming bacteria that enter the mucosal barrier through mucosal rents or increased mucosal permeability and produce the gas within the bowel wall. Indirect support for this theory was obtained by the successful treatment of PCI with antibiotics. However, the presence of aerogenic bacteria in the cysts has not yet been proven. Although Yale *et al*[17] reported that pneumatosis has been produced in germ-free rats by the injection of Clostridium species into the wall of the intestine, the isolation and cultivation of these organisms is rarely possible. Conversely, many of the patients who have pneumoperitoneum resulting from the rupture of cysts show no signs of peritonitis, prompting the theory that in this population, the gas is not caused by bacteria; (4) The chemical theory or nutritional deficiency theory: Malnutrition can prevent the digestion of carbohydrates and increased bacterial fermentation in the intestine, producing large volumes of gas leading to distention and ischemia and subsequently the submucosal dissection of gas. Recently, the development of PCI during the treatment with α-glucosidase inhibitors (α-GI) has been reported. The cessation of α-GI therapy is the key to the successful treatment of PCI[18,19], which supports the fourth theory; and (5) There have been some recent reports on PCI associated with chemotherapy, hormonal therapy and connective tissue disease that are not generally accepted[20-23].

Although many theories exist to explain the etiology and pathogenesis of PCI, no theory can explain the entire pathologic processes. Our experiments showed that many patients accompanied with pyloric obstruction, peptic ulcer, malnutrition and pulmonary diseases may support the theories of mechanical, pulmonary and nutritional deficiency (Table 2). Moreover, we also found that many of the patients came from highland areas, such as Qinghai, Sinkiang and Gansu (Table 1). The passage of intraluminal gas into the submucosa requires damage to the mucosa, which might be possible in these geographic areas. Further studies should be performed in the highland areas.

Although there are many symptoms of PCI, including abdominal pain, abdominal distention, diarrhea, mucous stool and bloody stool, none is disease specific (Figure 2A). The cysts may cause obstruction by internal or external compression of the bowel lumen when the cysts become larger. Complications associated with PCI occur in approximately 16.3% of cases and include intestinal obstruction or intestinal perforation (Table 2).

Approximately 85% of cases fall under secondary PCI[6], which results from other diseases. In these cases, the main symptoms and also the main treatments are related to the primary disease. Thus, some scholars do not think PCI is a disease by itself but rather a secondary manifestation of the reaction of the body to a variety of conditions; therefore, they believe that it does not have a single etiology[24].

In general, the diagnosis of PCI is based on endoscopy or plain radiography of the abdomen and is usually not difficult because the typical radiolucency appears as grape-like clusters or honeycomb-shaped shadows along the wall of the intestine. After the identification of PCI, a prompt further evaluation, including concomitant radiographic findings, of the patient should be conducted. Although only a few patients were diagnosed through an abdominal CT scan in our study, CT is a useful method for diagnosing PCI and is important because it provides

Table 3 Pneumatosis cystoids intestinalis therapies and their efficiency *n* (%)

Methods	*n*	Efficiency
Antibiotics	19	5 (26.3)
Oxygen	41	28 (68.3)
Endoscopy	12	12 (100)
Surgery	97	97 (100)
Conservative	15	14 (93.3)
Antibiotics + Oxygen	1	1 (100)
Oxygen + Endoscopic	53	53 (100)
Oxygen + Surgery	6	6 (100)

data on other abdominal pathologies[25-27]. Radiographic signs of bowel perforation or peritonitis and endoscopic signs of a tumor often indicate the need for emergency surgery[28]. Using two different preoperative imaging modalities to make a precise diagnosis is necessary.

The appropriate therapy is related to the underlying cause of PCI. The majority of patients (93.3%, Table 3) without pronounced symptoms were cured without any treatments. If the symptoms are pronounced, a conservative approach to treatment is allowed, such as gastrointestinal decompression, intestinal "rest", parenteral nutrition, and fluid and electrolyte supplementation. However, in contrast to the case reports, we found the efficiency of treatment by antibiotics was only 26.3%. The most efficient treatment was therapeutic alliance. Although oxygen was first used by Forgacs *et al*[16] in 1973, the optimal concentration, duration and effect of oxygen have not been established. The application of 200-300 mmHg PO_2 pressure for 1.5-2.5 h/d for 2-14 d or 55%-75% oxygen inhalation for 1-3 h/d for 2-5 d was suggested to lead to gas absorption within the cysts. Surgery is reserved either for cases of suspected inconvertible intestinal obstruction or perforation or cases with precancerous conditions[29]. The extremely high rate of surgical resection in China (40.6%) is associated with the lack of realization and the misdiagnosis of PCI as many cases can recover with non-operative management. Therefore, diagnosing PCI as early as possible and providing fast and adequate therapy to treat PCI are extremely important[30,31].

In conclusion, after recognizing the disease for almost three centuries, PCI is still a poorly understood phenomenon. Several theories explaining PCI and the variety of treatments reflect the lack of knowledge regarding the underlying pathophysiology. A long-term follow-up study is suggested to evaluate the long-term outcome of these therapies.

COMMENTS

Background

Pneumatosis cystoides intestinalis (PCI) is a rare disease characterized by the presence of multiple gas-filled cysts in the submucosa and/or subserosa of the intestinal wall. PCI is still a poorly understood entity, and nearly all of the studies for PCI are case reports.

Research frontiers

PCI is a rare disease that has not been unequivocally addressed. In this study, the authors systematically evaluated and demonstrated for the first time the characteristics of PCI patients in China.

Innovations and breakthroughs

For the first time, the authors determined the prevalence and the characteristics of PCI in Chinese people.

Applications

The study results suggest that PCI in Chinese patients presents in the large bowel more often than in the small intestine and more frequently in middle-aged males than in females. The majority of the cysts are found in the submucosa rather than the subserosa. High altitude residence may be associated with the PCI etiology. The majority of PCI can be safely managed by conservative treatment.

Peer review

The authors review PCI in the published literature from China and report on 239 cases. Stats on geography and altitude is hare to interpret - as population density may be more important - would incidence per 100000 population at high or low altitude be more helpful.

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BRIEF ARTICLE

Appropriate treatment of acute sigmoid volvulus in the emergency setting

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Abstract

AIM: To investigate an appropriate strategy for the treatment of patients with acute sigmoid volvulus in the emergency setting.

METHODS: A retrospective review of 28 patients with acute sigmoid volvulus treated in the Department of Colorectal Surgery, Changhai Hospital, Shanghai from January 2001 to July 2012 was performed. Following the diagnosis of acute sigmoid volvulus, an initial colonoscopic approach was adopted if there was no evidence of diffuse peritonitis.

RESULTS: Of the 28 patients with acute sigmoid volvulus, 19 (67.9%) were male and 9 (32.1%) were female. Their mean age was 63.1 ± 22.9 years (range, 21-93 years). Six (21.4%) patients had a history of abdominal surgery, and 17 (60.7%) patients had a history of constipation. Abdominal radiography or computed tomography was performed in all patients. Colonoscopic detorsion was performed in all 28 patients with a success rate of 92.8% (26/28). Emergency surgery was required in the other two patients. Of the 26 successfully treated patients, seven (26.9%) had recurrent volvulus.

CONCLUSION: Colonoscopy is the primary emergency treatment of choice in uncomplicated acute sigmoid volvulus. Emergency surgery is only for patients in whom nonoperative treatment is unsuccessful, or in those with peritonitis.

Key words: Sigmoid colon; Volvulus; Emergency; Colonoscopy

Core tip: Early and correct diagnosis of acute sigmoid volvulus is essential for appropriate treatment aimed at correcting abnormal pathophysiological changes and restoring intestinal transit caused by the volvulus. There is still much debate as to the ideal management of sigmoid volvulus. The results of this study suggest that colonoscopic decompression and derotation is the primary emergency treatment of choice in uncomplicated acute sigmoid volvulus and is a safe treatment modality for recurrent sigmoid volvulus. Emergency surgery is required for patients in whom nonoperative treatment is unsuccessful, or in those with peritonitis, bowel gangrene or perforation.

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INTRODUCTION

Acute sigmoid volvulus is defined as torsion of the sigmoid colon around its mesenteric axis, which leads to

acute large intestine obstruction, which, if left untreated, often results in life-threatening complications, such as bowel ischemia, gangrene, and perforation[1,2]. Early and correct diagnosis of this disease is essential for appropriate treatment aimed at correcting abnormal pathophysiological changes and restoring intestinal transit caused by the volvulus.

Despite significant progress in the treatment of this disease, no consensus has been reached[3-8]. Emergency surgery is the appropriate treatment for those who present with diffuse peritonitis, intestinal perforation or ischemic necrosis[9,10]. Nonoperative treatment is adopted if there is no evidence of these conditions. Barium enema, rectal tubes, rigid and flexible sigmoidoscopy as therapeutic methods have been adopted by clinicians[11,12]. Colonoscopy, besides being a therapeutic measure, allows the evaluation of colonic mucosa and therefore the presence or absence of signs of ischemia[13], and is effective in more than 70% of patients[14]. The initial management of clinically stable patients in good general condition with sigmoid volvulus is colonoscopic decompression as a first therapeutic option in the emergency setting in our hospital. The objectives of this study were to review our experience and the benefits of colonoscopy in the treatment of patients with acute sigmoid volvulus in the emergency setting.

MATERIALS AND METHODS

We performed a retrospective clinical data review of patients with acute sigmoid volvulus treated in the Department of Colorectal Surgery, Changhai Hospital, Shanghai, China, from January 2001 to July 2012. We included 28 patients who were diagnosed with acute sigmoid volvulus in the emergency department and then admitted to our department for treatment. Patient data included demographics, comorbidities, clinical manifestations, radiological investigations, colonoscopic findings and interventions, and clinical outcome.

Following the diagnosis of acute sigmoid volvulus, an initial colonoscopic approach was adopted if there was no evidence of diffuse peritonitis. If patients failed in the colonoscopic derotation, surgical intervention would be performed. Figure 1 illustrates the flowchart of patients who were admitted to our hospital with acute sigmoid volvulus.

RESULTS

Of the 28 patients with acute sigmoid volvulus, 19 (67.9%) were male and 9 (32.1%) were female. The mean age was 63.1 ± 22.9 years (range, 21-93 years). Six patients (21.4%) had a history of abdominal surgery. Seventeen patients (60.7%) had a history of constipation.

All patients presented with acute large intestine obstruction. The interval between the development of symptoms and hospitalization ranged from 15 h to 7 d (mean 37.3 ± 28.2 h). Clinical manifestations included abdominal pain in all patients (100%) and vomiting in 8 patients (28.6%). Abdominal examination revealed marked abdominal distension in 20 patients (71.4%). A visible intestinal loop was noted in 8 patients (28.6%). Following rectal examination, blood on the examining finger was absent in all patients (0%).

During the diagnostic period, all 28 patients underwent plain abdominal X-rays (100%), followed by computed tomography (CT) scan of the abdomen in 20 (71.4%). Blood chemistry and hematological profile were routinely studied. Abdominal radiographs showed multiple air/fluid levels and a dilated sigmoid colon in all patients (Figure 2). All patients were found to have positive sigmoid volvulus findings by CT scanning, such as a dilated sigmoid colon and a whirl pattern in the mesentery (Figure 3).

After early and effective resuscitation, colonoscopy was performed in all patients in order to complete decompression and derotation, and was successful in 26 (92.9%) patients. Emergency surgery was performed in a 41-year-old woman in whom colonoscopic derotation was unsuccessful. A 90-year-old man who did not receive derotation underwent immediate surgery due to a perforation with inlaid adipose tissue 50 cm from the edge of the anus (Figure 4).

There was no mortality or morbidity in the colonoscopic treatment group. One of two patients in the surgical treatment group died. Exploration revealed a 3-cm perforation in the sigmoid colon with omentum adhesion, and a dilated, dusky segment of the descending and transverse colon in this patient. Left hemicolectomy and end colostomy were performed. Postoperatively, he was treated in the intensive care unit but died 1 day later due to sepsis with progressive multiple organ failure.

The overall recurrence rate was 26.9% (7/26) after colonoscopic derotation. These patients underwent repeat colonoscopic detorsion without mortality or morbidity.

DISCUSSION

Acute sigmoid volvulus is the third most common cause of large bowel obstruction[15]. It has a wide geographic variation and it differs significantly between high-incidence countries and low-incidence countries[16]. This variation may be associated with differences in anatomy[17]. Acute sigmoid volvulus usually occurs in adult men. The mean age was found to be between 56 and 77 years and nearly one-third of all colonic emergencies in elderly patients are due to sigmoid volvulus[18]. In our patient group, age ranged from 21 to 93 years with a mean of 63.1 ± 22.9 years which showed that the Chinese population with acute sigmoid volvulus also included many younger patients. A preponderance in males compared with females was found and the ratio in our group was 2.1:1 (19 *vs* 9). This indicated that male preponderance in acute sigmoid volvulus is pronounced in the Chinese population.

The presence of a redundant and mobile sigmoid co-

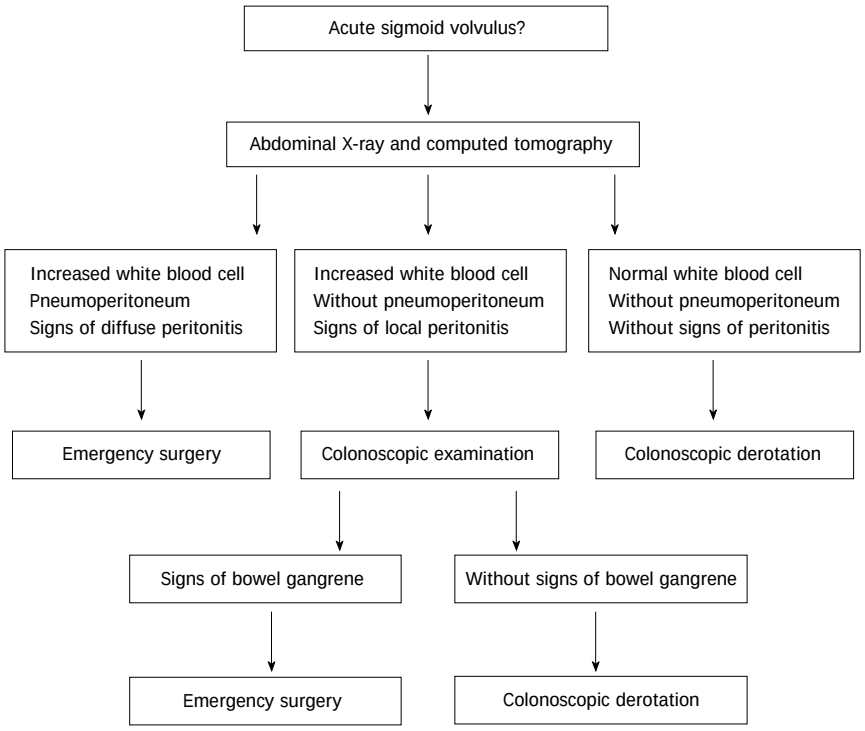

Figure 1 Flowchart of emergency therapeutic strategy for acute sigmoid volvulus.

lon, with a narrow base at the mesenteric root, is one of the major predisposing factors for sigmoid volvulus. Other predisposing factors, such as a high-fiber diet, constipation, previous abdominal surgery, pregnancy, diabetes, or neurological and psychiatric diseases such as dementia or schizophrenia have been described in the literature[19]. In our group, 60.7% of the patients had a history of chronic constipation, and over 15% suffered from diabetes or neurological diseases, and 21.4% of patients had a history of previous abdominal surgery.

The diagnosis of acute sigmoid volvulus is established by clinical and radiological findings. In the majority of patients, a thorough physical examination and abdominal radiographs are adequate to achieve the diagnosis. Typical symptoms include sudden abdominal pain and distension followed by constipation. The most common signs are abdominal tenderness and asymmetrical abdominal distention. Other findings include abnormal bowel sounds, abdominal tympany, a palpable abdominal mass, empty rectum, and dehydration[18]. Plain radiographs are diagnostic in 57%-90% of patients[20,21]. The classical sign of acute sigmoid volvulus is the coffee bean sign. Abdominal CT usually reveals a dilated colon with an air/fluid level and the "whirl sign", which represents twisted colon and mesentery[22].

Raveenthiran *et al*[23] recently provided more insight into the pathophysiology of acute sigmoid volvulus. Increasing intraluminal pressure impairs capillary perfusion following the occurrence of acute sigmoid volvulus. Mechanical obstruction due to twisting of mesenteric vessels and thrombosis of mesosigmoid veins contribute to ischemia. Ischemic injury in the mucosa occurs earlier than in other colonic layers and facilitates bacterial translocation and toxemia. A competent ileo-cecal valve converts the proximal colon into a second "closed loop". Increased intra-abdominal pressure results in the "abdominal compartment syndrome". Prompt and optimal correction of these pathophysiological features is vital to improve the prognosis of acute sigmoid volvulus.

The treatment of colonic volvulus remains controversial, and depends on the elected procedure and the most appropriate therapeutic approach in terms of the clinical status of the patient, the location of the problem, the suspicion or presence of peritonitis, bowel viability and the experience of the surgical team[19].

Emergency surgery is associated with significant mortality and morbidity. Kassi *et al*[24] reported that the mortality rate was 12% (n = 3) for Hartmann's procedure. Surgical site infections (42.86%) were the most common complications. Eleven (50%) of 22 patients had intestinal continuity restored. Bhatnagar *et al*[25] reported that the risk factors for mortality were: (1) age over 60 years; (2) presence of shock on admission; and (3) positive history of a previous episode of volvulus. With regard to the former two risk factors, special efforts are necessary by intensive care staff to monitor homeostatic disturbance and reduce mortality in older patients (> 60 years) and those presenting with shock at the time of admission. One of our patients died due to sepsis with progressive multiple organ failure on postoperative day 1.

Nonoperative detorsion is advocated as the primary treatment choice in uncomplicated acute sigmoid volvulus. Although rectal tubes, barium enemas or rigid sigmoidoscopy have been widely used, flexible sigmoid-

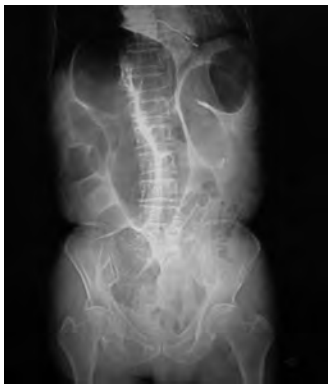

Figure 2 Plain abdominal X-ray film obtained in the supine position reveals gross dilatation of the colon.

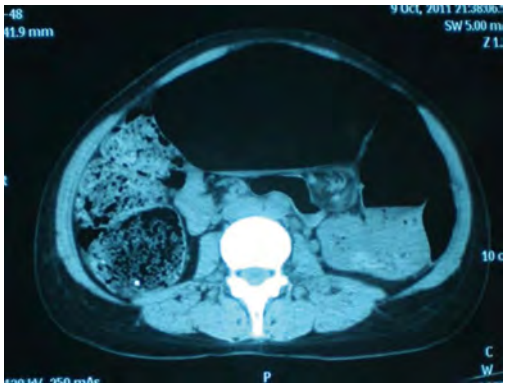

Figure 3 Computed tomography reveals dilated colon with an air/fluid level, as well as the "whirl sign" composed of mesentery and twisted colon.

oscopy is now the preferred nonoperative procedure. Nonoperative treatment is successful in 70%-91% of cases, with reported complication rates of 2%-4.7% in geriatric patients[26,27]. However, in our patients, the success rate of colonoscopic derotation was 92.9%. Compared with sigmoidoscopy, colonoscopy is readily used in our hospital for endoscopic derotation for several reasons. In addition to a superior success rate and safety profile, it is of adequate length to reach beyond the second constricting point. It also allows better visualization of the colonic mucosa and can guide the decompression and derotation procedure. Colonoscopic suctioning of the proximal colon facilitates quick recovery by removing bacterial toxins. Furthermore, the authors adopt modified double-operating colonoscopy which can help minimize the risk of perforation. Colonoscopic derotation simply converts an emergency into an elective procedure, which facilitates treatment of comorbidity and allows preparation of the bowel prior to definitive surgery. The results from the present study suggest that colonoscopy is a safe and effective treatment modality for acute sigmoid volvulus.

Following derotation, ischemia-reperfusion injury aggravates intestinal dysfunction, and even intestinal ulcer and perforation. Peritoneal exudate, high intestinal fluid accumulation, electrolyte disturbances, and hypoproteinemia lead to serious adverse consequences. Consequently,

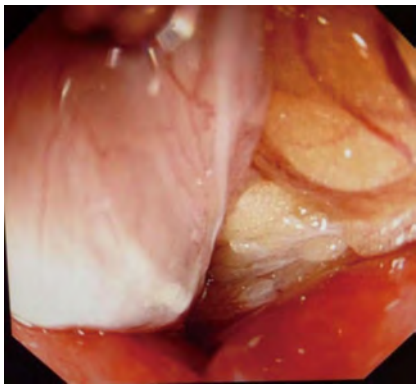

Figure 4 Colonoscopic examination shows a perforation with inlaid adipose tissue.

effective treatment following colonoscopic derotation is very important. Fluid resuscitation should be performed immediately. Vasodilator therapy should be continued, as the use of these agents can ameliorate intestinal tissue microcirculation. Broad-spectrum antibiotics are indicated to control bacterial translocation across the ischemic intestinal wall.

Colonoscopic derotation was followed by recurrence in 26.9% of our patients. These patients underwent successful repeat colonoscopic detorsion without mortality or morbidity. These results suggest that colonoscopy is the primary choice in the treatment of recurrent sigmoid volvulus, particularly in elderly patients who refuse elective surgery.

In conclusion, colonoscopic decompression and derotation is the primary emergency treatment of choice in uncomplicated acute sigmoid volvulus and is a safe treatment modality for recurrent sigmoid volvulus. Emergency surgery is reserved for gangrene and failed decompression, and in patients with a high recurrence rate it may be prudent to consider interval semi-elective resection and primary anastomosis several days after successful decompression.

COMMENTS

Background

Acute sigmoid volvulus is common worldwide, and leads to acute large intestine obstruction, which, if left untreated, often results in life-threatening complications, such as bowel ischemia, gangrene, and perforation. Despite significant progress in the treatment of this disease, no consensus has been reached.

Research frontiers

As the authors described, there is still considerable debate on the ideal management of acute sigmoid volvulus. In this study, the authors demonstrated that colonoscopy is the primary emergency treatment of choice in uncomplicated acute sigmoid volvulus. Surgery is reserved for patients in whom nonoperative treatment is unsuccessful, or in those with peritonitis.

Innovations and breakthroughs

Barium enema, rectal tubes, rigid and flexible sigmoidoscopy have been adopted by clinicians as therapeutic methods. Colonoscopy, besides being a therapeutic measure, allows evaluation of the colonic mucosa and therefore the presence or absence of signs of ischemia, and is effective in more than 70% of patients. The initial management of clinically stable patients in good general condition with sigmoid volvulus is colonoscopic decompression as a first therapeutic option with satisfactory results in the emergency setting.

Applications

By understanding abnormal pathophysiological changes associated with acute sigmoid volvulus, this study may represent a future strategy for therapeutic intervention in the treatment of this condition.

Terminology

Acute sigmoid volvulus is defined as torsion of the sigmoid colon around its mesenteric axis, which leads to acute large intestine obstruction, which, if left untreated, often results in life-threatening complications, such as bowel ischemia, gangrene, and perforation.

Peer review

The authors present a well-written case series describing their experience with sigmoid volvulus. There is still much debate as to the ideal management for this condition. Emergency surgery is reserved for gangrene and failed decompression, and due to a high recurrence rate, it may be prudent to consider interval semi-elective resection and primary anastomosis several days after successful decompression.

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BRIEF ARTICLE

Prevalence of minimal hepatic encephalopathy and quality of life evaluations in hospitalized cirrhotic patients in China

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Author contributions: Wang JY designed and organized the study, interpreted data and revised the paper; Zhang NP collected and interpreted data and drafted the manuscript; Wang JY, Chi BR, Mi YQ, Meng LN, Liu YD, Wang JB, Jiang HX, Yang JH, Xu Y, Li X, Xu JM, Zhang G, Zhou XM, Zhuge YZ, Tian DA and Ye J enrolled patients and provided evaluable data; Liu YL coordinated the study; all authors have approved the final version of the manuscript.

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Abstract

AIM: To investigate the prevalence of minimal hepatic encephalopathy (MHE) and to assess corresponding health-related quality of life (HRQoL) in hospitalized cirrhotic patients in China.

METHODS: This multi-center cross-sectional study included 16 teaching hospitals, which were members of "Hepatobiliary Cooperation Group, Society of Gastroenterology, Chinese Medical Association", from different areas of China carried out between June and October in 2011. All the eligible hospitalized cirrhotic patients (n = 538) were required to complete triplicate number connection tests combined with one digit symbol test

for diagnosing MHE. Patients' clinical examination data were complemented by a modified questionnaire assessing HRQoL. Written informed consent was obtained from each patient.

RESULTS: Male was predominant (68.6%) in 519 patients who met the criteria of the study, with a mean age of 49.17 ± 11.02 years. The most common cause of liver cirrhosis was chronic hepatitis B (55.9%). The prevalence of MHE was 39.9% and varied by Child-Pugh-Classification score (CPC-A: 24.8%, CPC-B: 39.4% and CPC-C: 56.1%, $P < 0.01$). MHE ($P < 0.01$) and higher CPC scores ($P < 0.01$) were associated with a high HRQoL scores (reflecting poorer quality of life). The prevalence of MHE was proportionate to CPC ($P = 0.01$) and high quality of life scores ($P = 0.01$).

CONCLUSION: Hospitalized cirrhotic patients have a high prevalence of MHE that is proportionate to the degree of liver function and HRQoL impairment.

Key words: Minimal hepatic encephalopathy; Health-related quality of life; China; Child-Pugh Classification; Liver cirrhosis

Core tip: This study showed that 39.9% of hospitalized patients with liver cirrhosis had minimal hepatic encephalopathy (MHE), and patients with Child-Pugh Classification-C had a high prevalence of MHE (56.1%) and increased health-related quality of life scores that reflected poorer life status. Increasing awareness of its adverse impact on life should be emphasized. Recommendations to screen for MHE may be applicable for evaluating the risks of driving and work accidents in patients with cirrhosis.

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INTRODUCTION

Hepatic encephalopathy (HE) is a serious complication of liver cirrhosis that represents a continuous spectrum of neurologic and neuropsychiatric abnormalities[1,2]. Minimal HE (MHE), the mildest form of HE[1,2], is defined as patients with normal mental and neurological examinations but with a number of neuropsychiatric and neuro-physiological defects identified by psychometric tests[3]. Patients with MHE have various subtle abnormalities in the cognitive functioning that detrimentally affects their fitness to drive[4] and handle complex mechanical machines[5]. In a study by Prasad *et al*[6], significant impairment was observed in HE patients' social interactions, alertness, emotional behavior, sleep, work, household management, recreation, and pastimes.

There are several methods of diagnosing MHE, including comprehensive neuropsychological examinations, standard psychometric batteries, neuro-physiological testing, and computerized testing[3]. However, there are no current guidelines for the standardized diagnosis of MHE. The Working Group on HE recommended that at least two of the following neuropsychologic tests should be used for diagnosing MHE: number connection test-A (NCT-A), NCT-B, block-design test (BDT), and the digit-symbol test (DST)[7]. The current definition of MHE is based on psychometric test results that are two SD more than normal on at least two psychometric tests. As there is no gold standard for diagnosis of MHE, the prevalence of MHE in patients with cirrhosis ranges from 30% to 80%[8-11]. Recently, the estimated prevalence of MHE varied from 29.2% to 57.1% in China[12-14]. Some studies only employed one neuropsychologic tests (NCT) for MHE diagnosis. Therefore, the exact prevalence of MHE is unknown in China.

MHE is associated with potential progression to HE, diminished quality of life, driving impairment that increases the risk of traffic accidents, and negative health-related quality of life (HRQoL)[15-17]. Quality of life (QoL) is a multidimensional index that comprehensively addresses all aspects of human well-being, including physical and cognitive capabilities, functional behavior, emotional status, and psychosocial adjustment. As compared with generic measures of impairment, disease-specific measures are more likely to be sensitive to small, yet clinically meaningful, differences in HRQoL. Our group[18] developed and verified a reliable and valid HRQoL instrument that measures the functional and health status of patients with MHE. That study also demonstrated that HRQoL in patients with MHE deteriorates as the disease becomes more severe, although the study had a small sample size and a limited regional scope.

This study investigated the prevalence of MHE in hospitalized cirrhotic patients from different areas of China, and HRQoL evaluations among them.

MATERIALS AND METHODS

Study population

A multi-center cross-sectional study was initiated by the Hepatobiliary Cooperation Group of Society of Gastroenterology, the Chinese Medical Association. The study was conducted in 16 teaching hospitals representing different areas of China (4 in the East, 3 in the West, 1 in the South, 4 in the North and 4 in the central region). All consecutive cirrhotic hospitalized patients aged between 18 and 70 years and without overt HE (OHE) were screened for MHE between June and October 2011. Cirrhosis was diagnosed based on available clinical data,

including laboratory tests, endoscopy, diagnostic imaging, or liver histology. Exclusion criteria included the presence or a history of OHE, a history of taking lactulose or any antibiotics, alcohol intake, gastrointestinal hemorrhage, or spontaneous bacterial peritonitis during the previous 6 wk, significant concurrent diseases such as heart, respiratory, or renal failure, and neurologic abnormalities such as Alzheimer's disease, Parkinson's disease, non-hepatic metabolic encephalopathy, electrolyte disorders, inability to perform psychometric tests or complete the questionnaire (caused by either insufficient knowledge of the Chinese language or poor vision). All patients provided written informed consent. Study protocols were approved by the ethics committees of the participating hospitals in accordance with the Principles of Declaration of Helsinki.

Physical examination, laboratory testing, and medical history documentation

Qualified physicians documented routine physical examinations and laboratory assessments that included biochemical tests (alanine aminotransferase, aspartate aminotransferase, bilirubin, albumin, creatinine, prothrombin time, serum potassium, serum sodium, and serum chloride), virological tests [hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), anti-HBe, anti-hepatitis C virus (HCV), hepatitis B virus (HBV) DNA levels, and HCV RNA levels], and diagnostic imaging [ultrasonography, computer tomography (CT), or magnetic resonance imaging], etiology of cirrhosis, a history of medication use and other medical histories. The Child-Pugh-Classification (CPC) scoring system was used to assess the severity of liver disease.

Psychometric testing

All patients underwent a series of psychometric tests, including triplicate NCT-A and one DST. The NCT-A measures cognitive motor abilities by having patients connect numbers, from 1 to 25 on printed paper, as quickly as possible. DST: Subjects are asked to insert symbols in the blank squares below the numbers using the key provided. The exercise is timed and the number correctly completed in 90 seconds recorded. Bao *et al*[19] established age-based normal parameters of psychometric measures for NCT-A and DST in China in 2006. Normative values for NCT-A and DST were based on those from healthy volunteers with the same geographical background as liver cirrhosis patients.

According to the normative parameters for NCT-A and DST established by Bao *et al*, diagnostic criteria for MHE were as follows: time greater than two SD from the mean for the NCT, and score less than two SD from the mean for the DST. For the NCT-A, diagnostic criteria were: > 34.3 s in patients aged < 35 years; > 45.7 s in patients aged 35-44 years; > 52.8 s in patients aged 45-54 years and > 61.9 s in patients aged > 55 years. Diagnostic criteria for the DST were: < 40.5 in patients < 35 years; < 35 in patients aged 35-44 years; < 28.5 in patients aged 45-54 years and < 26 in patients aged > 55 years. Patients with abnormal results from both psychometric tests were diagnosed as having MHE.

Assessment of HRQoL

A modified Chinese QoL questionnaire with 30 questions verified in Chinese populations in 2009 was used to assess all patients' HRQoL index[18] The domains included physical functioning (8 questions), psychological well-being (7 questions), symptoms/side effects (7 questions), social functioning (4 questions), and self-evaluation about general health (4 questions). Impact scores for each question ranged from 1 to 5. These scores increase as the QoL declines. The total QoL scores were obtained from the sum of each question's score.

Statistical analysis

Continuous variables were expressed as mean ± SD or median (range), where appropriate. Categorical variables were described as the number and proportion of each category. In order to determine relevant risk factors for MHE occurrence, characteristics such as age, gender, pre-existing ascites, variceal bleeding, occupation, driving, alcohol drinking, hepatitis B antigen status, and antiviral therapy for HBV-related cirrhosis were included in the univariate analysis. The χ^2 or Fisher's exact test was used for categorical variables, and the Mann-Whitney *U* test or analysis of variance (ANOVA), was performed as appropriate to determine associations for continuous data. All tested variables with *P* values < 0.5 were entered into logistic regression. All statistical testing was two-tailed at the 5% level. Software used for analysis was Statistical Package for Social Science (SPSS, version 14.0, SPSS Inc. Chicago, IL, United States) and Science Analysis Software (SAS, version 9.13; SAS Institute Inc., Cary, NC, United States).

RESULTS

Study patients

Of the patients screened (n = 538), 519 patients met the study's criteria for inclusion. Excluded patients included those who were older than 70 years (n = 7) and who did not complete all psychometric tests (n = 12). Liver cirrhosis was diagnosed based on diagnostic imaging (ultrasonography, CT and/or magnetic resonance imaging), histopathology, endoscopy, or clinical and laboratory data. Most patients (n = 356, 68.6%) were male with a mean age of 49.17 ± 11.02 years, and 100 (20.3%) of them had college degrees or higher levels of education. Included patients were diagnosed with liver cirrhosis for 2.54 ± 3.16 years. The most common causes of liver cirrhosis were chronic hepatitis B (CHB) (55.7%), followed by CHB accompanied by alcoholic liver disease (ALD) (11.8%), ALD (8.9%), chronic hepatitis C (CHC) (7.3%), and autoimmune hepatitis (AIH) (7.5%). According to the CPC scoring system, 161 (31.0%) patients were classified as CPC-A, 203 (39.1%) were as CPC-B, and 155 (29.9%) as CPC-C. Of 320 cirrhotic patients with CHB, 41.6% (n = 133) were HBeAg positive, 85.0% (n = 272)

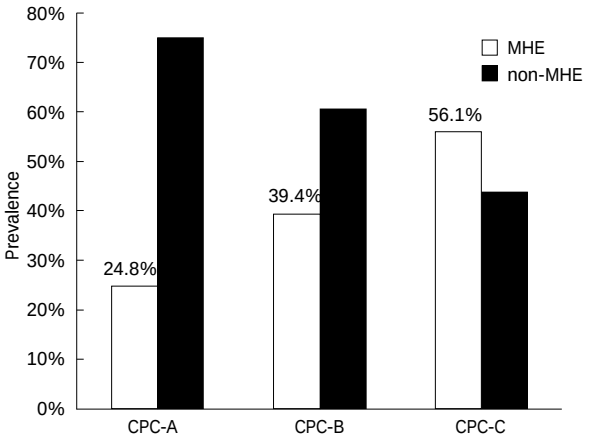

Figure 1 Prevalence of minimal hepatic encephalopathy for various Child-Pugh classes. $P < 0.01$ between minimal hepatic encephalopathy (MHE) and non-MHE. CPC: Child-Pugh classes.

were HBV DNA positive, and 202 (60.3%) had received antiviral treatment.

Prevalence and characteristics of MHE

Cirrhotic patients with concurrent positive NCT and DST results (n = 207, 39.9%) were diagnosed with MHE. The prevalence of MHE differed among CPC-A (24.8%), CPC-B (39.4%) and CPC-C (56.1%) patients (CPC-A *vs* CPC-B, $P < 0.05$; CPC-A *vs* CPC-C, $P < 0.01$; CPC-B *vs* CPC-C, $P < 0.01$) (Figure 1). Older patients and patients with lower levels of education, a history of prior ascites had a higher prevalence of MHE (Table 1). Compared to patients without MHE, those with MHE had lower levels of serum albumin ($P = 0.01$), sodium ($P = 0.01$), potassium ($P = 0.04$), and platelet count ($P = 0.03$); higher levels of serum bilirubin ($P < 0.01$) and blood ammonia ($P = 0.02$); and longer prothrombin times ($P = 0.01$). Many (24.3%) MHE patients were still driving at the time of diagnosis.

There were no statistical differences in HBeAg status ($P = 0.30$) or HBV-DNA levels ($P = 0.19$), duration of HBV infection ($P = 1.00$), antiviral therapy ($P = 0.17$), or duration of antiviral treatment ($P = 0.54$) between patients with and without MHE.

Evaluation of HRQoL

Compared to cirrhotic patients without MHE, patients diagnosed with MHE had higher scores (more dysfunctions) for physical functioning (20.09 ± 6.26 *vs* 18.10 ± 6.02, $P < 0.01$), symptom/side effects (14.98 ± 5.88 *vs* 13.35 ± 5.61, $P < 0.01$), and psychological well-being (15.93 ± 6.62 *vs* 14.80 ± 5.44, $P = 0.04$) (Table 2). Pooled HRQoL scales were higher in MHE patients than in non-MHE ones (69.12 ± 20.40 *vs* 63.89 ± 18.85, $P < 0.01$). Patients with CPC-C had higher HRQoL scores (71.61 ± 21.01) than those with CPC-A (61.13 ± 17.24) and CPC-B (65.50 ± 19.31), $P < 0.01$, which reflect poorer QoL (Table 3).

Table 1 Characteristics of the study population with and without minimal hepatic encephalopathy *n* (%)

Characteristics	MHE	Non-MHE	*P* value
Gender			
Male	140 (67.6)	216 (69.2)	0.77
Female	67 (32.4)	96 (30.8)	
Mean age (yr), mean ± SD	51.56 ± 9.70	47.58 ± 11.55	< 0.01
Level of education			
Grade six or less	93 (44.9)	34 (10.9)	< 0.01
Junior high school	64 (39.9)	76 (24.4)	
Senior high school/vocational school	32 (15.5)	94 (30.1)	
College degree or more	12 (5.8)	88 (28.2)	
Unknown	6 (2.9)	20 (6.4)	
Driving			
Yes	50 (24.2)	89 (28.5)	0.22
No	144 (69.6)	193 (61.9)	
Unknown	13 (6.3)	30 (9.6)	
Primary etiology for chronic liver disease			
Hepatitis B virus	117 (56.5)	172 (55.5)	0.18
Hepatitis B virus and alcohol	21 (10.1)	40 (12.9)	
Alcohol	30 (14.5)	19 (6.1)	
Hepatitis C virus	11 (5.3)	27 (8.7)	
Hepatitis B and C virus	2 (1.0)	1 (0.3)	
Autoimmune hepatitis	12 (5.8)	27 (8.7)	
Other	14 (6.8)	24 (7.7)	
History of prior variceal bleeding			
Yes	43 (20.7)	82 (26.3)	0.21
No	158 (76.3)	226 (72.4)	
Unknown	6 (2.9)	4 (1.3)	
History of prior ascites			
Yes	131 (63.3)	139 (44.6)	0.00
No	66 (31.9)	168 (53.8)	
Unknown	10 (4.8)	5 (1.6)	
Duration of liver cirrhosis (yr), mean ± SD	2.28 ± 3.06	2.73 ± 3.21	0.25

MHE: Minimal hepatic encephalopathy.

Comparison of one single psychometric test and combined psychometric tests

We employed combined NCT and DST tests as "gold standard" in this study. Consistency of diagnosis between one single psychometric test (NCT or DST) and combined psychometric tests was assessed by Kappa statistics (Table 4). Agreement between DST and combined NCT and DST was good, with a Kappa coefficient around 0.98 (95%CI: 0.97-0.99) for diagnosing MHE. Agreement between NCT and combined tests was fair (Kappa value 0.24, 95%CI: 0.19-0.29).

DISCUSSION

This study was the first nationwide investigation of the prevalence of MHE among hospitalized cirrhotic patients in China. The study locations are dispersed in different parts of China, including east, west, north, south and central regions, covering 16 hospitals located in 10 provinces and 3 municipalities under direct administration of the central government. Because each teaching hospital in the capital city of a province provides service

Table 2 Health-related quality of life scales for patients with and without minimal hepatic encephalopathy

	MHE	Non-MHE	*P* value
Physical functioning (8 questions)	20.09 ± 6.26	18.10 ± 6.0	< 0.01
Psychological well-being (7 questions)	15.93 ± 6.62	14.80 ± 5.44	0.04
Symptom/side effects (7 questions)	14.98 ± 5.88	13.35 ± 5.61	< 0.01
Social functioning (4 questions)	9.67 ± 2.73	9.66 ± 2.65	0.95
Self-evaluation regarding general-health (4 questions)	9.74 ± 2.73	9.43 ± 2.57	0.21
Total pooled score (30 questions)	69.12 ± 20.40	63.89 ± 18.85	< 0.01

MHE: Minimal hepatic encephalopathy.

Table 3 Health-related quality of life scales for various Child-Pugh classes

	CPC-A	CPC-B	CPC-C	*P* value
Physical functioning	16.77 ± 5.07	18.94 ± 6.35	20.82 ± 6.33	< 0.01
Psychological well-being	14.43 ± 5.46	15.03 ± 5.93	16.39 ± 6.38	0.02
Symptom/side effects	12.12 ± 5.33	13.56 ± 5.21	16.45 ± 6.05	< 0.01
Social functioning	9.03 ± 2.53	9.60 ± 2.75	10.37 ± 2.58	< 0.01
Self evaluation about general-health	9.20 ± 2.59	9.46 ± 2.74	10.02 ± 2.52	0.02
Total pooled score	61.13 ± 17.24	65.50 ± 19.31	71.61 ± 21.01	< 0.01

CPC: Child-Pugh-Classification.

Table 4 Consistency of diagnosis between one single psychometric test and combined psychometric tests

	NCT and DST		Kappa value (95%CI)	*P* value
	MHE	Non-MHE		
NCT				
MHE	207	223	0.24 (0.19-0.29)	< 0.01
Non- MHE	0	89		
DST				
MHE	207	4	0.98 (0.97-0.99)	0.05
Non- MHE	0	308		

NCT: Number connection test; DST: Digit-symbol test; MHE: Minimal hepatic encephalopathy.

to patients from the entire province, the study population could well represent cirrhotic patients throughout China.

China has the greatest burden of chronic liver disease in the world due to an epidemic of viral B hepatitis. Although the exact nationwide prevalence of liver cirrhosis in China is unknown, a reasonable estimate suggests that up to 1% of the entire population could have histological evidence of cirrhosis[20,21]. The prevalence of MHE in Chinese cirrhotic patients was reported to be 51.3% by Zeng *et al*[22]. However, their study only included local patients and lacked assessment of cognitive impairments and decreased quality of life. Other studies also reported varying and higher than 50% MHE prevalences among cirrhotic patients[10,23-25]. Our study showed that the nationwide prevalence of MHE in hospitalized cirrhotic patients was 39.9%. These discrepancies were due to the different criteria used to diagnose MHE and inter-population variations. The absence of a gold standard for determining MHE is a major challenge for attaining consistency among studies.

Impairments in visuospatial function, attention, response time, and inhibition are specific to MHE in the absence of other neurocognitive disorders; the psychometric HE score (PHES) was specifically designed to detect these impairments. The PHES comprises 5 different tests: the NCT-A, NCT-B, DST, the line-tracing test, and the serial dotting test. NCT-A and NCT-B evaluate concentration, mental tracking, and visuomotor speed. The DST evaluates psychomotor and visuomotor speed with attention on speed and accuracy. According to the consensus of the Working Group on HE[26], if the entire PHES cannot be completed, at least two of the following tests are recommended for the diagnosis of MHE: NCT-A, NCT-B, block design test, and DST.

In this study, we combined two age-based psychometric tests (NCT-A and DST)[19] as "gold standard" to diagnose MHE. Consistency of MHE diagnosis between one single psychometric test (NCT or DST) and combined psychometric tests was assessed by Kappa statistics. Agreement between DST and combined NCT and DST was good, with a Kappa coefficient around 0.98 for diagnosing MHE. This good agreement indicates that DST is equally good as combined NCT and DST[27-29]. However, single NCT, which showed a higher prevalence of MHE, did not have good agreement with combined test. Therefore, in clinical practice, DST can be used as the first test for screening MEH so as to avoid a high rate of false positive diagnosis.

One of the limitations of neuropsychological test is that the results can be influenced by age, educational level, and learning effects. We used three parallel versions of NCT-A to avoid the effects of education. Yet the limitations of our study were that (1) the normality of the NCT-A and DST scores used was not adjusted by educational level; and (2) half of the patients in our study had lower educational levels, which might have influenced the neuropsychological test results.

Furthermore, the prevalence of MHE reported in other studies was higher in cirrhotic patients with CPC-B, CPC-C, advanced age, alcoholism, a previous episode of overt HE, and portosystemic shunts[30]. None of the patients in our study had previous episodes of OHE or histories of portosystemic shunt surgery. Groeneweg *et al*[31] found that cirrhotic patients with normal liver function (CPC-A) had a low prevalence (15%) of MHE, while MHE was present in half of the patients with advanced cirrhosis (CPC-B/C). Our study confirmed that the prevalence of MHE in CPC-C patients was the highest (56.5%). The results demonstrated that cirrhotic patients with MHE had impaired liver function, including reduced hepatic biosynthetic, excretory and/or detoxification capacity, hyponatremia, lower platelet count, and high

blood ammonia.

Patients with MHE had impaired perception, memory, learning, expression (language, constructive abilities, and voluntary motor control), mental activity (attention and mental speed), and executive function[30,32]. There are many aspects of English-published HRQoL instruments which cannot be adapted well to the Chinese due to the differences in cultures and language. In order to define and assess HRQoL appropriately in the Chinese patients, our group developed a modified Chinese questionnaire of HRQoL that was verified in a Chinese population in 2009[18]. The questionnaire was administered to a cohort of patients with varying types and stages of cirrhosis for assessment of its reliability and discriminant validity. As liver disease becomes more severe, the questionnaire documents deterioration in patients HRQoL[18]. Our study confirmed that severity of liver function impairment, based on CPC scoring system, was associated with HRQoL. Patients with CPC-C had higher HRQoL scores and compromised life status compared to patients with CPC-A/B. Patients with MHE had high health-related QoL scores that reflect poorer QoL.

Patients with MHE have higher physical functioning and symptom/side effects scores than patients without MHE. These two domains of the questionnaire also have a higher test-retest reliability (0.94 and 0.96) than other domains[18]. Our study results retested the discriminant validity of the questionnaire for distinguishing among groups with varying CPC classes. However, the validity of this instrument for evaluating the efficacy of MHE treatment needs to be established.

Physicians formerly agreed that it was unnecessary to screen for and treat MHE in cirrhotic patients without a history of OHE[33]. However, given increased knowledge of the impact of MHE, great emphasis on OHE has recently been shifted towards covert HE[34]. Because of psychomotor defects, patients meeting the criteria for MHE have been shown to have reduced driving skills, who are more likely to suffer from falls and develop episodic HE more frequently[4,35,36]. Our study found that 24.2% of MHE patients were driving at the time of diagnosis. Due to the potential risks, there is a need to assess the presence of MHE in cirrhotic patients who drive. Similarly, recommendations for MHE screening of cirrhotic patients may be valuable in reducing the risk of work-related accidents, especially while handling machinery[30,37].

Compared to OHE, there are fewer randomized clinical trials about the treatment of MHE and these trials have smaller case numbers. Some studies show that treatments using lactulose and/or rifaximin can improve the cognitive abilities, QoL[6,38], and driving ability[39] of patients with MHE. Yet the effects of these drugs on MHE patients' ability to work or risk of falling remain unproven. The duration of treatment and choice of medication also remain unclear[40]. Therefore, high quality studies are needed to assess whether patients suffering from liver cirrhosis and MHE require a specific treatment.

In conclusion, our study showed that 39.9% of hospitalized patients with liver cirrhosis had MHE, and this was associated with severe liver function and QoL impairment. Cirrhotic patients with CPC-C had a high prevalence of MHE and increased HRQoL scores that reflected poorer life status. The modified Chinese HRQoL questionnaire performed well in this study. The HRQoL scale results indicated that the questionnaire was suitable for evaluating cirrhotic patients in clinical practice in China. Recommendations to screen for MHE using NCT-A combining DST tests may be applicable for evaluating the risks of driving and work accidents in patients with cirrhosis. In clinical practice, DST can be considered as the first screening test of MHE due to the good agreement between DST and combined psychometric tests.

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COMMENTS

Background

China has the greatest burden of chronic liver disease in the world due to an epidemic of viral B hepatitis. Yet the exact nationwide prevalence of liver cirrhosis in China is unknown. Furthermore, minimal hepatic encephalopathy (MHE) is associated with potential progression to Hepatic encephalopathy (HE), diminished quality of life (QoL), driving impairment that increases the risk of traffic accidents, and negative health-related QoL (HRQoL).

Research frontiers

This is a first multicenter nationwide study to investigate the burden of MHE in hospitalized patients with cirrhosis in China. No such data was reported before.

Innovations and breakthroughs

This study was the first nationwide investigation of the prevalence of MHE among hospitalized cirrhotic patients in China. The study locations are dispersed in different parts of China, including east, west, north, south and central regions, covering 16 hospitals located in 10 provinces and 3 municipalities under direct administration of the central government. Because each teaching hospital in the capital city of a province provides service to patients from the entire province, the study population could well represent cirrhotic patients throughout China.

Applications

The results showed that 39.9% of hospitalized patients with liver cirrhosis had MHE, and patients with Child-Pugh-Classification score-C had a high prevalence of MHE (56.1%) and increased health-related QoL scores that reflected poorer life status. Increasing awareness of its adverse impact on life quality should be emphasized. Recommendations to screen for MHE may be applicable for evaluating the risks of driving and work accidents in patients with cirrhosis.

Terminology

HE is a serious complication of liver cirrhosis that represents a continuous spectrum of neurologic and neuropsychiatric abnormalities. MHE, the mildest form of HE, is defined as patients with normal mental and neurological examinations but with a number of neuropsychiatric and neuro-physiological defects identified by psychometric tests.

Peer review

In this paper, the authors investigated the prevalence of MHE, and assessed corresponding HEQoL in hospitalized cirrhotic patients in China. This is an interesting study regarding the prevalence and clinical features of MHE in China. The article showed a progress in the study of HE in cirrhotic patients. It is an

innovative job of HE study and data is reliable.

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BRIEF ARTICLE

Laparoscopic splenic hilum lymph node dissection for advanced proximal gastric cancer: A modified approach for pancreas- and spleen-preserving total gastrectomy

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Abstract

AIM: To investigate the feasibility and optimal approach for laparoscopic pancreas- and spleen-preserving splenic hilum lymph node dissection in advanced proximal gastric cancer.

METHODS: Between August 2009 and August 2012, 12 patients with advanced proximal gastric cancer treated in Nanfang Hospital, Southern Medical University, Guangzhou, China were enrolled and subsequently underwent laparoscopic total gastrectomy with pancreas- and spleen-preserving splenic hilum lymph node (LN) dissection. The clinicopathological characteristics, surgical outcomes, postoperative course and follow-up data of these patients were retrospectively collected and analyzed in the study.

RESULTS: Based on our anatomical understanding of peripancreatic structures, we combined the characteristics of laparoscopic surgery and developed a modified approach (combined supra- and infra-pancreatic approaches) for laparoscopic pancreas- and spleen-preserving splenic hilum LN dissection. Surgery was completed in all 12 patients laparoscopically without conversion. Only one patient experienced intraoperative bleeding when dissecting LNs along the splenic artery and was handled with laparoscopic hemostasis. The mean operating time was 268.4 min and mean number of retrieved splenic hilum LNs was 4.8. One patient had splenic hilum LN metastasis (8.3%). Neither postoperative morbidity nor mortality was observed. Peritoneal metastasis occurred in one patient and none of the other patients died or experienced recurrent disease during the follow-up period.

CONCLUSION: Laparoscopic total gastrectomy with pancreas- and spleen-preserving splenic hilum LN dissection using the modified approach for advanced proximal gastric cancer could be safely achieved.

Key words: Proximal stomach; Stomach neoplasm; Laparoscopy; Lymph node excision; Splenic hilum

Core tip: Pancreas- and spleen-preserving splenic hilum lymph node dissection in laparoscopic total gastrectomy is challenging. Even though a small number of skilled laparoscopic surgeons have demonstrated the safety and feasibility of this procedure, most surgeons adopt only the suprapancreatic approach. However, exposure and dissection of splenic hilum lymph nodes posterior to the splenic artery, especially its inferior branch is sometimes difficult and unpredicted injury or bleeding is more likely to occur if only through the suprapancreatic approach. We combined the supra- and infra-pancreatic approaches to better expose the posterior

splenic artery lymph nodes at the splenic hilum and dissect more safely.

Mou TY, Hu YF, Yu J, Liu H, Wang YN, Li GX. Laparoscopic splenic hilum lymph node dissection for advanced proximal gastric cancer: A modified approach for pancreas- and spleen-preserving total gastrectomy. *World J Gastroenterol* 2013; 19(30): 4992-4999 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/4992.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.4992

INTRODUCTION

The metastatic rate of splenic hilum lymph nodes (LNs) has been reported to range from 8% to 21% in advanced proximal gastric cancer[1-6], and the removal of splenic hilum LNs might bring about potential survival benefit for these patients. Accordingly, splenic hilum LN dissection is recommended in the surgical treatment for advanced proximal gastric cancer[7].

Traditionally, the dissection of splenic hilum LNs and nodes along the splenic artery (SA) is achieved through pancreatosplenectomy or pancreas-preserving splenectomy. However, it has been suggested that the combined resection of pancreas and/or spleen would significantly increase postoperative morbidity and mortality rather than improve prognosis, as well as decrease immunological function[8-12]. As an alternative, pancreas- and spleen-preserving splenic hilum LN dissection might decrease postoperative morbidity without compromising oncological principles[13].

With the rapid development of minimally invasive surgery, the application of laparoscopic surgery for gastric cancer is gradually gaining popularity[14-16]. However, due to the tortuous splenic vessels and possibility of parenchymal injury to the spleen or pancreas, it is still a challenging and technically demanding procedure for conducting laparoscopic pancreas- and spleen-preserving splenic hilum LN dissection. Only a few experienced laparoscopic surgeons have suggested its safety and feasibility[17-19], and most of them adopted the suprapancreatic approach to perform pancreas- and spleen-preserving splenic hilum LN dissection without using the infrapancreatic approach near pancreatic tail, while this method might not facilitate the dissection of LNs posterior to the splenic hilum.

Based on our anatomical understanding of peripancreatic fascia and spaces, we attempted a novel strategy combining supra- and infra-pancreatic approaches to perform laparoscopic pancreas- and spleen-preserving splenic hilum LN dissection in total gastrectomy for treating advanced proximal gastric cancer. Herein, detailed procedure and preliminary results are presented.

MATERIALS AND METHODS

Patients

Between August 2009 and August 2012, 112 patients with endoscopically biopsy-proven proximal gastric cancer underwent laparoscopic total gastrectomy in Nanfang Hospital, Southern Medical University. Among them, twelve consecutive patients underwent laparoscopic pancreas- and spleen-preserving splenic hilum LN dissection with curative intent.

Surgical indications

The indications for this procedure were as follows: (1) tumors were located at the upper- or middle-third of the stomach without distant metastasis; (2) tumors penetrated over the mucosa layer without invading adjacent structures; and (3) no gross involvement of the gastrosplenic ligament or LN number 4sb, at the splenic hilum or along the SA. Preoperative staging was confirmed by endoscopic ultrasound, abdominal high-resolution multidirectional computed tomography (CT), and positron emission computed tomography if necessary.

All surgical procedures were performed by Dr. Li GX, who had experience of over 500 laparoscopic gastrectomies for gastric cancer. All patients were given details about the operative procedure and potential risks before operation and provided written informed consent. This study was approved by the Ethics Committee of Nanfang Hospital.

Surgical procedures

The regional LNs were numbered according to the Japanese Classification of Gastric Carcinoma (JCGC) guidelines and LN dissection was done with laparoscopic ultrasonic shears [laparoscopic coagulation shears (LCSs); Ethicon Endo-Surgery, Cincinnati, OH, United States].

Under general anesthesia, the patient was placed in the supine position with legs set apart in a reverse Trendelenburg position. The surgeon stood on the patient's left side, the assistant surgeon on the patient's right side, and the camera operator stood between the patient's legs. After pneumoperitoneum was established with CO_2 insufflated at a pressure of 12 mmHg, five working ports were introduced (Figure 1)[20]. Exploration of the abdominopelvic cavity was conducted to exclude distant metastasis and carcinomatosis.

The greater omentum was divided along the border of the transverse colon toward the inferior pole of the spleen. By dividing the gastrocolic ligament, the lesser sac was entered. The stomach was then overturned cephalad and the left gastroepiploic vessels were located at the boundary between the gastrocolic ligament and the gastrosplenic ligament, which were then divided at their roots (Figure 2A). By separating the gastrosplenic ligament up to the left side of the esophageal hiatus, the short gastric vessels were divided just adjacent to the spleen and the upper part of the greater curvature was mobilized. LN numbers 4sa and 2 were dissected. The right gastroepiploic vein was identified by tracing proximally along the gastrocolic trunk or dissecting the mesogastrium inferior to the gastric antrum off the transverse mesocolon, which was then ligated and divided at its origin. The right gastroepiploic artery was usually identified

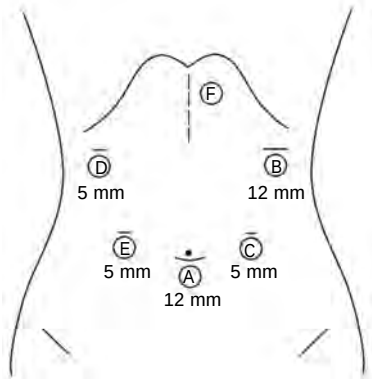

Figure 1 Positions of trocars. The trocars were inserted into the abdomen in the order A-E. Position F stands for the 4-5 cm midline minilaparotomy incision for reconstruction.

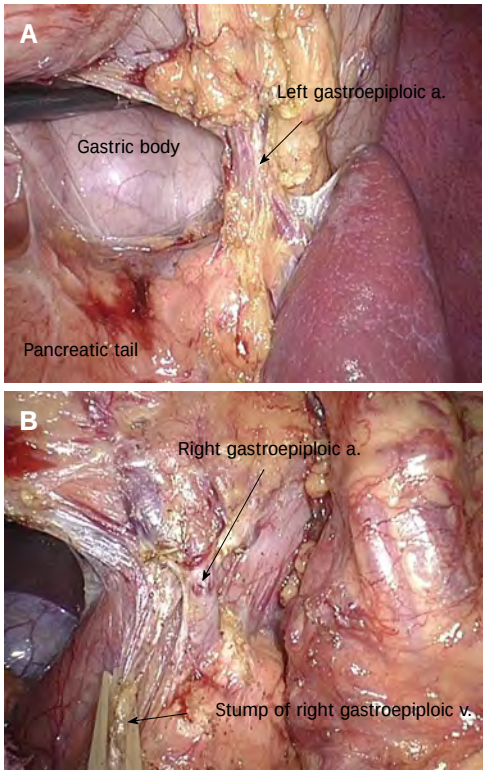

Figure 2 Gastroepiploic artery. A: Dividing left gastroepiploic artery (arrow); B: Dividing right gastroepiploic vessels (arrow). a: Artery; v: Vein.

next to the vein, which was also divided to allow the removal of LN numbers 4d and 6 (Figure 2B). After overturning the gastric antrum cranially, the gastropancreatic fold was exposed. The gastroduodenal artery was usually located in the groove between the duodenum and pancreatic head, which was a clue to trace the celiac trunk and its branches. By following the common hepatic artery, the proper hepatic artery was traced. The right gastric artery was located in the hepatoduodenal ligament as a small branch running from the proper hepatic artery to the supra-pylorus. By ligating the right and left gastric arteries and veins at origin and dissecting the tissues around the proper hepatic artery, common hepatic artery and celiac

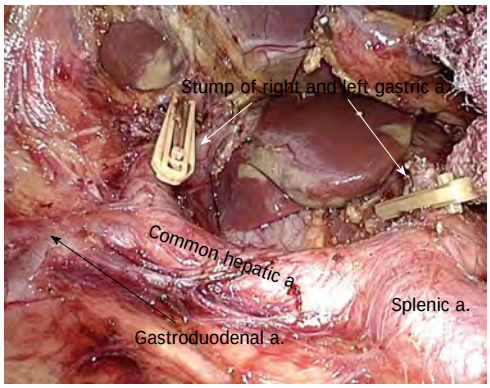

Figure 3 Tracing gastroduodenal artery to locate celiac trunk and its branches (arrows). a: Artery.

trunk, the right side of the suprapancreatic LNs (numbers 5, 7, 8a, 9 and 12a) were removed *en bloc* (Figure 3).

By retracting the pancreas meticulously in the caudal direction, the surgeon could dissect the soft tissue off the superior margin of the pancreatic body and tail in order to enter the retropancreatic space, thus uncovering the proximal SA (Figure 4A). From this step, in order to facilitate this manipulation, the surgeon changed his operating position and stood between the patient's legs. By opening the artery sheath and skeletonizing the SA from the proximal portion towards the distal portion, LN number 11p could be removed. When the bifurcation was reached, two secondary branches of the SA could be seen in most cases. The superior branch coursed towards the superior pole of the spleen and the inferior one coursed directly towards the splenic hilum. The pancreatic tail was mobilized using the infrapancreatic approach to enter the retropancreatic space (Figure 4B). The superior and inferior branches of the SA were then skeletonized until they reached the splenic parenchyma (Figure 5). Meanwhile, the remaining short gastric vessels originating from the SA were further ligated and divided. By skeletonizing the SA, fatty tissues bearing LN numbers 10 and 11d were removed, and all vessels in the splenic hilum area were saved with the preservation of both the pancreas and the spleen.

The duodenum was transected 2 cm distal to the pylorus using an endoscopic linear stapler (Echelon 60 Endopath Stapler; Ethicon Endo-Surgery, Guaynabo, Puerto Rico, United States). Subsequently, the phrenoesophageal and both vagus nerves were divided, along with the removal of LN number 1. The transaction of the esophagus and Roux-en-Y esophagojejunostomy were carried out extracorporeally through a 4-5-cm midline minilaparotomy just below the xiphoid process using a circular stapler. An end-to-side jejunojejunostomy was performed by hand suture.

RESULTS

The clinicopathological characteristics of the patients are shown in Table 1. Surgical outcomes and postoperative

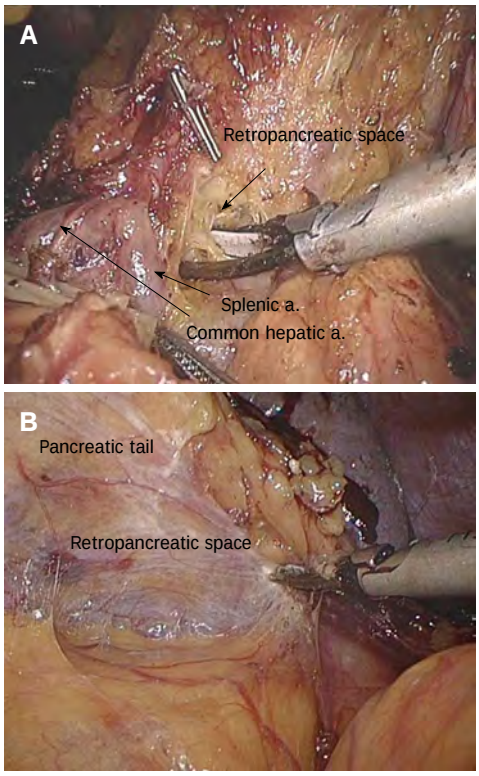

Figure 4 Entering retropancreatic space. A: Near the superior margin of the pancreas (arrows); B: Near the lower margin of the pancreatic tail. a: Artery.

course are summarized in Tables 2 and 3. There were nine male and three female patients, with a mean age of 60.6 years (range, 45-75 years). The mean body mass index was 21.5 kg/m^2 (range, 19.1-25.6 kg/m^2).

Laparoscopic total gastrectomy with pancreas- and spleen-preserving splenic hilum LN dissection was successfully performed in all 12 patients without conversion to open procedure. Only one patient experienced intraoperative bleeding during the skeletonization of the inferior branch of the SA. Pathological findings showed that tumor penetrated into the subserosal layer (T3) in only one patient and into the serosa without invasion to adjacent structures (T4a) in the other 11 patients. In accordance with the American Joint Committee on Cancer (AJCC) cancer staging manual, 7th edition, the TNM stages were distributed as follows: one stage ⅡA, three stage ⅡB, four stage ⅢA, two stage ⅢB, and two stage ⅢC. The mean number of retrieved splenic hilum LNs per patient was 4.8 (range, 2-8) and only one patient had splenic hilum LN metastasis (8.3%). Postoperatively, neither morbidity nor mortality was observed (Table 3).

At a median follow-up of 21 mo (range, 1-37 mo), one patient had peritoneal metastasis after 12 mo of surgery and died 6 mo later. None of the other patients died or experienced recurrent disease during the follow-up period.

DISCUSSION

Splenic hilum LN involvement was reported to range between 8% and 21%[1-6] and was identified as an important prognostic factor for gastric carcinoma in previous studies[4,6,9,21]. Splenic hilum LN involvement rate correlates with the depth of tumor invasion over the mucosal layer[2,3], the tumor is classified as Bormann's type Ⅲ or Ⅳ[3,5,22], the tumor is located at the greater curvature[5], and the tumor size is > 5 cm[4]. Thus, splenic hilum LN dissection should be conducted in patients with advanced proximal gastric cancer, especially those whose tumor has the above mentioned properties.

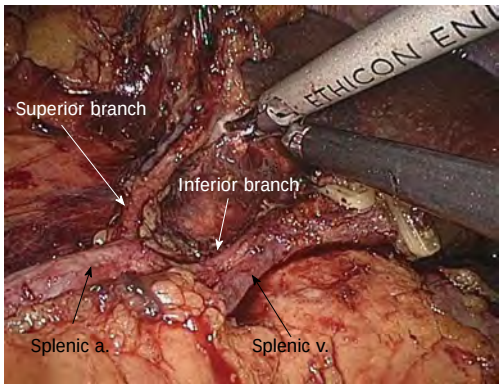

Figure 5 Skeletonizing the branches of the splenic artery (arrows). a: Artery; v: Vein.

For the complete removal of splenic hilum LNs, in traditional open surgery, extended total gastrectomy including pancreatosplenectomy was once recommended as the classic procedure by some surgeons[23,24]. However, combined resection of the distal pancreas is associated with increased postoperative complications, including acute pancreatitis, pancreatic fistula, abdominal abscess, and postoperative diabetes, which may even adversely affect survival. As a result, total gastrectomy with pancreas-preserving splenectomy has been proposed by other surgeons[11,25]. Other studies have demonstrated that splenectomy may result in higher morbidity and mortality, and has no significant survival benefit[1,9,10,22,26]. Accordingly, pancreas- and spleen-preserving total gastrectomy has been attempted in open surgery[13], although it is still controversial.

Laparoscopic gastrectomy, as an alternative to traditional open surgery for early gastric cancer, has been suggested to produce comparable morbidity and mortality, as well as long-term survival as open gastrectomy, while possessing the benefits of minimally invasive approaches[27-32]. With respect to the above reasons, laparoscopic distal gastrectomy has gradually gained popularity for the treatment of early gastric cancer located in the lower portion of the stomach[14-16,32]. However, only a few studies have reported the application of laparoscopic total gastrectomy in advanced proximal gastric cancer[33-35]. With the development of laparoscopic devices and accumulation of experiences, a small number of skilled laparoscopic surgeons in high-volume specialized centers have attempted to extend the indications to advanced proximal gastric cancer using the strategy of splenic hilum LN dis-

Table 1 Clinicopathological characteristics of patients

Patient No.	Gender	Age (yr)	BMI (kg/m^2)	Tumor location	Tumor size (cm)	Tumor depth[1]	TNM stage[1]	No. of retrieved LN[2]	No. of metastatic LN[2]	No. of retrieved splenic hilum LN[2]	No. of metastatic splenic hilum LNc
1	Male	60	19.1	U	5.0	T4a	ⅢC	21	7	3	0
2	Male	73	24.8	U	6.0	T4a	ⅢB	18	5	4	0
3	Male	61	20.6	U	8.5	T4a	ⅢA	34	2	8	0
4	Male	62	20.2	U	7.0	T3	ⅡA	39	0	4	0
5	Male	59	20.8	U	4.5	T4a	ⅡB	16	0	3	0
6	Female	54	20.4	M	5.0	T4a	ⅢA	20	1	5	0
7	Female	57	24.0	U	5.0	T4a	ⅢA	20	1	7	0
8	Male	57	19.1	U	8.0	T4a	ⅢC	28	21	6	3
9	Male	61	25.6	U	3.5	T4a	ⅢA	16	2	4	0
10	Male	63	21.1	M	2.9	T4a	ⅡB	24	0	5	0
11	Female	75	21.8	U	5.5	T4a	ⅢB	19	3	2	0
12	Male	45	20.6	M	4.0	T4a	ⅡB	35	0	6	0

[1]"Tumor depth" and "Tumor node metastasis (TNM) stage" were in accordance with the AJCC cancer staging manual-7th edition; [2]Lymph nodes (LN). BMI: Body mass index.

Table 2 Surgical outcomes, postoperative course and follow-up data of patients

Patient No.	Operating time (min)	Estimated blood loss (mL)	Time to first flatus (POD)	Time to soft diet (POD)	Hospital stay (POD)	Follow-up (mo)	Follow-up outcome
1	230	50	5	10	10	37	No recurrence, alive
2	352	300	6	10	13	37	No recurrence, alive
3	180	100	4	8	11	36	No recurrence, alive
4	314	100	5	7	10	28	No recurrence, alive
5	278	100	4	8	12	24	No recurrence, alive
6	305	100	2	9	11	24	No recurrence, alive
7	298	300	5	8	12	24	No recurrence, alive
8	260	150	2	4	8	18	Peritoneal metastasis, death
9	280	200	3	5	6	6	No recurrence, alive
10	223	50	2	6	7	5	No recurrence, alive
11	221	150	3	4	5	4	No recurrence, alive
12	280	200	3	7	8	1	No recurrence, alive

POD: Postoperative days.

Table 3 Surgical outcomes and postoperative courses of laparoscopic pancreas- and spleen-preserving splenic hilum lymph nodes dissection

Items	mean ± SD (range)
Operating time (min)	268.4 ± 48.0 (180-352)
Estimated blood loss (mL)	150.0 ± 85.3 (50-300)
No. of retrieved LN	24.2 ± 7.9 (16-39)
No. of metastatic LN	3.5 ± 5.9 (0-21)
No. of retrieved splenic hilum LN	4.8 ± 1.8 (2-8)
No. of metastatic splenic hilum LN	0.3 ± 0.9 (0-3)
Time to first flatus (POD)	3.7 ± 1.4 (2-6)
Time to soft diet (POD)	7.2 ± 2.1 (4-10)
Hospital stay (POD)	9.4 ± 2.6 (5-13)
Intraoperative complication	1 (8.3%)
Postoperative complication	0
Mortality	0

LN: Lymph nodes; POD: Postoperative days.

section in pancreas- and spleen-preserving total gastrectomy[17-19,36].

The major difficulties of this laparoscopic procedure lie in the complicated variations of the SA supplying the spleen with its variable branching. The greatest challenges to surgeons are the high probability of injuries to the splenic vessels, unpredicted avulsion of the splenic capsule, skillful manipulation of endoscopic devices in a limited space, and injuries to the splenic hilum during skeletonization of the splenic vessels. Our strategy to deal with these difficulties was based on our thorough understanding of anatomy under laparoscopic view[20] and team cooperation. The SA is located in the retropancreatic space, coursing near the superior margin of the pancreas and usually dividing into two terminal branches near the pancreatic tail[37-39].

The inferior branch of the SA courses directly into the splenic hilum, therefore, the exposure and dissection of the LNs posterior to it are sometimes difficult. From our past experience, if the vascularization and dissection was continued leftward only through the suprapancreatic approach, bleeding and unpredicted injury were more likely to occur due to the exposure limit. Thus, in our clinical practice, the suprapancreatic approach was adopted for the vascularization of the SA trunk, its superior branch, and the upper hemisphere of its inferior branch. Then, the lower margin of the pancreatic tail was mobilized. Since the retropancreatic space was filled with loose

connective tissue near the lower margin of the pancreatic tail[40], exposure of the lower hemisphere could easily be achieved with the assistant turning the pancreatic tail cephalad. The vascularization of the inferior branch was continued until coming across the upper hemisphere, in other words, the inferior branch of the SA was skeletonized both through the supra- and infra-pancreatic approaches. The splenic pedicle was also freed, allowing for the complete removal of the posterior splenic hilum LNs.

Our strategy for laparoscopic pancreas- and spleen-preserving splenic hilum LN dissection is different from that in previous reports in the literature. The hand-assisted technique was adopted by Uyama *et al*[17], taping the SA was applied by Hur *et al*[19], and Hyung stood at the patient's right side and skeletonized the distal portion of the SA as soon as completing division of the gastrosplenic ligament[18]. To our knowledge, all these surgeons adopted the suprapancreatic approach. However, one similarity we noted was that when approaching the splenic hilum, meticulous manipulation was required to avoid injury. In Hyung's report, preoperative assessment of the splenic vascular anatomy was conducted with CT in collaboration with radiologists[18]. In our study, we experienced an episode of major intraoperative bleeding during dissection of the inferior branch of the SA in one of our patients. We applied endoscopic gauze to compress the bleeding area and identified the bleeding point. A hemo-lock was then used to clip onto the artery surface, involving the bleeding point without fully clamping the whole artery. Successfully, the bleeding was finally controlled after some maneuvers. In retrospect, even if this attempt was not successful, splenectomy could be safely conducted because the splenic pedicle was freed. Given these aspects, the average operating time was increased to 268.4 min, and the time was especially longer for the first three cases; nevertheless, our average operating time was still in congruent with that in the previous reports[17-19]. Although the patients in our study suffered from a more advanced stage of gastric carcinoma, the early follow-up results showed satisfactory survival.

In our study, only one out of 12 patients had splenic hilum LN metastasis. Interestingly, this patient had an overall high percentage of positive LNs, experienced peritoneal metastasis at 12 mo after surgery and died 6 mo later. This finding might suggest that splenic hilum LN involvement is always associated with highly advanced proximal gastric cancers, and poorer prognosis in these patients might be predicted. Similarly, Shin *et al*[4] found that splenic hilum LN metastasis had a poor prognosis. However, due to the limitation of our relatively small sample size, the correlation between splenic hilum LN metastasis and oncological outcomes needs to be further confirmed.

Our retrospective study also had several limitations, including patient selection bias and relatively small sample size. However, to the best of our knowledge, this is a modified approach for conducting laparoscopic pancreas- and spleen-preserving splenic hilum LN dissection. The detailed procedure might be useful for surgeons who wish to conduct similar laparoscopic surgery.

In conclusion, using the strategy of combining supra- and infra-pancreatic approach to extend the retropancreatic space in experienced hands, laparoscopic total gastrectomy with pancreas- and spleen-preserving splenic hilum lymph nodes dissection for the treatment of advanced proximal gastric cancer in selected patients could be safe and feasible. However, long-term follow-up and randomized clinical trials to evaluate its surgical safety and oncological efficacy are needed.

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COMMENTS

Background

Laparoscopic gastrectomy, as a minimally invasive alternative treatment to traditional open surgery in treating gastric cancer, is gaining popularity worldwide. For advanced gastric cancer, radical surgery should accomplish adequate lymph node (LN) dissection (D2 lymphadenectomy) according to oncological principles. Splenic hilum LN dissection should be included in the D2 lymphadenectomy when treating advanced proximal gastric cancer.

Research frontiers

Traditional removal of splenic hilum LNs was achieved through combined resection of the pancreas and/or spleen. However, it has been suggested that combined resection would increase postoperative morbidity and mortality and not significantly benefit patient survival. Thus, pancreas- and spleen-preserving total gastrectomy was subsequently attempted in open and laparoscopic surgery.

Innovations and breakthroughs

In laparoscopic total gastrectomy, pancreas- and spleen-preserving splenic hilum LN dissection is challenging because of the tortuous splenic vessels and possibility of parenchymal injury to the spleen or pancreas. To date, only a small number of skilled laparoscopic surgeons in high-volume specialized centers can achieve splenic hilum LN dissection in pancreas- and spleen-preserving total gastrectomy, and most of them only adopt the suprapancreatic approach. This method might not facilitate the dissection of LNs posterior to the splenic hilum and might cause unpredicted injury to splenic vessels. Thus, they modified this strategy by combining both supra- and infra-pancreatic approaches to better expose the posterior splenic artery LNs at the splenic hilum, and dissect more safely.

Applications

Using the strategy of combining supra- and infra-pancreatic approach to extend the retropancreatic space in experienced hands, laparoscopic total gastrectomy with pancreas- and spleen-preserving splenic hilum lymph nodes dissection for the treatment of advanced proximal gastric cancer in selected patients could be safe and feasible. The indications for laparoscopic surgery could be extended to advanced proximal gastric cancer. The detailed procedure described here might be useful for laparoscopic surgeons. However, due to the limited sample size, further long-term follow-up results and randomized controlled trials are needed to ascertain its surgical safety and oncological efficacy.

Terminology

In advanced gastric cancer, the tumor penetrates the mucosal layer of the stomach wall. In advanced proximal gastric cancer, the tumor is located in the upper or middle third of the stomach. Splenic hilum LNs are the LNs located adjacent to the splenic artery distal to the pancreatic tail, those on the roots of the short gastric arteries, and those along the left gastroepiploic artery proximal to its first gastric branch, according to the Japanese Classification of Gastric Carcinoma guidelines.

Peer review

The authors described of clinical impact of laparoscopic splenic hilum lymph node dissection for advanced proximal gastric cancer based on the strategy combining supra- and infra-pancreatic approach for pancreas- and spleen-preserving total gastrectomy. It is well written.

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BRIEF ARTICLE

Stent-grafts for the treatment of TIPS dysfunction: Fluency stent *vs* Wallgraft stent

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Abstract

AIM: To evaluate the clinical efficacy of an expanded polytetrafluoro-ethylene-covered Fluency stent compared with that of a polyethylene terephthalate-covered Wallgraft stent for the management of transjugular intrahepatic portosystemic shunt (TIPS) dysfunction.

METHODS: A retrospective review of patients who underwent TIPS revision with stent-grafts between May 2007 and June 2011 was conducted. The patients were divided into two groups according to the stent-grafts implanted: the Fluency stent (Bard Incorporated, Karlsruhe, Germany) and the Wallgraft stent (Boston Scientific, Galway, Ireland). The primary patency rates were calculated and compared using the Kaplan-Meier method.

RESULTS: A total of 73 patients were evaluated in this study: 33 with Fluency stents and 40 with Wallgraft stents. The primary patency rates at 12 and 24 mo were 91% and 85%, respectively, in the Fluency stent group and 78% and 63%, respectively, in the Wallgraft stent group. The primary shunt patency rates after TIPS revision were significantly better with the Fluency stent than with the Wallgraft stent (P = 0.033).

CONCLUSION: TIPS revision with the Fluency stent has higher medium-term patency rates than that with the Wallgraft stent.

Key words: Expanded polytetrafluoroethylene-covered stent-grafts; Transjugular intrahepatic portosystemic shunt dysfunction; Revision; Fluency

Core tip: There are few data on the clinical use of expanded polytetrafluoroethylene-covered stent-grafts for the management of transjugular intrahepatic portosystemic shunt (TIPS) dysfunction in the literature. The present study was designed to retrospectively evaluate the clinical efficacy of Fluency stent compared with Wallgraft stent in the treatment of TIPS dysfunction. And the results demonstrated that TIPS revision with the Fluency stent has higher medium-term patency rates than that with the Wallgraft stent.

Luo XF, Nie L, Wang Z, Tsauo J, Liu LJ, Yu Y, Zhou B, Tang CW, Li X. Stent-grafts for the treatment of TIPS dysfunction: Fluency stent *vs* Wallgraft stent. *World J Gastroenterol* 2013; 19(30): 5000-5005 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/5000.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.5000

INTRODUCTION

Transjugular intrahepatic portosystemic shunts (TIPS) have been increasingly used for the management of portal hypertension complications in patients with cirrhosis[1,2]. However, shunt dysfunction is a major drawback of TIPS. The primary patency rate after 24 mo has been reported to be 40%-60% when bare stents were used[1]. Consequently, regular shunt surveillance and reintervention are required to maintain shunt patency.

TIPS dysfunction is the result of acute thrombosis within the stent or of pseudointimal hyperplasia within the TIPS tract in the liver parenchyma or along the outflow hepatic vein[3]. Both phenomena may be associated with a biliary fistula. Several experimental and clinical studies have shown that TIPS creation with an expanded polytetrafluoroethylene (ePTFE)-covered Viatorr stent can remarkably improve the long-term shunt patency[4-6]. Similarly, encouraging results have been obtained when the Viatorr stent was used for the treatment of TIPS dysfunction[7,8].

However, the Viatorr stent is not available in many countries. The Fluency stent, which is a non-dedicated ePTFE-covered stent-graft, has been utilized to establish a transjugular intrahepatic portosystemic shunt[9]. Currently, there is no relevant report on the clinical use of the Fluency stent for the management of TIPS dysfunction in the literature. In this study, we retrospectively evaluated the clinical efficacy of the Fluency stent and of the Wallgraft stent in TIPS revision.

MATERIALS AND METHODS

Patient selection

This retrospective study was approved by the ethics committees of West China Hospital, Sichuan University. Between May 2007 and June 2011, patients who underwent TIPS revision by implantation of stent-grafts were analyzed. Patients were excluded if they already had a previous TIPS revision. Patients who underwent the insertion of a parallel shunt due to failed original shunt revision were also excluded. Thus, a total of 73 patients were evaluated in this study. This study group was further divided into two subgroups according to the stent-grafts received: 33 patients who underwent TIPS revision by implantation of the Fluency stent and 40 patients who underwent TIPS revision by implantation of the Wallgraft stent.

TIPS revision procedure

Written consent was obtained from each patient before the procedure. All procedures were performed by two experienced interventional radiologists. The patients were prepared and draped in the angiographic suite using local anesthesia. After puncturing the right internal jugular vein, a standard 10-F TIPS set (Cook Incorporated, Bloomington, United States) was introduced into the inferior vena cava. Once the previous shunt was accessed through the sheath using an angled hydrophilic guidewire (Terumo Company, Fijinomiya, Japan), a 5-F Cobra catheter (Terumo Company) was advanced into the superior mesenteric or splenic vein. Portography was performed with the 5-F catheter placed in the portal region, and the portosystemic pressure gradient (PPG) was measured. After dilating the stenotic or occluded shunt with a 10 mm angioplasty balloon catheter (Cordis, LJ Roden, the Netherlands), we advanced the 10-F sheath into the main portal vein. The stent-graft delivery set, which was either a Fluency stent or a Wallgraft stent, was then introduced into the sheath. The sheath was withdrawn into the inferior vena cava, and the stent-graft was released to cover the entire length of the shunt up to the junction of the hepatic vein and the inferior vena cava. Shunt venography was performed, and the PPG was measured again. Additional shunt dilation or an additional stent-graft implantation was performed if necessary. Patients with a PPG higher than 12 mmHg despite sufficient dilation of the shunt received prophylactic embolization of the varices with metal coils.

Medication

All patients received a single prophylactic dose of a second-generation cephalosporin 1 h before the procedure. Intravenous heparin (3000 U) was administered immediately after successful TIPS revision, except for those patients with a coagulation disorder. Subsequently, antiplatelet therapy with aspirin was maintained for life.

Follow-up

All patients were evaluated by the same medical team in the gastroenterology clinic according to the follow-up schedule. Doppler duplex ultrasonography (US) was performed 24 h and 1, 3 and 6 mo after the procedure, followed by every 6 mo thereafter or whenever recurrent TIPS dysfunction was suspected clinically. TIPS dysfunction was suspected on the US if the intrastent flow velocity was less than 60 cm/s or higher than 120 cm/s or if there was a change in the direction of the flow in the intrahepatic portal branches compared with previous US findings. TIPS dysfunction was defined as a shunt narrowing of more than 50%, a PPG higher than 12 mmHg or both. Primary patency was defined as the interval of time without an intervention.

Statistical analysis

The results were expressed as the mean ± SD. The 12 and 24 mo primary patency rates were analyzed and compared using the Kaplan-Meier method. The results were compared with the log-rank test. A P value of less than 0.05 was considered statistically significant. All calculations were performed using SPSS version 20.0 software for Windows.

RESULTS

The patient characteristics at the time of the TIPS revision and indications for TIPS revision are documented and summarized in Table 1. Technical success was achieved in all 73 patients, and no severe complications

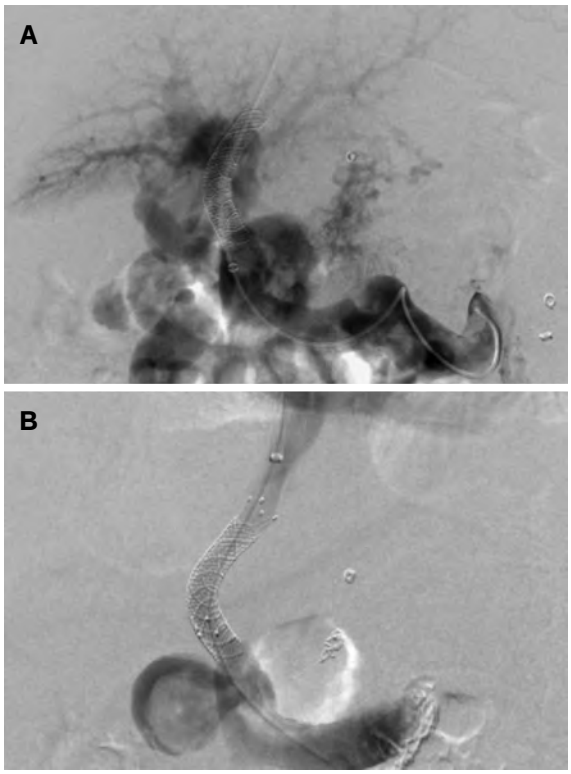

Figure 1 Portal venograms in a 52-year-old woman with hepatitis B cirrhosis who was treated with transjugular intrahepatic portosystemic shunt for recurrent variceal bleeding. A: Portography after crossing the previous shunt shows a patent splenic vein and the occluded main portal vein with cavernous transformation; B: After implantation with a Fluency stent, the portosystemic pressure gradient was reduced from 31 to 11 mmHg.

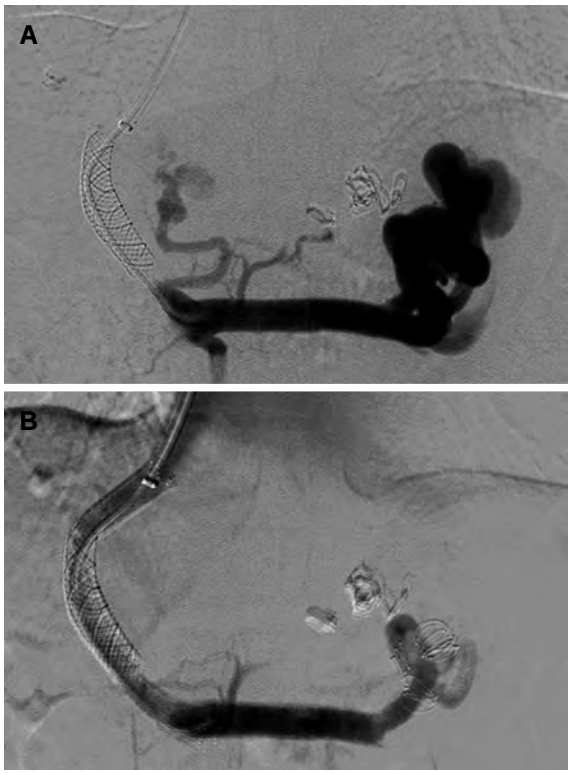

Figure 2 Portal venograms in a 45-year-old man with hepatitis B cirrhosis who had undergone transjugular intrahepatic portosystemic shunt for repeated variceal bleeding. A: The anteroposterior portal venogram obtained 5 mo after the initial transjugular intrahepatic portosystemic shunt procedure shows the complete occlusion of the stent and opacification of a massive spontaneous splenorenal shunt; B: Embolization of the splenorenal shunt was performed to maintain sufficient portal flow to keep the stent open. The portal venogram after Wallgraft stent placement reveals a wide patent shunt. The portosystemic pressure gradient decreased from 37 to 10 mmHg.

occurred (Figures 1 and 2). Four patients had transient discomfort in the upper abdominal area. A total of 15 patients developed new hepatic encephalopathy after the procedure, including nine patients with the Fluency stent and six with the Wallgraft stent. Eleven of these patients were successfully managed by protein restriction and lactulose administration. One patient was treated with shunt reduction by implantation of an additional Fluency stent, which raised the PPG from 7 to 10 mmHg. Hepatic encephalopathy failed to improve in the remaining three patients.

In the 33 TIPS revisions with the Fluency stent, the mean PPG was reduced from 18.5 ± 4.9 mmHg (range: 13-47 mmHg) to 7.4 ± 5.7 mmHg (range: 5-15 mmHg) after the procedure. A single stent-graft was used in 32 cases, and two stent-grafts were used in the other case because the stent was not terminated at the hepatocaval junction. All Fluency stents measured 10 mm in diameter. A PPG value below 12 mmHg after TIPS revision was not achieved in five patients, even though no obvious shunt stenosis was observed. Prophylactic embolization of the varices with metal coils was then performed in these patients.

In the 40 TIPS revisions with the Wallgraft stent, the mean PPG was reduced from 20.8 ± 3.7 mmHg (range: 12-51 mmHg) to 8.6 ± 3.1 mmHg (range: 6-17 mmHg) after the procedure. A single Wallgraft stent was used in 38 patients. Two patients were implanted with an additional stent because the shunt was not extended to the inferior vena cava. All Wallgraft stents measured 10 mm in diameter. The stent-grafts were placed after shunt dilation with 4 mm × 10 mm angioplasty balloons. In three patients, the PPG was not reduced to below 12 mmHg. These three patients received prophylactic embolization of the varices with metal coils. Another patient was treated with embolization of a massive splenorenal shunt to maintain sufficient portal flow in the stent (Figure 2).

The mean follow-up time in the Fluency stent group was 27.7 ± 11.6 mo (range: 3.5-46.5 mo). Of the 33 patients in this group, seven patients developed recurrent TIPS dysfunction during this period. Three patients presented with gastrointestinal hemorrhage, and the other four were diagnosed by US findings (Table 2). Angioplasty and subsequent implantation of a second stent-graft were performed in four of these patients, resulting in the restoration of blood flow within the shunt. One patient underwent orthotopic liver transplantation without revision. The other two patients refused TIPS revision. The primary shunt patency rates after TIPS revision with the Fluency stent were 91% at 12 mo and 85% at 24 mo.

The mean follow-up time in the Wallgraft stent group

Table 1 Patient demographics

Characteristics	Fluency endoprosthesis	Wallgraft endoprosthesis	*P* value
Patients (*n*)	33	40	
Age (yr)	51.4 ± 5.7	50.2 ± 8.5	0.491
Sex (male/female)	25/8	29/11	0.752
Etiology			0.242
Hepatitis B	21	31	
Alcohol	7	4	
Other	5	5	
Child-Pugh classification			0.564
A	6	8	
B	19	25	
C	8	7	
TIPS indication			0.946
Variceal bleeding	25	31	
Ascites	6	5	
Other	2	4	
Indications for TIPS dysfunction			0.762
Abnormal US findings	12	15	
Variceal bleeding	18	23	
Ascites	3	2	

TIPS: Transjugular intrahepatic portosystemic shunt; US: Ultrasonography.

Table 2 Long-term outcome of patients

	Fluency endoprosthesis	Wallgraft endoprosthesis	*P* value
Patients (*n*)	33	40	
Hepatic encephalopathy	9	6	0.196
Variceal rebleeding	7	19	0.020
Shunt dysfunction			
12 mo	3	9	0.722
24 mo	4	15	0.039

was 25.6 ± 13.2 mo (range: 3-48 mo). Of the 40 patients in this group, 19 patients developed recurrent TIPS dysfunction during this period. Eight patients presented with gastrointestinal hemorrhage, three presented with recurrent ascites, and the remaining eight were diagnosed by US findings (Table 2). Angioplasty and subsequent implantation of a second stent-graft were performed in the nine patients who had shunt restenosis or occlusion. Five patients were solely treated with angioplasty because the portography did not show any stenosis or portal vein thrombosis. Three patients were not revised because of overt hepatic encephalopathy. One patient underwent orthotopic liver transplantation. The primary shunt patency rates after TIPS revision with the Wallgraft stent were 78% at 12 mo and 63% at 24 mo. The primary shunt patency rates after TIPS revision were significantly better with the Fluency stent than with the Wallgraft stent (Log-rank test, $P = 0.033$, Figure 3).

DISCUSSION

The causes of TIPS dysfunction include acute thrombosis and pseudointimal hyperplasia in the parenchymal tract of the shunt or in the outflow hepatic vein[3,10]. During TIPS creation with bare stents, the dilatation of the liver parenchymal tract may cause laceration of the bile ducts, resulting in biliary-TIPS fistulas, which have been observed frequently in patients with acute thrombosis or recurrent shunt occlusions. The fibrotic or inflammatory healing response to the trauma of shunt creation induces the fibroblasts from adjacent liver stroma to differentiate into myofibroblasts and to then migrate through the stent mesh into the shunt lumen[3,10]. The overgrowth of pseudointimal hyperplasia could be responsible for stenosis or occlusion within the parenchymal tract.

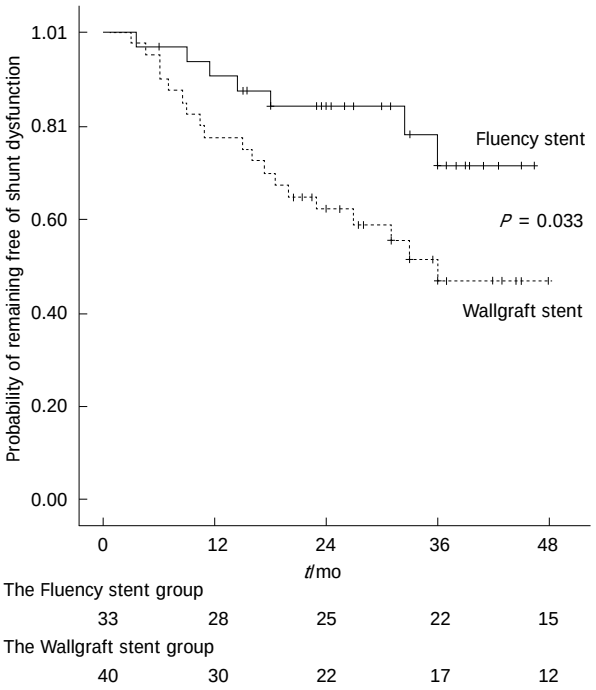

Figure 3 The probability of remaining free of shunt dysfunction.

The preliminary stent position within the outflow hepatic vein plays an important role in TIPS patency. The turbulence and shear stress from increased shunt flow could provoke the acceleration of pseudointimal hyperplasia and predispose the patient to shunt dysfunction. Additionally, late shortening of the self-expanding stent-grafts may occur, leaving the outflow hepatic vein at risk of subsequent intimal hyperplasia or the recoiling of an unsupported outflow section of the parenchymal tract[11]. Clark *et al*[12] demonstrated that the initial stent position within the hepatic venous outflow was predictive of shunt patency, with TIPS extending to the junction of the hepatic vein and the inferior vena cava having longer lifespans than shunts terminating in the hepatic vein. In this study, the primary patency rates at 12 mo were 36% ± 10% among patients with the outflow portion of the stent-grafts terminating in the hepatic vein and 58% ± 8% among patients with the outflow portion of the stent-grafts terminating at the hepatocaval junction[12]. Based on previous studies and our experience, we were careful about bridging the complete tract to the inferior vena cava in the present series.

A few experimental and clinical studies have verified the application of ePTFE-covered stent-grafts in *de novo*

TIPS creation and TIPS revision[4,7,8]. ePTFE was utilized as a cover material for stent-grafts, separating the blood flow within the shunt from the liver parenchyma and from the injured outflow hepatic vein. After TIPS creation with the ePTFE-covered stent-graft, the shunt flow was maintained by inhibiting the overgrowth of pseudointimal hyperplasia in the parenchymal tract or along the outflow hepatic vein. Echenagusia et al described the application of ePTFE-covered stent-grafts in the treatment of TIPS stenosis or occlusion in 12 patients. After TIPS revision, the primary patency rates were 100% at 12 mo and 88.8% at 24 mo[7]. More recently, Jirkovsky et al reported a clinical study in which 121 episodes of dysfunctional TIPS were evaluated retrospectively. The primary patency rates after 12 and 24 mo were 49.7% and 25.3%, respectively, in conventional angioplasty, 74.9% and 64.9%, respectively, with bare stents, 75.2% and 64.5%, respectively, with ePTFE-covered stent-grafts and 88.1% and 80.8%, respectively, with Viatorr stent-grafts. These results showed a tendency favoring ePTFE-covered stent-grafts, especially the Viatorr stent[13]. Unfortunately, the Viatorr stent is not commercially available in mainland China.

Compared with previously published reports, our study has a relatively large number of patients who underwent TIPS revision (n = 73). In our series, the primary shunt patency rates at 12 and 24 mo were 91% and 85%, respectively, with the Fluency stent and 78% and 63%, respectively, with the Wallgraft stent. After TIPS revision by implantation of a second stent-graft, the primary shunt patency rates were significantly better with the Fluency stent than with the Wallgraft stent. The long-term patency of the Fluency stent compared well with the previously published results of ePTFE-covered stent-grafts[13]. To the best of our knowledge, TIPS revision with angioplasty alone may have barely satisfactory short-term patency but results in a high incidence of recurrent shunt dysfunction. Lining the dysfunctional TIPS with a second stent-graft not only enables the restoration of shunt function but may also improve the configuration of the TIPS to prevent future restenosis.

The Fluency stent is a PTFE-encapsulated grid-like cylinder composed of a biocompatible nickel-titanium alloy. The deployment of a Fluency stent is easy because the position of this type of stent-graft is very precise. Based on our data, one patient (3%) with the Fluency stent and two patients (5%) with the Wallgraft stent required additional stent-graft implantation, partly due to previous stent malposition. The Fluency stent is one of the two commercially available stent-grafts in mainland China, the other being the Wallgraft stent[9,14]. Wu *et al*[9] performed a retrospective study on shunt patency in patients treated with TIPS creation using the Fluency stent. The rates of recurrent bleeding, shunt occlusion, hepatic encephalopathy and mortality were 0.03%, 0.0%, 16.7% and 0% after 6.16 ± 3.89 mo of follow-up. Although the follow-up time was not long enough, these results suggested that the Fluency stent was effective in TIPS creation and had a favorable patency rate. The Wallgraft stent is a polyethylene terephthalate-covered stent-graft and has been considered unsuitable for initial TIPS creation according to experimental studies[12,15]. However, the role of the Wallgraft stent in TIPS revision remains unknown.

There are several limitations of the present study that warrant consideration. This study is a single-institution, retrospective study, which increases the likelihood of systematic bias. Ideally, a randomized two-arm clinical trial should be designed to compare the Fluency stent with the Wallgraft stent. Additionally, the decision on the stent-graft selected for revision was based on the operator`s preference.

This study represents one of the largest published case series of patients with dysfunctional TIPS who underwent shunt revision with stent-grafts. Our results suggest that completing TIPS revision with the Fluency stent is safe and effective. Although large prospective studies with longer follow-up periods are needed, our analysis indicates that TIPS revision using the Fluency stent provided better shunt patency than that using the Wallgraft stent in the medium-term. Based on our institutional experience and on results from the literature, the ePTFE-covered Fluency stent could be a valuable solution for TIPS revision.

COMMENTS

Background

Transjugular intrahepatic portosystemic shunts (TIPS) has been widely used in the treatment of complications of portal hypertension. However, shunt dysfunction is a main defect of TIPS. Previously clinical studies have demonstrated that de novo TIPS creation and shunt revision with an expanded polytetrafluoroethylene (ePTFE)-covered stent could improve the long-term shunt patency.

Research frontiers

In this study, the authors demonstrated that TIPS revision with the ePTFE-covered Fluency stent has higher medium-term patency rates than that with the polyethylene terephthalate-covered Wallgraft stent which are commercially available in mainland China.

Innovations and breakthroughs

There are few data on the clinical use of Fluency stent or Wallgraft strent for the management of TIPS dysfunction in the literature. This is believed to be the first study to report that TIPS revision with Fluency stent could provide better second shunt patency.

Applications

Considering TIPS dysfunction would still be an improtant clinical issue in the near future, this study may illustrate a useful management strategy in the treatment of TIPS dysfunction.

Terminology

The Fluency stent is a polytetrafluoroethylene-encapsulated grid-like cylinder composed of a biocompatible nickel-titanium alloy.

Peer review

An interesting publication in which authors show that TIPS revision with the Fluency stent has higher medium-term patency rates than that with the Wallgraft stent. This study will be of interest and the paper is clearly written.

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BRIEF ARTICLE

Predicting a novel pathogenicity island in *Helicobacter pylori* by genomic barcoding

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Abstract

AIM: To apply a new, integrated technique for visualizing bacterial genomes to identify novel pathogenicity islands in *Helicobacter pylori* (*H. pylori*).

METHODS: A genomic barcode imaging method (converting frequency matrices to grey-scale levels) was designed to visually distinguish origin-specific genomic regions in *H. pylori*. The complete genome sequences of the six *H. pylori* strains published in the National Center for Biotechnological Information prokaryotic genome database were scanned, and compared to the genome barcodes of *Escherichia coli* (*E. coli*) O157:H7 strain EDL933 and a random nucleotide sequence. The following criteria were applied to identify potential pathogenicity islands (PAIs): (1) barcode distance distinct from that of the general background; (2) length greater than 10000 continuous base pairs; and (3) containing genes with known virulence-related functions (as determined by PfamScan and Blast2GO).

RESULTS: Comparison of the barcode images generated for the 26695, HPAG1, J99, Shi470, G27 and P12 *H. pylori* genomes with those for the *E. coli* and random sequence controls revealed that *H. pylori* genomes contained fewer anomalous regions. Among the *H. pylori*-specific continuous anomalous regions (longer than 20 kbp in each strain's genome), two fit the criteria for identifying candidate PAIs. The bioinformatic-based functional analyses revealed that one of the two anomalous regions was the known pathogenicity island *cag*-PAI, this finding also served as proof-of-principle for the utility of the genomic barcoding approach for identifying PAIs, and characterized the other as a novel PAI, which was designated as *tfs3*-PAI. Furthermore, the *cag*-PAI and *tfs3*-PAI harbored genes encoding type IV secretion system proteins and were predicted to have potential for functional synergy.

CONCLUSION: Genomic barcode imaging represents an effective bioinformatic-based approach for scanning bacterial genomes, such as *H. pylori*, to identify candidate PAIs.

Key words: *Helicobacter pylori*; Genome analysis; Pathogenicity islands; Genomic bar coding

Core tip: The genomic barcoding technology was recently developed to increase the accuracy of genome analysis, and has facilitated the identification of origin-

specific genomic regions of both eukaryotic and prokaryotic lifeforms. In this study, we applied the genomic barcode imaging approach to screen for pathogenicity islands (PAIs) in *Helicobacter pylori* using the six strains for which the complete genome sequences have been published and performing comparison to a common *Enterobacter* species. Bioinformatic-based functional analysis not only provided proof-of-principle (identifying the known *cag*-PAI) but also identified a novel PAI (designated as *tsf3*-PAI).

Wang GQ, Xu JT, Xu GY, Zhang Y, Li F, Suo J. Predicting a novel pathogenicity island in *Helicobacter pylori* by genomic barcoding. *World J Gastroenterol* 2013; 19(30): 5006-5010 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/5006.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.5006

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative pathogen that colonizes the stomachs of over half the world's population[1,2]. Despite being one of the most common chronic infections among humans, it often remains undiagnosed until an unknown trigger causes manifestation of gastric diseases (*e.g.*, gastritis[3], ulcers[4], and gastric carcinoma[5]) with varying degrees of symptom severity and outcome. Extensive research efforts have been dedicated to understanding the molecular mechanisms of *H. pylori* pathogenesis, and have identified several (*bona fide* and putative) classes of virulence factors, including adhesins[6,7], cytotoxins[8], and lipopolysaccharide (LPS)[9]. While LPS has received the majority of research attention in the *H. pylori* field, due to its prevalence among pathogenic bacteria and its well-characterized interactions with the Toll-like receptor 4 of the host innate immune system, systematic investigations of the cytotoxins have also elucidated the host-pathogen signaling interactions leading to pathogenic changes in the infected tissues. For example, the vacuolating cytotoxin (VacA) has been shown to induce apoptosis in epithelial cells, and the cytotoxin-associated antigen (CagA) has been shown to counteract the VacA-induced apoptosis to promote survival of infected host cells and facilitate stomach colonization[10].

Recent evidence has suggested that pathogenicity islands (PAIs) in the bacterial genome play an important role in pathogenesis[11,12]. PAIs are defined as large DNA fragments that have been acquired through horizontal transfer and which bear multiple genes encoding bacterial factors with virulence functions[13]. The genes located on each PAI serve as molecular markers for clinical testing to diagnose bacterial pathogens, estimate their pathogenic potential, and predict treatment response (*i.e.*, antibiotic resistance)[14]. Therefore, genomic scanning to determine the PAI profile of *H. pylori* will not only provide insights into the molecular evolution and pathogenic mechanisms of this important human pathogen but also identify putative targets for effective molecular therapies.

The advent of high-throughput sequencing technologies has allowed for the complete genome sequences of a large number of prokaryotes; in conjunction with the rapid accumulation of such minable data in publicly available databases, various *in silico* methods have been developed to detect PAIs[15,16]. Most of these methods depend on finding aberrant G + C content and/or bias in codon usage[17] among various genera and species. Yet, this approach produces a high frequency of false negative results due to post-transfer changes that naturally accumulate in the transferred fragments over the course of evolution in a new environment.

In our previous studies, we addressed the limitations of the *in silico* methods. It was found that when genome scanning was performed using a fixed window size of at least 1000 bp, the frequency of each κ-nucleotide sequence ($2 < \kappa < 7$) was highly stable across a whole genome[18]. As a result, we represented the κ-nucleotide sequence frequency distributions across a whole genome as a 2-D barcode-like image, which was designated as a genomic barcode. By visualizing the barcodes of each genome, we were able to easily identify those sequences of foreign origin, such as horizontally transferred genes[18].

In the current study, we applied the genomic barcode imaging technique to scan the *H. pylori* genome for PAIs. Both known (serving as a proof-of-principle finding) and novel PAIs were detected.

MATERIALS AND METHODS

Genome sequence data

Complete genomes of the 26695, HPAG1, J99, Shi470, G27 and P12 strains of *H. pylori*, as well as those of *Escherichia coli* (*E. coli*) O157:H7 strain EDL933 (serving as a control for comparative analysis), were downloaded from the National Center for Biotechnological Information FTP server (ftp://ftp.ncbi.nih.gov/genomes/Bacteria/) in January 2012. In addition, a random nucleotide sequence was generated by a K-order Markov chain model for use as an additional control.

Generation of genomic barcode images

Each genome was partitioned into non-overlapping fragments of 1000 bp and a 4-nucleotide-based barcode was calculated for each genome. Specifically, the barcode for each genome is a matrix of N (4) columns and genome length/M rows, so that N (4) = 136, with the i^{th} value being the combined frequency of the i^{th} 4-nucleotide and its reverse complement in this fragment. The κ-nucleotide frequencies were then converted to grey-scale levels to visualize the overall barcode image profile for the whole genome. Darker grey levels represent lower frequencies.

Identification of PAIs

The following criteria were applied to identify potential PAIs: (1) barcode distance distinct from that of the general background; (2) length greater than 10000 continu-

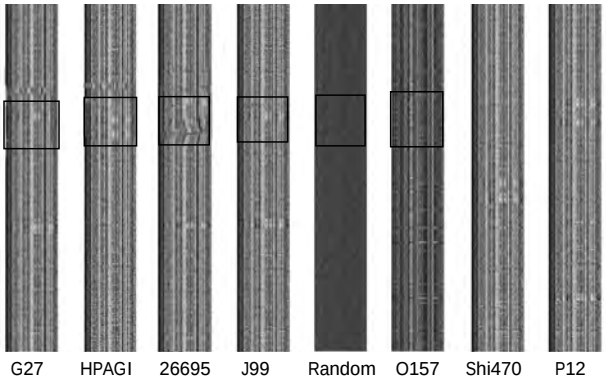

Figure 1 2-D barcode images of genomes of *Helicobacter pylori* strains J99, G27, 26695, HPAG1, P12, and Shi470, *Escherichia coli* O157:H7 strain EDL933, and a random sequence. The y-axis represents the genome axis from top-down, with each pixel representing a fragment of n = 1000 bp; the x-axis represents the 4-nucletide frequencies. The abnormal barcode regions are demarcated by a rectangle.

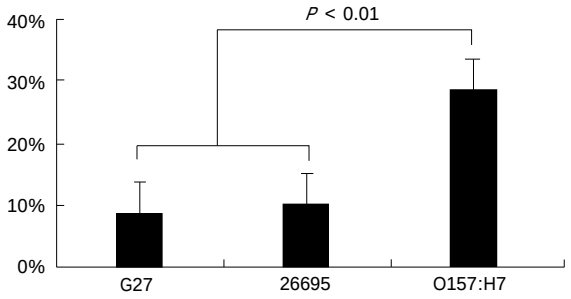

Figure 2 Fraction of anomalous fragments detected by genomic barcode imaging of *Helicobacter pylori* strains G27 and 26695, and *Escherichia coli* O157:H7 strain EDL933.

ous base pairs; and (3) containing genes with known virulence-related functions (as determined by PfamScan[19] and Blast2GO[20]).

Statistical analysis

The distance between two barcodes was calculated as the Euclidean distance between the corresponding 136-dimensional vectors. The distance database was built using Microsoft Excel spreadsheet software, and SPSS 13.0 statistical software was employed for analysis of the data using descriptive methods and the χ^2 test.

RESULTS

Visualization of H. pylori genomes based on genomic barcode images

Each genome was partitioned into a series of non-overlapping fragments of 1000 bp, and the combined frequencies of each 4-nucleotide/reverse complement were calculated. The frequency matrices converted to grey-scale are shown in Figure 1. The unique barcode image for each of these microbial genomes represents the underlying base composition. The 2-D barcode images of the *H. pylori* strains were similar to one another but distinct from that

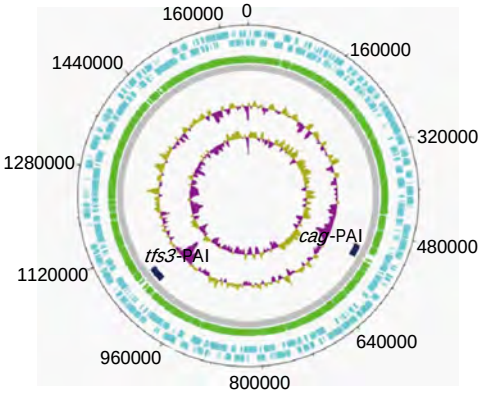

Figure 3 Circular representation of the *Helicobacter pylori* 26695 chromosome. The outermost (first) concentric circle denotes the predicted coding regions on the plus strand. The second concentric circle denotes the predicted coding regions on the minus strand. The third concentric circle denotes the predicted coding regions on both strands. The fourth concentric circle denotes the buffer zone. The fifth concentric circle denotes the predicted pathogenicity island (PAI) candidates. The sixth concentric circle denotes the guanine and cytosine (GC) content. The seventh concentric circle denotes the GC content. The figure was created using GenVision from DNASTAR.

of *E. coli*, demonstrating the close relationship of strains from the *H. pylori* species. It should be noted that no barcode structure was able to be produced for the random nucleotide sequence, indicating that the genomic barcode is an inherent property of the microbial genome.

Identification of H. pylori-specific genomic regions

While the genomes of different *H. pylori* strains possessed the conserved κ-nucleotide frequency producing the visual barcode, some regions appeared to have an abnormal structure. As shown in Figure 1, an abnormal band was apparent across the barcode image of the corresponding genome. In principle, these regions may have been acquired through horizontal gene transfer or derived from phage-mediated gene conversion.

The percentage of the anomalous regions in each genome are shown in Figure 2. As expected, the *H. pylori* strains contain fewer anomalous regions than *E. coli* ($P < 0.01$).

Identification of PAIs in H. pylori

We collected continuous anomalous fragments, longer than 20 kbp in each genome, and kept only those specific for most *H. pylori* genomes. In addition, some anomalous fragments found only in some *H. pylori* genomes, but subdivided into a number of discrete smaller segments in another *H. pylori* genome, were excluded from further analysis since such fragments may have resulted from frequent recombination events[21,22]. As a result of this procedure, two specific genome regions were selected as potential PAI candidates. Figure 3 and Table 1 show the position of these two candidate PAIs in *H. pylori*.

The bioinformatic-based functional analyses revealed that one of the two anomalous regions was the known pathogenicity island *cag*PAI, this finding also served as proof-of-principle for the utility of the genomic barcoding approach for identifying PAIs, and characterized the

Table 1 Pathogenicity island candidates in sequenced *Helicobacter pylori* genomes

	Size	GC	Barcodedistance	ORF
Wholegenome	1.5-1.7 Mbp	38.0% ± 0.2%	114.3 ± 14.9	-
cag-PAI	35 kbp	35.4% ± 0.8%	134.6 ± 20.1	20.0 ± 0.6
tfs3-PAI	30 kbp	33.0% ± 0.8%	138.0 ± 20.0	17.0 ± 3.0

PAI: Pathogenicity island; ORF: Open reading frame; GC: Guanine and cytosine.

other as a novel PAI, which was designated as *tfs3*-PAI and was located at the 3' end of the *Ser*-tRNA gene.

Identification of genes in cag-PAI and tfs3-PAI and prediction of the pathogenic role for each

We verified that the genes located in *cag*-PAI encode components of the type Ⅳ secretion system (T4SS), as characterized by previous studies[22-24].

Compared with *cag*-PAI, *tfs3*-PAI displayed some sequence variability due to rearrangements. The *tfs3*-PAI consisted of three distinct domains separated by mobile genetic elements. The first module contained the largest number of genes and encoded mobile sequence elements including a transposase (IS605), which is an essential element for a PAI. The second module encoded homologous genes of *tfs3* gene clusters, which formed a T4SS. The function of the *tfs3* gene cluster is not yet known, but it may play a role in bacterial conjugation and host cell signaling complementary to that of the *cag*-PAI-encoded system, which indicates a functional synergy. Most genes of the third module encoded hypothetical genes; as these genes have no orthologs in the databases, it is not clear at this point how many of them are in fact pseudogenes. It worth noting that *tfs3*-PAI consists of 17 open reading frames, six of which encode homologous genes of the T4SS. Therefore, this region may be related to pathogenesis in gastroduodenal diseases, and may represent a useful target for new vaccines and antibiotics.

DISCUSSION

The first potential PAI was *cag*-PAI, a well-known pathogenicity island in *H. Pylori*[25]. This approximately 35 kbp cluster of genes was acquired through horizontal transfer from an unknown extraneous source and integrated into the *H. pylori* chromosome. It is known that, compared to Enterobacteriaceae, *H. pylori* has less opportunity to obtain foreign genes by horizontal transfer since only a few bacterial species colonize human stomachs. Indeed, a previous microarray-based study of a larger strain collection suggested that up to 10% of all genes in an individual isolate may be accessory genes[26], which corroborates our finding.

T4SS is one of at least six specialized secretion systems characterized in bacteria. Usually consisting of 12 components, T4SS plays various functions in transporting a wide range of components, from single protein to protein-protein complexes and protein-DNA complexes. Moreover, T4SS facilitates injection of bacteria-encoded effectors into host cells during the infection process. The *cag*-PAI-encoded secretion systems have been implicated in modulation of bacteria-host interactions, interference with host signal-transduction pathways, and promotion of apoptosis, to name a few[22-24].

Pathogenesis of *H. pylori* is a multi-stage process. It is likely that multiple bacterial and host mechanisms are involved; however, a long-standing dogma of infectious biology claims that PAIs of *H. pylori* are stable entities and could be robustly correlated with disease progression or outcome. Screening and functional analysis of PAIs in *H. pylori*, as developed and demonstrated in this study, will aid in the development of more accurate and timely diagnosis and improved control of this common pathogen.

COMMENTS

Background

Recent evidence suggests that pathogenicity islands (PAIs) play an important role in bacterial pathogenesis. Scanning of PAIs in the *Helicobacter pylori* (*H. pylori*) genome will provide insights into the molecular evolution and pathogenic mechanisms of this important human pathogen but also identify putative targets for effective molecular therapies.

Research frontiers

Auhtors have applied the genomic barcode imaging technique to scan PAIs in *H. pylori*. Bioinformatic-based functional analysis not only provided proof-of-principle (identifying the known *cag*PAI) but also identified a novel PAI (designated as *tsf3*-PAI).

Innovations and breakthroughs

A novel PAI, *tsf3*-PAI, was detected in *H. pylori* using the genomic barcode imaging technique. Bioinformatic-based functional analysis revealed that *tsf3*-PAI encodes a type Ⅳ secretion system (T4SS) which may functionally synergize with the T4SS encoded by *cag*-PAI.

Applications

The genomic barcode imaging technique is useful for identifying known and novel PAIs in bacterial genomes. The PAIs identified in this study may be related to the manifestation of *H. pylori*-induced gastroduodenal diseases, and may represent useful targets of new molecular therapies or vaccines.

Terminology

The genomic barcode is generated by measuring the κ-nucleotide sequence frequency distributions across a whole genome using a fixed window size of at least 1000 bp. The 2-D barcode-like image is generated by converting the frequency matrices to grey-scale levels.

Peer review

This manuscript applied genomic barcodes to screen for PAIs in *H. Pylori*, which showed that genomic barcode technique is more usefulness and accuracy tool for genome analysis so far. The proof-of-principle work showed that one known and one novel PAI could be detected using this technique.

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BRIEF ARTICLE

Milligan-Morgan hemorrhoidectomy with anal cushion suspension and partial internal sphincter resection for circumferential mixed hemorrhoids

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Abstract

AIM: To identify a more effective treatment protocol for circumferential mixed hemorrhoids.

METHODS: A total of 192 patients with circumferential mixed hemorrhoids were randomized into the treatment group, where they underwent Milligan-Morgan hemorrhoidectomy with anal cushion suspension and partial internal sphincter resection, or the control group, where traditional external dissection and internal ligation were performed. Postoperative recovery and complications were monitored.

RESULTS: The time to wound healing was 12.96 ± 2.25 d in the treatment group shorter than 19.58 ± 2.71 d in the control group. Slight pain rate was 58.3% in the treatment group higher than 22.9% in the control group; moderate pain rate was 33.3% in the treatment group lower than 56.3% in the control group severe pain rate was 8.4% in the treatment group lower than 20.8% in the control group. No edema rate was 70.8% in the treatment group higher than 43.8% in the control group; mild local edema rate was 26% in the treatment group lower than 39.6% in the control group obvious local edema was 3.03% in the treatment group lower than 16.7% in the control group. No stenosis rate was 85.4% in the treatment group higher than 63.5% in the control group; moderate stenosis rate was 14.6% in the treatment group Lower than 27.1% in the control group severe anal stenosis rate was 0% in the treatment group lower than 9.4% in the control group.

CONCLUSION: Milligan-Morgan hemorrhoidectomy with anal cushion suspension and partial internal sphincter resection is the optimal treatment for circumferential mixed hemorrhoids and can be widely applied in clinical settings.

Key words: Milligan-Morgan hemorrhoidectomy; Mixed hemorrhoids; Anal cushion; Internal sphincter

Core tip: We treated 96 patients with circumferential mixed hemorrhoids using Milligan-Morgan hemorrhoidectomy with anal cushion suspension and partial internal sphincter resection, and compared their clinical outcomes with those undergoing traditional hemorrhoidectomy. The differences are significant in favor of the modified Milligan-Morgan technique in terms of time to wound healing, anal stenosis, wound pain, edema and other complications. This approach can be widely applied in clinical practice.

Lu M, Shi GY, Wang GQ, Wu Y, Liu Y, Wen H. Milligan-Morgan hemorrhoidectomy with anal cushion suspension and partial internal sphincter resection for circumferential mixed hemorrhoids. *World J Gastroenterol* 2013; 19(30): 5011-5015 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/5011.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.5011

INTRODUCTION

The treatment of circumferential mixed hemorrhoids is challenging for medical providers. Despite a number of reports on the surgical options at home and abroad, no effective treatment method is currently available[1]. The traditional "external dissection and internal ligation" or Milligan-Morgan technique, is the mainstream treatment for mixed hemorrhoids. However, with larger and more hemorrhoids involved in the circumferential type, surgery becomes more complicated and is inevitably associated with a range of post-operative complications[2]. The most common and serious complication is anal stenosis and incontinence, which results in significant pain in affected patients[3-6]. We treated 96 patients with circumferential mixed hemorrhoids using Milligan-Morgan hemorrhoidectomy with anal cushion suspension and partial internal sphincter resection, and compared their clinical outcomes with those undergoing traditional hemorrhoidectomy. The differences were significant in favor of the former group, as shown below.

MATERIALS AND METHODS

General information

A select group of 192 patients with circumferential mixed hemorrhoids treated in our department from August 2010 to November 2012 were randomized into two groups, with 96 patients in each group. The 96 patients in the treatment group underwent Milligan-Morgan hemorrhoidectomy with anal cushion suspension and partial internal sphincter resection, while the patients in the control group were treated with the traditional Milligan-Morgan technique. The patients comprised 98 men and 94 women aged 26 to 65 years, with a mean age of 48.5 years. Disease duration ranged between 7 and 48 mo, with an average of 26.5 mo. The two groups were comparable as there were no significant differences in terms of age, gender, and disease duration.

Methods

Treatment group: Each patient was placed in the lateral position after caudal or spinal anesthesia for routine disinfection and draping. Hemorrhoids of higher grades situated in the 3, 7 and 11 o'clock positions were treated in most cases. A V-shape was made from the body of an external hemorrhoid to the anal margin using scissors. With complete exposure after anal dilatation, each internal hemorrhoid was slightly pulled towards the outside with forceps for high suspension and ligation. To improve the effect of suspension, the rectal mucosa 1-2 cm above the internal hemorrhoid was lifted with tissue forceps, clamped with the base of the hemorrhoid that was carried by curved forceps, and ligated with a 10-gauge silk suture at 0.5 cm away from the dentate line, without injuring it. The internal hemorrhoids at the base were individually ligated in the same way, keeping the ligated bodies at different levels of the anal canal with sufficient mucosal bridges between them to prevent postoperative anal stenosis. Each external hemorrhoid was then lifted with forceps and dissected along the V-shaped incision. Subcutaneous varicose veins were stripped off and bleeding was managed appropriately. All external hemorrhoids were treated in the same way. Sufficient flaps were retained between each incision to avoid anal stenosis due to anal skin defects. Finally, the lower edge of the internal sphincter was divided and cut off at the interscalene in the 3 or 9 o'clock position. A second-generation cephalosporin was administered for 2 d after the procedure to prevent infection.

Control group: The traditional external dissection and internal ligation method was used to treat the patients in this group. Postoperative treatment was the same as that in the treatment group.

Outcome evaluation

The therapeutic efficacy was evaluated according to the Diagnosis and Efficacy Standards in Traditional Chinese Medicine issued by the State Administration of Traditional Chinese Medicine of China[7].

The severity of anal stenosis was classified as: (1) no stenosis: index finger can pass smoothly; stool passes easily, without pain or discomfort; mild stenosis: index finger can pass with difficulty; stool passes relatively easily, without obvious pain; (2) moderate stenosis: only the first joint of the index finger can pass; stool passes with difficulty and anal pain; and (3) severe anal stenosis: only the little finger can pass through; stool passes with difficulty and obvious anal pain.

The degree of pain was classified as: grade 0: no pain; grade 1: mild pain; the anal pain is slight and tolerable without disturbing normal life and sleep; no significant changes in mood; grade 2 (moderate): obvious pain; the anal pain is so intense that analgesics are required, normal life and sleep are compromised, and mood changes are present, such as irritability; the condition is still under control with typical analgesics; grade 3 (severe): severe pain; the pain is intolerable and seriously disturbing normal life and sleep, causing autonomic dysfunction; analgesic drugs are necessary. The degree of anal margin edema was classified into: grade 0: no edema; grade 1: mild local edema, without affecting normal activity; and grade 2: obvious local edema, restricting normal activity.

Statistical analysis

The data were processed using SPSS 16.0 for statistical analysis with both the χ^2 test and t test. A P value of less

than 0.05 was considered statistically significant.

RESULTS

Following treatment: the time to wound healing was 12.96 ± 2.25 d in the treatment group shorter than 19.58 ± 2.71 d in the control group, the difference was statistically significant (t = 32.52, P = 0.000). Slight pain rate was 58.3% in the treatment group higher than 22.9% in the control group, the difference was statistically significant (χ^2 = 24.961, P = 0.000); moderate pain rate was 33.3% in the treatment group lower than 56.3% in the control group, the difference was statistically significant (χ^2 = 10.194, P = 0.001); severe pain rate was 8.4% in the treatment group lower than 20.8% in the control group, the difference was statistically significant (χ^2 = 6.021, P = 0.014). No edema rate was 70.8% in the treatment group higher than 43.8% in the control group, the difference was statistically significant (χ^2 = 14.389, P = 0.000); mild local edema rate was 26% in the treatment group lower than 39.6% in the control group, the difference was statistically significant (χ^2 = 3.993, P = 0.046); obvious local edema was 3.03% in the treatment group lower than 16.7% in the control group, the difference was statistically significant (χ^2 = 9.872, P = 0.002). No stenosis rate was 85.4% in the treatment group higher than 63.5% in the control group, the difference was statistically significant (χ^2 = 12.084, P = 0.001); moderate stenosis rate was 14.6% in the treatment group lower than 27.1% in the control group, the difference was statistically significant (χ^2 = 4.547, P = 0.033); severe anal stenosis rate was 0% in the treatment group lower than 9.4% in the control group, the difference was statistically significant (χ^2 = 7.461, P = 0.006).

DISCUSSION

Arising above or beneath the dentate line, the mixed hemorrhoid is a condition where the internal and external hemorrhoidal plexuses of veins merge, presenting the features of both internal hemorrhoid and external hemorrhoid[8]. The circumferential mixed hemorrhoids, as the severe type of the disease, are particularly difficult in surgical treatment. The conventional Milligan-Morgan[9-11] hemorrhoidectomy has been the globally recognized "golden standard" for circumferential mixed hemorrhoids[12]. However, it is limited by severe pain[13,14] after surgery, prolonged wound healing time, and complications such as anal stenosis[15]. In fact, it is an extremely painful procedure for the patients, Anal function and feeling fine and other defects be affected[16]. Based on these results, there were significant differences in favor of the modified Milligan-Morgan technique in terms of time to wound healing, anal stenosis, wound pain, edema and other complications. Anal stenosis is a critical factor that compromises the treatment effect and wound healing, resulting in significant inconvenience to the patient's life and work. Hence, caution should be taken to reduce the risk of this complication. It is proved that reduction of the anal cushions may trigger anal incontinence[17]. However, the conventional Milligan-Morgan hemorrhoidectomy can not achieve the ring-shaped resection of all external hemorrhoids, and anal stenosis can easily occur due to excessive resection. In addition, the massive damage of the dentate line can result in the change of anal function[18]. The modified Milligan-Morgan technique is designed to minimize anal stenosis, anal margin edema, incision pain, and other undesirable factors. During surgery, the anal cushions can be retained by suspending and ligating internal hemorrhoids and mucosa above the dentate line, minimizing injury to the anal cushions[19] and the dentate line. Anal cushions are the normal structures above the dentate line. They have certain immune and endocrine functions, and can effectively cause anal reflex, ensuring normal continence and defecation[20,21]. Bowel movement is induced at the dentate line, which should be preserved during surgery. Destruction of this area may lead to prolapse and incontinence of anal cushions, thus protection of anal cushions and the dentate line is essential for normal anal function after surgery[22-24]. Using the modified operation, only lesions are removed, and normal anal cushions are retained, ensuring normal function of the anus. Sufficient skin bridges and mucosal bridges are required between adjacent incisions to avoid the formation of a mucosal tension band, which may lead to anal stenosis. In addition, as part of the anorectal smooth muscle, the internal sphincter is prone to spasm due to its contractile properties, resulting in spastic pain after surgery and subsequently worsened edema, affecting wound healing. Therefore, the internal sphincter is partially resected during surgery to relieve persistent spasm and reduce the pressure of the sphincter to decrease the anal resting pressure and restore normal blood and lymph circulation. In this way, postoperative anal margin edema can be reduced or avoided, ensuring less pain and better wound healing. Based on our experience, retention of anal cushions and partial resection of the internal sphincter results in considerable benefits in the treatment of mixed hemorrhoids, including: (1) definite efficacy, less invasiveness, faster healing after surgery, and a shorter treatment course; (2) reduced sequelae, less damage to the anal cushions, and maximal retention of the anal canal, anal function and normal structure; (3) fewer complications, effectively reducing the incidence of postoperative hemorrhage, edema, urinary retention and anal stenosis; (4) suspension and high ligation of internal hemorrhoids can elevate the anal cushions that have moved downward in a similar way to the procedure for prolapse and hemorrhoids; this simple and cost-effective operation can be applied in various hospitals of different levels; and (5) with the preserved and elevated anal cushions, external hemorrhoids are also significantly reduced, making it possible for uncompromised, refined postoperative management.

In conclusion, Milligan-Morgan hemorrhoidectomy with anal cushion suspension and partial internal sphinc-

ter resection is the optimal treatment for circumferential mixed hemorrhoids, as it effectively removes hemorrhoids while retaining the anal canal anatomy and physiology, complying with the physiological function, pathological changes and anorectal dynamics of the anus[25]. In addition, this surgical method overcomes the drawbacks of traditional techniques by eliminating continued spasm of the internal sphincter, and reducing postoperative pain, edema, anal stenosis and other complications. This modified technique is a valuable approach in the treatment of circumferential mixed hemorrhoids resulting in a shorter time to wound healing, improved quality of surgery and can be widely applied in clinical settings.

COMMENTS

Background

The treatment of circumferential mixed hemorrhoids is a challenging procedure for medical providers. Despite a number of reports on the surgical options at home and abroad, no effective treatment method is currently available. The traditional "external dissection and internal ligation" or Milligan-Morgan technique, is a mainstream treatment for mixed hemorrhoids. With larger and more hemorrhoids involved in the circumferential type, however, the surgery becomes more complicated and is inevitably associated with a range of post-operative complications.

Research frontiers

The authors treated 96 patients with circumferential mixed hemorrhoids using Milligan-Morgan hemorrhoidectomy with anal cushion suspension and partial internal sphincter resection, and compared their clinical outcomes with those undergoing traditional hemorrhoidectomy. The differences are significant in favor of the modified Milligan-Morgan technique in terms of time to wound healing, anal stenosis, wound pain, edema and other complications. This approach can be widely applied in clinical practice.

Innovations and breakthroughs

Milligan-Morgan hemorrhoidectomy with anal cushion suspension and partial internal sphincter resection is an optimal treatment of circumferential mixed hemorrhoids, which effectively removes hemorrhoids while retaining the anal canal anatomy and physiology, complying with the physiological function, pathological changes and anorectal dynamics of the anus. Meanwhile, it overcomes the drawbacks of traditional techniques by eliminating continued spasm of the internal sphincter, and reducing postoperative pain, edema, anal stenosis and other complications.

Applications

The modified Milligan-Morgan hemorrhoidectomy provides a valuable approach to the treatment of circumferential mixed hemorrhoids with shortened time to wound healing, and improved the quality of surgery, which can be widely applied in clinical settings.

Terminology

The traditional "external dissection and internal ligation" or Milligan-Morgan technique, is a mainstream treatment for mixed hemorrhoids. PPH stands for "procedure for prolapse and hemorrhoids". With the new PPH or stapled hemorrhoidectomy there is no need to cut out the hemorrhoid tissue at the anus.

Peer review

In this manuscript, the authors made a good study of 96 cases with circumferential mixed hemorrhoids. The paper is well written and interesting for the readers.

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CASE REPORT

Bone metastasis from early gastric cancer following non-curative endoscopic submucosal dissection

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Abstract

A 67-year-old male underwent endoscopic submucosal dissection (ESD) to treat early gastric cancer (EGC) in 2001. The lesion (50 mm × 25 mm diameter) was histologically diagnosed as poorly differentiated adenocarcinoma, with an ulcer finding. Although the tumor was confined to the mucosa with no evidence of lymphovascular involvement, the ESD was regarded as a non-curative resection due to the histological type, tumor size, and existence of an ulcer finding (as indicated by the 2010 Japanese gastric cancer treatment guidelines, ver. 3). Despite strong recommendation for subsequent gastrectomy, the patient refused surgery. An alternative follow-up routine was designed, which included five years of biannual clinical examinations to detect and measure serum tumor markers and perform visual assessment of recurrence by endoscopy and computed tomography scan after which the examinations were performed annually. The patient's condition remained stable for eight years, until a complaint of back pain in 2010 prompted further clinical investigation. Bone scintigraphy indicated increased uptake. Histological examination of biopsy specimens taken from the lumbar spine revealed adenocarcinoma resembling the carcinoma cells from the EGC that had been treated previously by ESD, and which was consistent with immunohistochemical findings of gastrointestinal tract cancer. Thus, the diagnosis of bone metastasis from EGC was made. The reported rates of EGC recurrence in surgically resected cases range 1.4%-3.4%, but among these bone metastasis is very rare. To our knowledge, this is the first reported case of bone metastasis from EGC following a non-curative ESD and occurring after an eight-year disease-free interval.

Key words: Endoscopic submucosal dissection; Early gastric cancer; Non-curative resection; Bone metastasis; Late recurrence

Core tip: This case report provides the first description of an adult male with bone metastasis from early gastric cancer following an eight-year disease-free interval after endoscopic submucosal dissection (ESD). Although the original tumor was confined to the mucosa, with no evidence of lymphovascular involvement, the ESD was regarded as a non-curative resection based upon the tumor's histological type and size, and existence of an ulcer finding. If any patient, who is otherwise fit, initially refuses surgery and requests a contraindicated ESD, efforts should be made to persuade the patient to undergo a gastrectomy with lymph-node dissection.

Kawabata H, Oda I, Suzuki H, Nonaka S, Yoshinaga S, Katai H, Taniguchi H, Kushima R, Saito Y. Bone metastasis from early

gastric cancer following non-curative endoscopic submucosal dissection. *World J Gastroenterol* 2013; 19(30): 5016-5020 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/5016.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.5016

INTRODUCTION

Endoscopic resection remains the preferred treatment modality for cases of early gastric cancer (EGC) with a negligible risk of lymph-node metastasis[1-3]. The 2010 Japanese gastric cancer treatment guidelines (ver. 3) provide absolute indications and expanded indications for performing endoscopic resections, including endoscopic submucosal dissection (ESD), to treat these cases[4]. In contrast, EGC cases with possible risk of lymph-node metastasis generally require surgical treatment.

Here, we describe our clinical experience with a case of bone metastasis from EGC that developed after an eight-year disease-free interval following non-curative ESD. To our knowledge, this case represents the first of its kind to be reported in the literature. In this particular case, ESD was clinically contraindicated because the EGC consisted of an undifferentiated type adenocarcinoma with an ulcer finding, but was performed in accordance with the patient refusing surgical intervention despite our strong recommendation to the contrary.

CASE REPORT

In 2001, a 58-year-old man was admitted to our hospital upon detection of EGC by screening endoscopy. Physical examination and laboratory data revealed no abnormalities, and the patient had no relevant personal or family history. The endoscopic findings indicated a superficial depressed type (0-Ⅱc) EGC confined to the mucosa (in terms of invasion depth), along with an ulcer (50 mm) located in the gastric angle (Figure 1). Histological examination of the biopsied specimens indicated poorly- to moderately-differentiated adenocarcinoma. Ultrasound and computed tomography (CT) examinations revealed no metastasis.

Based on the 2010 Japanese gastric cancer treatment guidelines[4], endoscopic resection was not indicated for this lesion. The patient was informed of the possible risk of lymph-node metastasis and the need for a surgical resection; however, the patient refused surgery and the ESD was performed in October 2001 (Figure 2). Histological examination of the resected tissues revealed the tumor (50 mm × 25 mm diameter) to be primary composed of poorly-differentiated adenocarcinoma and confirmed the proximal ulcer (Figure 3). The tumor was confined to the mucosa, with no evidence of lymphovascular involvement. Both the vertical and horizontal margins were free of tumor cells. However, a slight tear was observed inside the lesion and was considered to have occurred incidentally during the ESD.

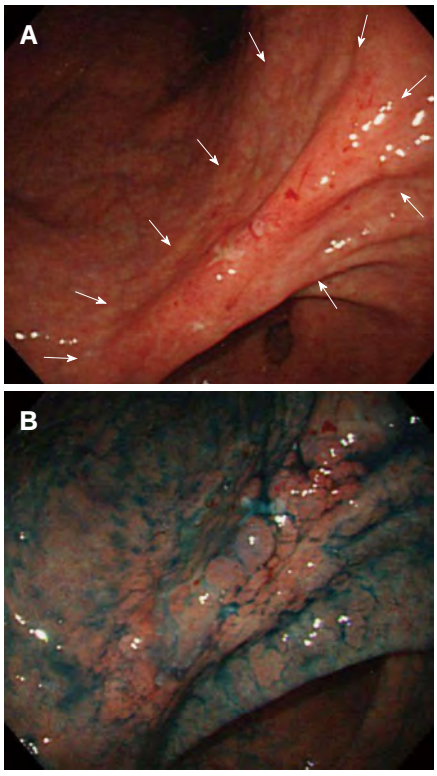

Figure 1 Endoscopic findings upon initial admission for early gastric cancer. A: Conventional endoscopy showing a superficial depressed type lesion, 50 mm in size with an ulcer scar located in the gastric angle (arrows); B: Chromoendoscopy with indigo-carmine dye (blue) showing the lesion margin.

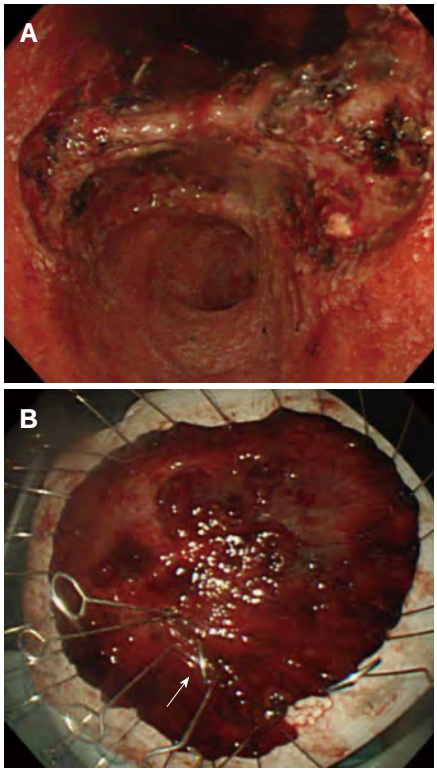

Figure 2 *In situ* locale of the lesion and gross appearance of the resected tumor. A: Intraoperative image taken during the endoscopic submucosal dissection (ESD) procedure. ESD was performed in October 2001; B: A tear (arrow) was present inside the lesion and likely was incident to the ESD procedure.

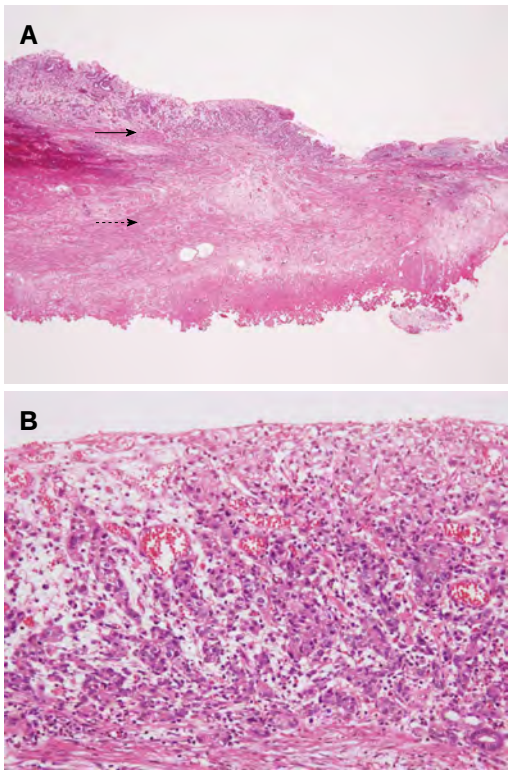

Figure 3 Histological findings of the endoscopic submucosal dissection-resected lesion tissues. A: The tumor is confined to the mucosa (solid arrow) and an ulcer scar (dashed arrow) (magnification: × 20); B: The tumor is composed of poorly differentiated adenocarcinoma with signet ring cells (magnification: × 100).

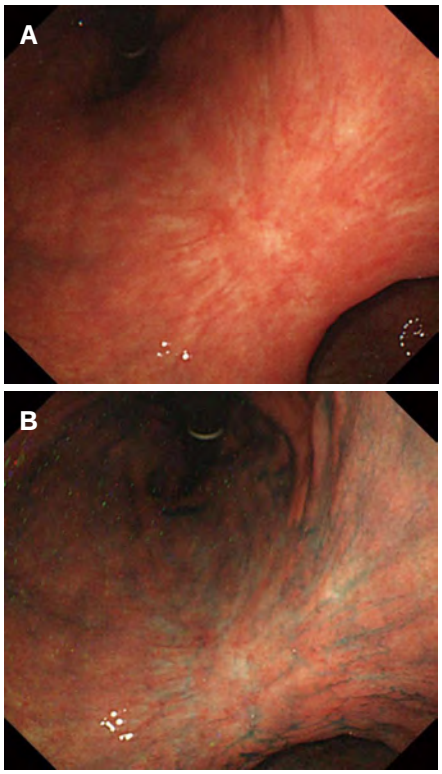

Figure 4 No endoscopic evidence of recurrence was found at the endoscopic submucosal dissection site eight years after endoscopic submucosal dissection. A: Conventional endoscopy; B: Chromoendoscopy with indigocarmine dye. Endoscopic examination provided no indications of recurrence at the endoscopic submucosal dissection site.

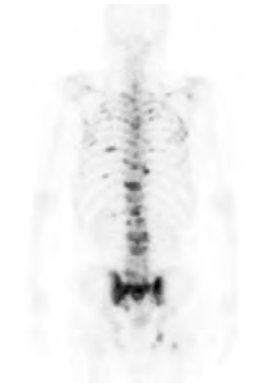

Figure 5 Bone scintigraphy results from the eight-year follow-up showing increased uptake in the spine and pelvic bone.

Since the ESD was regarded as a non-curative resection, we repeated our strong recommendations for gastrectomy, but the patient continued to refuse. As a result, we designed a monitoring strategy in which the patient would present to clinic biannually for testing of serum tumor markers and endoscopic and CT examinations; after five years with no remarkable findings, the clinical follow-ups were decreased to once a year. The patient's condition remained stable, with no evidence of recurrence, for eight years after the ESD.

In April 2010, however, the patient presented with a complaint of back pain. Serum testing revealed a remarkably enhanced level of alkaline phosphatase (1172 IU/L, normal range: 120-340 IU/L), but endoscopic examination provided no indications of recurrence at the ESD site (Figure 4). Bone scintigraphy indicated increased uptake in the spine and pelvic regions (Figure 5). Histological examination of biopsied specimens taken from the lumbar spine revealed a poorly-differentiated adenocarcinoma that resembled the carcinoma cells in the original ESD-treated EGC (Figure 6A). Immunohistochemical examination showed the tumor cells were positive for caudal-type homeobox transcription factor 2, which is consistent with gastrointestinal tract cancer (Figure 6B). There were no findings of malignancy in any other organs. Thus, the diagnosis was made of bone metastasis from the EGC following a non-curative ESD after an eight-year disease-free interval.

The patient received chemotherapy (methotrexate + fluorouracil), but developed disseminated intravascular coagulopathy and died in August 2011.

DISCUSSION

This is the first report of bone metastasis from EGC after an eight-year disease-free interval following non-curative ESD. This case presents several aspects for discussion, including contraindicated ESD, metastasis limited to bone, and late recurrence.

A recent report of EGC indicated a lower survival

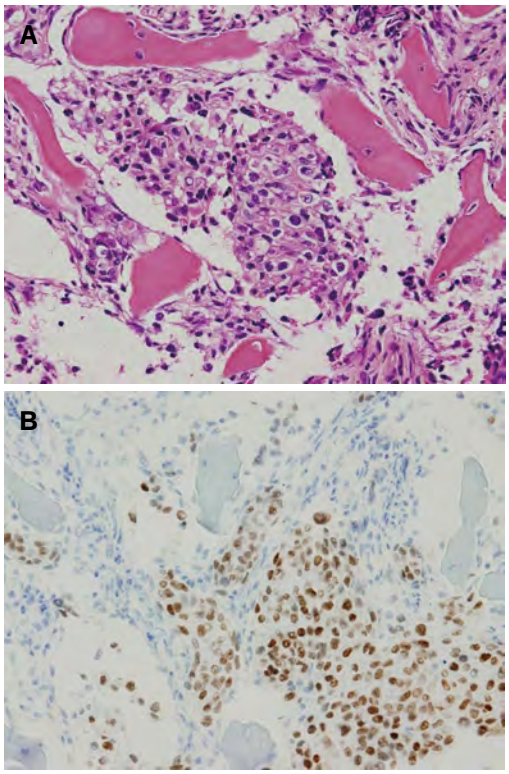

Figure 6 Histological findings of the biopsy specimen taken from the lumbar spine. A: The biopsy specimen taken from the lumbar spine revealed a poorly differentiated adenocarcinoma histologically resembling the carcinoma cells in the early gastric cancer treated previously by endoscopic submucosal dissection; B: Using immunohistochemical staining, the tumor cells were positive for caudal-type homeobox transcription factor 2.

rate after contraindicated ESD because of clinically diagnosed submucosal invasion and concluded that ESD was ineffective in such cases[5]. Although the case described herein did not involve clinically diagnosed submucosal invasion, ESD was contraindicated because of a possible risk of lymph-node metastasis due to the undifferentiated type adenocarcinoma with an ulcer finding. The risk of lymph-node metastasis has been reported as 13.4% for undifferentiated type EGC with ulcer finding, and as 5.9% in undifferentiated intramucosal cancer[6]. Bone metastasis is a hematogenous metastasis, so it may not have been avoided in the present case even if the patient had undergone a gastrectomy with lymph-node dissection. Nonetheless, we believe that gastrectomy would likely have provided some benefit to the patient, who was fit for surgery.

Bone metastasis is relatively common in patients with advanced breast, lung and prostate cancer, but not in gastric cancer patients (occurring in only 0.99%-2.1% of advanced cases)[7,8]. Recurrence of EGC has been reported to be in the range of 1.4%-3.4% for surgically resected cases, but among these bone metastasis is very rare[9]. In a previous investigation of 1452 EGC patients treated with curative resection, Lee *et al*[10] found that 21 patients (1.4%) experienced recurrence, including 4 (19.0%) with local lymph-node recurrence, 2 (9.5%) with peritoneal dissemination, 9 (42.9%) with distant metastasis, and 6 (28.6%) with mixed type recurrence. Similarly, Kobayashi *et al*[9] reviewed Japanese case reports to determine the characteristics of bone metastasis in EGC for this patient population. Among the tumors, 51.4% had infiltrated the mucosa and 48.6% had infiltrated the submucosa, 65.0% of the metastases were metachronous and 35.0% were synchronous, 18.8% of the primary tumors were differentiated and 81.2% were undifferentiated, and lymph-node metastasis was present in 55.2% of the patients.

In our case, the metastasis that was diagnosed after a lengthy interval (eight years) following non-curative ESD was only found in bone. One possible explanation of such a late recurrence involves the concept of tumor dormancy, a state in which cancer cells exist with no to low-level activity concomitant to normal, healthy tissues until the dormancy is disturbed by an instigating event, such as infection or immunosuppression[11]. In such a case, the activated cancer cells will have a negative impact on the prognosis of the cancer patient, regardless of the duration of the disease-free interval. Recurrence of gastric cancer is generally believed to occur within five years of the primary surgery, but there are some reports of very late recurrence, including bone metastasis after surgery, and particularly involving EGC cases[9,12-14]. It may be necessary, therefore, to follow-up EGC patients for more than five years, especially those who were treated by ESD in lieu of surgical resection or those with expanded indications.

The number of patients who undergo ESD instead of surgery has increased in recent years, in part because the indications have been expanded[3,5,15,16]; however, it is important to apply the indications properly. If any patient, who is otherwise fit, initially refuses surgery and requests a contraindicated ESD, all efforts should be made to persuade the patient to undergo a gastrectomy with lymph-node resection in order to optimize the patient prognosis.

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CASE REPORT

Mucocele of the appendix due to endometriosis: A rare case report

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Abstract

Mucocele of the appendix due to endometriosis is extremely rare, and there are only 10 previously reported cases in the English literature. We report a case of mucocele of the appendix due to endometriosis and provide the first review of the literature. A 43-year-old woman was admitted to the hospital because of recurrent right lower abdominal pain during her menstrual periods. Colonoscopy revealed submucosal tumor-like elevations of the appendiceal orifice. Computed tomography and magnetic resonance imaging of the abdomen suggested cystic lesions near the appendix. Consequently, mucocele of the appendix was suspected preoperatively. An open ileocecal resection was performed. Multiple cystic lesions were observed around the appendix. The cystic lesions contained mucus. Histopathological examination was consistent with a mucocele of the appendix due to endometriosis. The postoperative course was uneventful. We present the first review of the literature to clarify the clinical features.

Key words: Mucocele; Appendix; Endometriosis

Core tip: We report an extremely rare case of mucocele of the appendix due to endometriosis. Although it is uncommon, preoperative diagnosis of the mucocele is important; however, diagnosis is difficult using imaging modalities. We report a more accurate diagnostic possibility using preoperative imaging modalities, such as colonoscopy, ultrasonography, computed tomography, and magnetic resonance imaging. Furthermore, this report is important because it is the first review of the literature for mucocele of the appendix due to endometriosis.

Tsuda M, Yamashita Y, Azuma S, Akamatsu T, Seta T, Urai S, Uenoyama Y, Deguchi Y, Ono K, Chiba T. Mucocele of the appendix due to endometriosis: A rare case report. *World J Gastroenterol* 2013; 19(30): 5021-5024 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/5021.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.5021

INTRODUCTION

Mucocele of the appendix is an uncommon disease. It is observed in 0.2%-0.3% of appendectomies and 8%-10% of appendiceal tumors[1]. According to the modern classification[2,3], mucocele of the appendix includes four histological groups: simple mucocele, mucosal hyperplasia, mu-

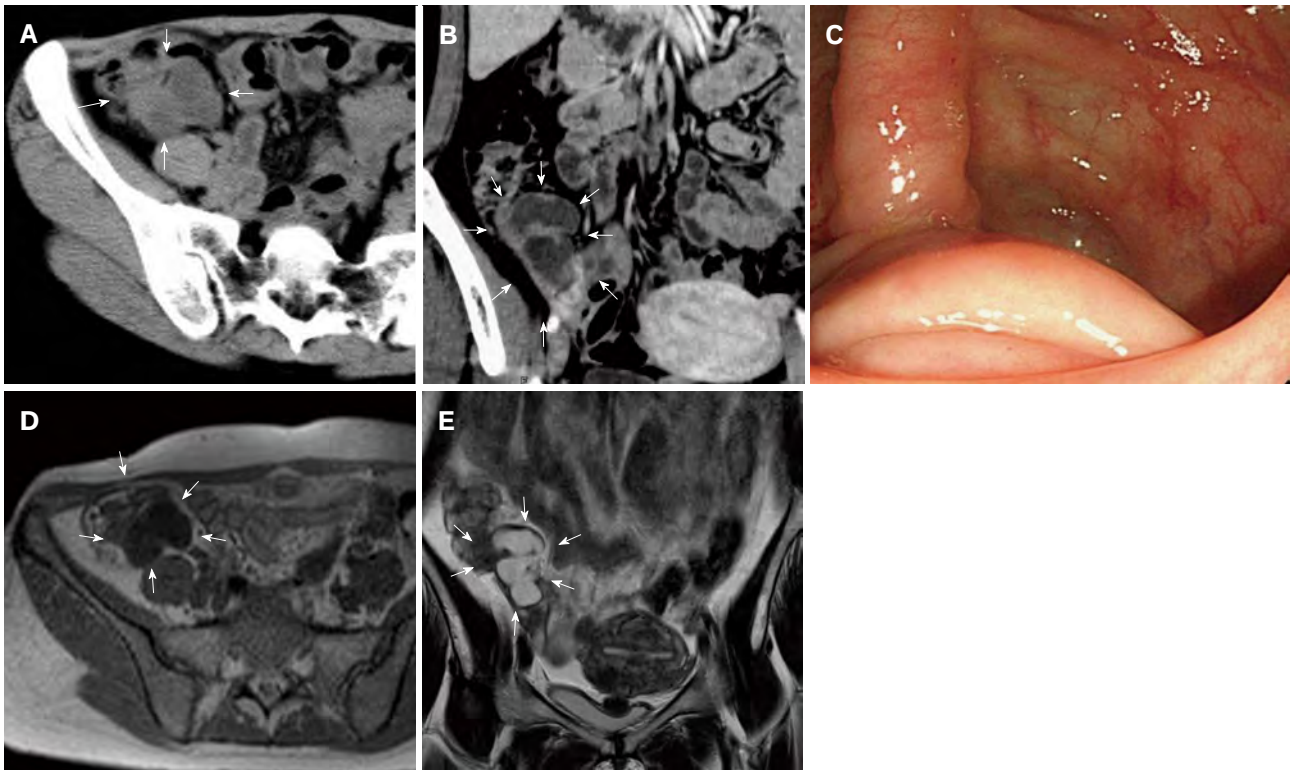

Figure 1 Preoperative image of the mucocele. A: Computed tomography (CT) (axial image); a tumor was detected (arrows); B: CT (coronal image); a tumor was detected (arrows); C: Colonoscopy; submucosal tumor-like elevations of the appendiceal orifice; D: Magnetic resonance imaging (MRI) (T1-WI, axial image); E: MRI (T2-WI, coronal image).

cinous cystadenoma, and mucinous cystadenocarcinoma. Simple mucocele is caused by mucus distention secondary to an obstruction of the appendix due to fecaliths, post-inflammatory scarring, or rarely, endometriosis. There are only 10 previously reported cases of mucocele of the appendix due to endometriosis in the English literature[4-12]. We present a rare case of mucocele of the appendix due to endometriosis and a review of the literature.

CASE REPORT

A 43-year-old woman was admitted to the hospital because of recurrent right lower abdominal pain during her menstrual periods. On physical examination, she presented with mild right lower abdominal tenderness without rigidity. Her blood and urine tests were normal. Abdominal computed tomography (CT) revealed low-density lesions near the appendix. Colonoscopy revealed submucosal tumor-like elevations of the appendiceal orifice. A biopsy was performed and result was negative. Subsequent magnetic resonance imaging (MRI) revealed hyperintensity on T2-weighted imaging (WI) (Figure 1). Consequently, mucocele of the appendix was diagnosed preoperatively. Her recurrent right lower abdominal pain during menstrual periods suggested the involvement of endometriosis. Our patient was offered open surgical resection because malignancy could not be ruled out. During surgery, several cystic lesions were observed around the appendix. Features of endometriosis were not observed in the pelvis, the uterus, or the rest of the abdominal cavity. An open ileocecal resection was performed. Multiple cystic lesions were observed around the appendix. The contents of the cystic lesions consisted of mucus (Figure 2). Histopathological examination indicated that the cysts were a simple type of mucocele and that endometriosis and smooth muscle hypertrophy were present in the muscle layer of the appendix around the mucocele (Figure 3). Consequently, we reached a diagnosis that the mucocele of the appendix was due to endometriosis. The postoperative course was uneventful.

DISCUSSION

A mucocele of the appendix is a rare lesion. It occurs in 0.2%-0.3% of all appendectomies performed and 8%-10% of all resected appendiceal tumors[1]. Endometriosis of the appendix is also a rare lesion, and is observed in 0.054%-0.8% of all appendectomies performed[13-15]. Finally, mucocele of the appendix due to endometriosis is extremely rare.

According to the modern classification[2,3], mucocele of the appendix includes four histological groups: simple mucocele, mucosal hyperplasia, mucinous cystadenoma, and mucinous cystadenocarcinoma. A simple mucocele is characterized by degenerative epithelial changes due to obstruction and distention of the appendix. This type represents 20%-30% of cases. A simple mucocele is caused by mucus distention secondary to an obstruction of the appendix due to fecaliths, post-inflammatory scarring, or rarely, endometriosis. Mucosal hyperplasia is similar to a hyperplastic colon polyp histologically. This type represents

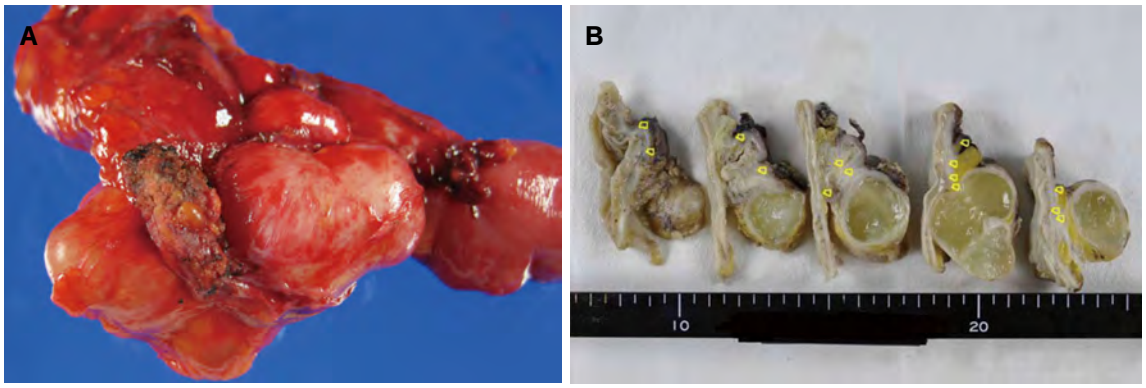

Figure 2 Macroscopic appearance of the resected specimen. A: Resected specimen contained thin-walled cystic masses; B: Cystic masses contained yellow mucin. Endometriosis was observed around the cyst (circle).

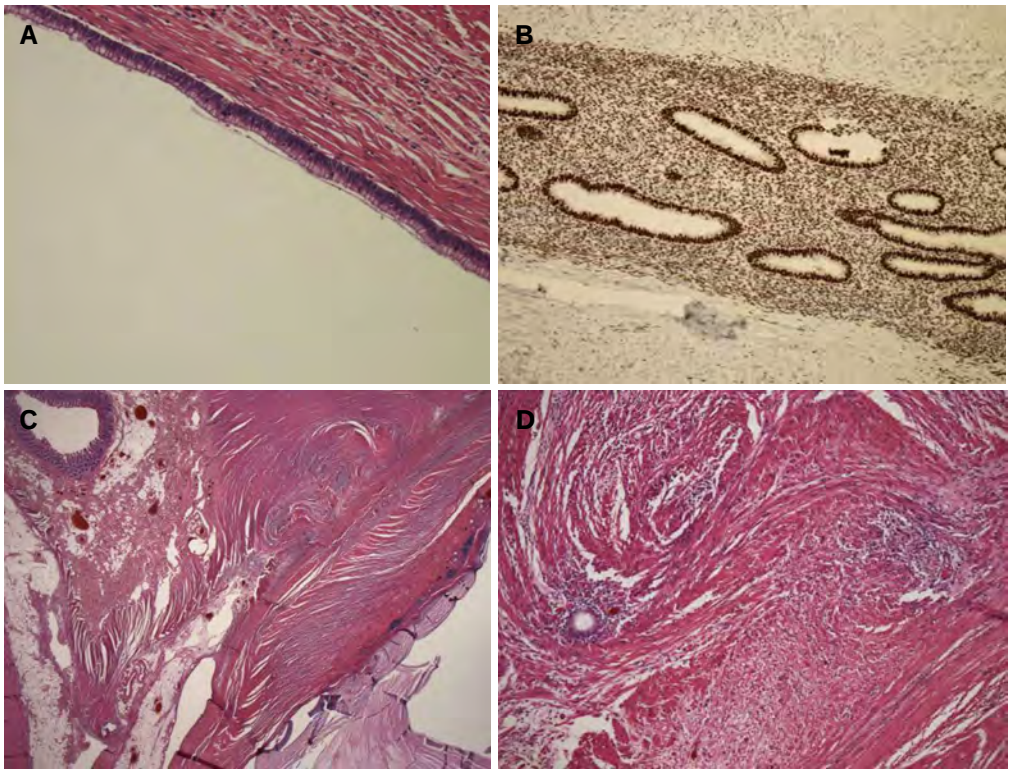

Figure 3 Pathological findings and immunohistochemical staining. A: Appendiceal mucosa was observed on the cyst wall; B: Estrogen receptor; C, D: Obstructive lesion of the appendiceal lumen. Endometriosis and smooth muscle hypertrophy were observed in the muscle layer (hematoxylin/eosin staining, C: × 20; D: × 100).

20%-30% of cases. Mucinous cystadenoma is a neoplasm that presents with a tubular or papillary pattern, with mucus production and adenomatous epithelium. This type represents 30%-50% of cases. Mucinous cystadenocarcinoma differs from cystadenoma because glandular and stromal invasion is involved. Previously, it was believed that only mucinous cystadenocarcinoma could cause pseudomyxoma peritonei (PMP), but it has recently been reported that other types of mucocele can cause PMP. Ruiz-Tovar *et al*[3] presented a case of simple mucocele that was apparently not perforated and developed PMP.

Consequently, the preoperative diagnosis of mucocele of the appendix is crucial. Colonoscopy, ultrasonography (USG), CT and MRI are used for diagnosis. On colonoscopy, the appearance of the appendiceal orifice at the center of the mound has been called the "volcano sign". USG can be used to differentiate between acute appendicitis and mucocele. Dilatation of the appendiceal lumen to ⩾ 15 mm suggests mucocele with 83% sensitivity and 92% specificity. CT offers better specificity in establishing a diagnosis of mucocele. The typical features are cystic masses that are well circumscribed with low attenuation. Wall calcifications are observed in 50% of cases, and they strongly suggest mucocele. In addition, enhancing nodules in the mucocele wall suggest cystadenocarcinoma. MRI is also useful in establishing a diagnosis of mucocele. On MRI, mucocele appears as a cystic mass with low to intermediate signal intensity on T1-WI and high

signal intensity on T2-WI. These findings can be attributed to the high protein content of a mucocele[16]. In our case, the CT, MRI and colonoscopy imaging results were compatible with these findings.

When mucocele of the appendix is diagnosed preoperatively, open surgery is favored over laparoscopy to prevent rupture of the mucocele, which may induce PMP. If mucocele is detected during a laparoscopic procedure, the patient must undergo conversion to open surgery.

Appendiceal endometriosis is diagnosed pathologically. Glandular tissue, endometrial stroma, and hemorrhage are typically assessed in patients who present with endometriosis[17]. Approximately half of the cases of endometriosis of the appendix involve the body and half involve the tip of the appendix. Muscular and seromuscular involvement occurs in two-thirds of patients, and the serosal surface is involved in one-third of patients. The mucosa is not involved and the submucosa is involved in one-third of patients. There was no relationship between the location of the endometriotic foci and the symptoms of our patient, who had endometriosis with muscular involvement.

Hapke *et al*[5] noted that the progression of mucocele of the appendix due to endometriosis consists of the following steps. Endometriosis results in smooth muscle hypertrophy of the appendix, including the muscularis mucosa, with obstruction of some of the gland crypts. These obstructions lead to local increased mucin production from multiple small cysts. Ultimately, several of these small cysts coalesce, resulting in a single layer cyst that can be dissected through the submucosa proximally. In our case, multiple small cysts were observed surrounding the appendix. These findings confirm the proposal of Hapke *et al*[5].

There are only 10 previously reported cases of a similar condition in the English literature[4-12]. We reviewed these reported cases and the present case. The mean age at presentation was 34 years (22-56 years). The patients presented various symptoms: four had recurrent abdominal pain during menstrual periods; three had chronic pelvic pain; two had acute abdominal pain with vomiting; one had increasing menorrhagia (she had complicated uterine myomas); and another had no symptoms. Preoperative diagnosis of mucocele was made in five cases; diagnostic laparoscopy was performed in five cases; and in one case, it was found by chance during surgery. Open surgery was performed in six cases, and two experienced rupture during surgery. The mean tumor size was 2.5 cm (1.3-5.5 cm). Nine cases had a single cyst, and two had multiple cysts. One case was complicated with intussusception and another with ureteric obstruction.

In summary, we report a rare case of mucocele of the appendix due to endometriosis and provide the first review of the literature.

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CASE REPORT

Anticoagulation and delayed bowel resection in the management of mesenteric venous thrombosis

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Author contributions: Kim HK, Chun JM and Huh S performed surgical operation; Kim HK organized the report; Kim HK and Chun JM wrote paper.

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Abstract

Acute mesenteric venous thrombosis is potentially lethal because it can result in mesenteric ischemia and, ultimately, bowel infarction requiring surgical intervention. Systemic anticoagulation for the prevention of thrombus propagation is a well-recognized treatment modality and the current mainstay therapy for patients with acute mesenteric venous thrombosis. However, the decision between prompt surgical exploration *vs* conservative treatment with anticoagulation is somewhat difficult in patients with suspected bowel ischemia. Here we describe a patient with acute mesenteric venous thrombosis who presented with bowel ischemia and was treated with anticoagulation and delayed short-segment bowel resection.

Key words: Thrombosis; Mesenteric vein; Anticoagulation; Small intestine; Resection

Core tip: Recently, we experienced a patient with acute mesenteric venous thrombosis who presented with bowel ischemia and was treated with anticoagulation and delayed short-segment bowel resection. The decision between prompt surgical exploration or conservative treatment with anticoagulation in patients with suspected bowel ischemia is difficult and one of the main purpose is the preservation of bowel. So, in equivocal patients, anticoagulation for potentially reversible bowel ischemia and delayed bowel resection for stricture if developed could be an appropriate management technique to prevent or limit future bowel resection.

Kim HK, Chun JM, Huh S. Anticoagulation and delayed bowel resection in the management of mesenteric venous thrombosis. *World J Gastroenterol* 2013; 19(30): 5025-5028 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/5025.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.5025

INTRODUCTION

Acute mesenteric venous thrombosis (MVT) accounts for 5%-10% of acute mesenteric ischemia, although it is rare in the absence of abdominal malignancy or liver cirrhosis[1,2]. Acute MVT is potentially lethal because it can result in intestinal ischemia and, ultimately, intestinal infarction requiring surgical intervention. Although intestinal gangrene resulting from mesenteric venous occlusion was first reported by Elliot[3], it was only after the detailed publication of Warren *et al*[4] in 1935 that MVT became known as a distinct clinical entity related to mesenteric ischemia.

Recent advances and widespread use of diagnostic modalities, in particular computed tomography (CT), have facilitated the early detection of MVT before laparotomy; systemic anticoagulation as early treatment for MVT prevents thrombus propagation and has resulted in a decrease in the reported mortality rate[5,6]. Nevertheless, the decision between laparotomy *vs* conservative treatment with anticoagulation can be difficult in patients with suspected bowel ischemia. Complicating matters, the

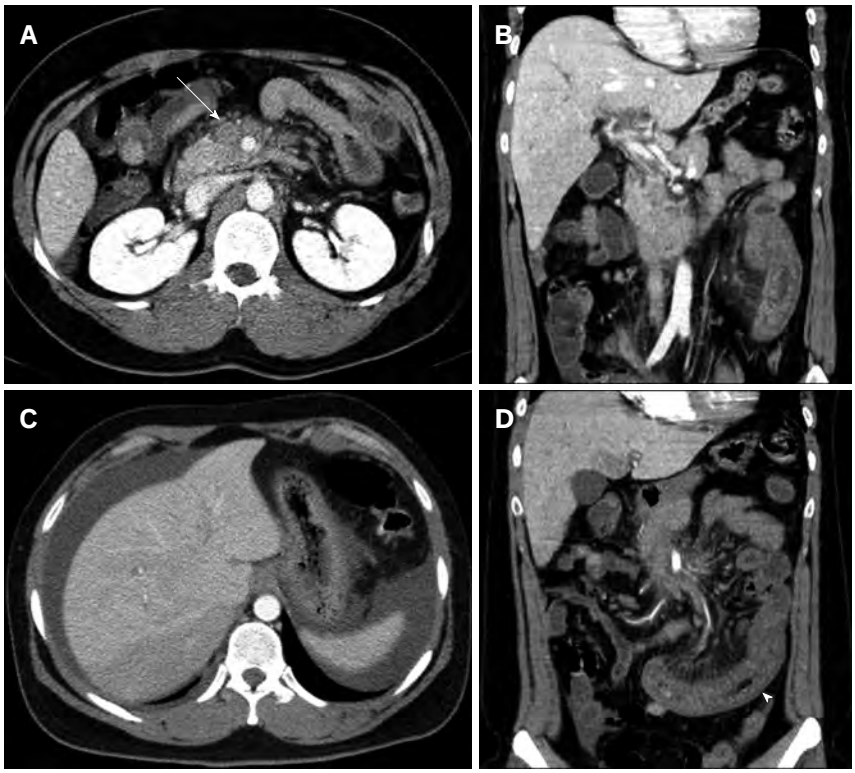

Figure 1 Abdominal computed tomography demonstrates an acute mesenteric venous thrombosis at the time of initial presentation. A: A thrombus (arrow) and perivenous infiltration at the proximal superior mesenteric vein; B: extension into the portal vein; C: An abnormal fluid collection around the liver and spleen; D: The affected small bowel (arrow head) with long-segment wall thickening and decreased enhancement.

border between ischemic bowel and viable bowel is often diffuse when exploratory laparotomy is performed in the acute stage. Because the viability of bowel is difficult to determine, overly aggressive bowel resection may result with consequent short bowel syndrome. We describe herein a patient with acute MVT and bowel ischemia who was treated with prompt anticoagulation and delayed short-segment bowel resection.

CASE REPORT

A 25-year-old male was referred to our emergency department from an outside hospital after experiencing 3 d of abdominal pain. The pain was gradually increasing in intensity and was squeezing and continuous in nature; he also noted blood-tinged stools. On abdominal examination, the patient complained of epigastric and left lower-quadrant tenderness to palpation, and the bowel sounds were decreased. Laboratory evaluation showed leukocytosis with a left shift (white blood cell count, 14100/mm^3; segmented neutrophils, 94.5%). The erythrocyte sedimentation rate was 29.0 mm/h, the C-reactive protein level was 18.61 mg/dL, the platelet count was 258000/mm^3, the hemoglobin level was 14.8 g/dL, the international normalized ratio (INR) was 1.22, the activated partial thromboplastin time was 39.6 s, and the D-dimer level was increased to 1344 μg/dL (normal range, < 340 μg/dL). Hypercoagulability testing, including protein S, protein C, and antithrombin-Ⅲ, was within normal limits. None of the following were detected: factor V Leiden mutation, prothrombin G20210A mutation, activated protein C resistance, anticardiolipin antibodies, antiphospholipid antibodies, or lupus anticoagulant. CT demonstrated complete thrombosis of the superior mesenteric vein (SMV) and partial thrombosis of the portal vein. An abnormal intraperitoneal fluid collection was noted around the liver and the pouch of Douglas, and the affected small bowel had an edematous and thickened wall with decreased enhancement, suggesting bowel ischemia (Figure 1).

The initial treatment included intravenous fluid administration, prophylactic antibiotics, bowel rest, and nasogastric-tube bowel decompression, with close monitoring for signs of bowel necrosis. Anticoagulation was started at the time of diagnosis with low molecular weight heparin (Enoxaparin, 1 mg/kg subcutaneous injection twice daily). The patient's abdominal pain gradually decreased over 1 wk of conservative management. With continuing parenteral nutrition and anticoagulation, his clinical condition stabilized 18 d after the initiation of treatment and he started oral intake with sips of water. He was able to progress to some oral food intake, however intermittent abdominal discomfort and fullness occurred when he tried to increase his oral intake further. At this point, we were concerned about the possibility of bowel stricture and discussed with him the likely ultimate need for surgical exploration. The patient strongly desired a longer period of conservative management, so after 5 wk in the hospital he was discharged on oral anticoagulation.

During the follow-up period, his INR was regulated at the outpatient clinic within the optimal range of 2.0 to

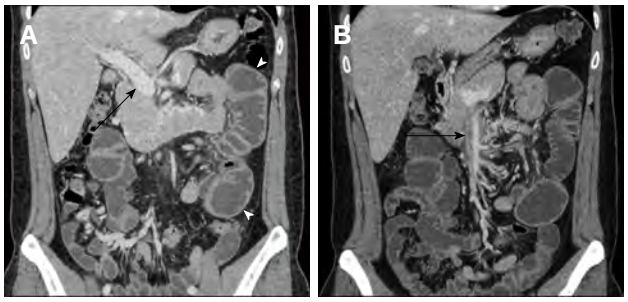

Figure 2 Abdominal computed tomography at second admission. A: Dilated proximal jejunal loop (arrow heads) and resolution of thrombus in the main portal vein (arrow); B: Remnant thrombus in superior mesenteric vein (arrow).

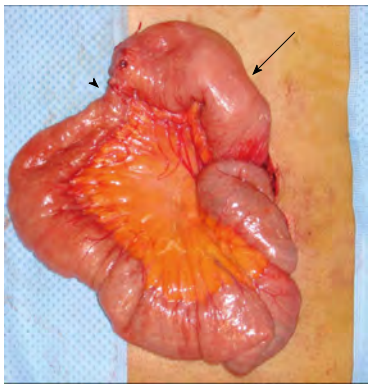

Figure 3 Intraoperative findings: sequelae of the mesenteric venous thrombosis. A dilated proximal jejunum (arrow) and short-segment stricture (arrow head) are noted.

3.0. Unfortunately, he returned to our emergency department 5 wk after discharge with abdominal pain, distension, and vomiting. A small bowel ileus was detected on plain abdominal radiography, and CT showed dilation of the proximal jejunal loop. The thrombus of the main portal vein had resolved, and the SMV demonstrated a remnant thrombus with obliteration; mesenteric congestion with development of collateral circulation was also present (Figure 2). We suspected bowel stricture due to the previous MVT with bowel ischemia and his complaint of abdominal distension, along with a weight loss of 8 kg over 2 mo. He received bowel decompression through a nasogastric tube and underwent scheduled laparotomy 2 d after the second admission. At surgery, a short-segment stricture of the distal upper jejunum with proximal dilatation was noted; the bowel color was normal (Figure 3). We performed segmental resection of 6 cm of the small bowel and a functional side-to-side anastomosis with staplers. The patient's postoperative course was uneventful and he was discharged 1 wk after surgery. He receives regular follow-up at the outpatient clinic and has been taking oral anticoagulation, without a recurrence, for 2 years.

DISCUSSION

Currently, MVT is recognized as a multifactorial disorder predisposed by some genetic and acquired risk factors. Several genetic and acquired risk factors such as factor V Leiden mutation, prothrombin G20210A mutation, protein S deficiency, protein C deficiency, antithrombin-Ⅲ deficiency, activated protein C resistance, and antiphospholipid syndrome have been reported to be associated with MVT. In our case, we tried to find out risk factors mentioned above, but we could not find any risk factors including a local inflammatory disease. The JAK-2 V617F mutation, which is associated with myeloproliferative disorder, has been recently reported to be associated with splanchnic vein thrombosis in a number of series[7,8]. Therefore, determination of this mutation may contribute to the search for genetic determinant of MVT, and further research will define the role and clinical significance of this mutation.

The management of acute MVT has changed over recent decades, however there is no consensus on its optimal management, especially in patients with bowel ischemia. Currently, systemic anticoagulation for the prevention of thrombus propagation is a well-recognized treatment modality and the mainstay of treatment in patients with acute MVT. Abdu *et al*[5], in their literature review involving 372 patients, reported that the addition of anticoagulation to previous treatment modalities improves survival rates and reduces recurrence rates in patients with MVT. However, they still recommend prompt surgical intervention. Since the 1990s, several studies have reported the feasibility of non-operative management for acute MVT[2,9-11]. Brunaud *et al*[2] determined that the morbidity, mortality, and survival rates are similar in surgical and non-surgical groups, with a shorter length of hospital stay in patients who avoid surgery. They also reported non-transmural infarction in 83% of resected specimens in the surgical group and concluded that the non-operative approach, when indicated, could avoid the resection of small bowel that is macroscopically infarcted but potentially curable with anticoagulation.

There are 2 potential difficulties in the management of patients with acute-stage MVT. The first is the decision between prompt surgical exploration or conservative treatment with anticoagulation, and the second is the difficulty in confirming bowel viability if surgical exploration is conducted. Most of the literature on the subject considers surgical exploration to be indicated if there are signs of peritoneal irritation at presentation. However, Brunaud *et al*[2] discussed the finding that peritoneal signs may not strictly correlate with the severity of bowel ischemia and suggested that new criteria, such as bowel-wall thickness and bowel-wall enhancement on the arterial phase of CT, need further evaluation. Our patient complained of abdominal pain and had tenderness to palpation, with the CT findings of an abnormal fluid collection and edematous bowel with wall thickening. However, conservative management with bowel rest and anticoagulation did not lead to transmural infarction of the affected bowel (bowel gangrene or perforation). We agree that further research into alternate criteria for surgical exploration, perhaps specific CT findings, is necessary

to improve management in these patients.

Limited areas of infarcted bowel can be surgically resected and anastomosed without significant morbidity, however when extensive ischemia is present, surgeons should try to conserve as much bowel as possible. Unfortunately, the border between ischemic bowel and viable bowel is often diffuse, making viability difficult to determine. Bowel viability is typically assessed using Doppler examination and the clinician's judgment; when the diagnosis is in doubt, a second-look operation is usually planned and performed[12,13]. Currently, numerous other techniques for assessing intestinal viability are available[14], however there is no consensus regarding their clinical usefulness. Further studies are needed to determine the value of various methods in the diagnosis of MVT and to standardize these methods.

After patients recover from the acute stage of MVT, the development of a small bowel stricture is among the possible complications during the chronic stage[15,16]. Arguably, conservative management with anticoagulation requires a more prolonged treatment period and increases patient discomfort due to the diet restrictions and need for hospitalization. Therefore, in patients with limited acute-stage bowel involvement on CT, early surgical exploration could be a more appropriate treatment, eliminating the above-mentioned shortcomings of conservative treatment. Nevertheless, the more important consideration in these patients is the possibility of recurrence. Though the reported MVT recurrence rate seems to be low while patients are receiving anticoagulation[17], recurrence is still possible, and its surgical treatment may lead to catastrophic sequelae such as short bowel syndrome.

In summary, surgical exploration in acute MVT is appropriate to be limited to the patients with definite signs of bowel infarction; in equivocal patients, anticoagulation for potentially reversible bowel ischemia could be an appropriate management technique to prevent or limit future bowel resection. Of course, patients undergoing conservative management need to be closely observed for evidence of clinical deterioration.

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EDITORIAL

Ursodeoxycholic acid therapy in gallbladder disease, a story not yet completed

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Abstract

Gallstone disease represents an important issue in the healthcare system. The principal non-invasive non-surgical medical treatment for cholesterol gallstones is still represented by oral litholysis with bile acids. The first successful and documented dissolution of cholesterol gallstones was achieved in 1972. Since then a large number of investigators all over the world, have been dedicated in biochemical and clinical studies on ursodeoxycholic acid (UDCA), demonstrating its extreme versatility. This editorial is aimed to provide a brief review of recent developments in UDCA use, current indications for its use and, the more recent advances in understanding its effects in terms of an anti-inflammatory drug.

Key words: Gallbladder; Cholesterol gallstones; Ursodeoxycholic acid

Core tip: Ursodeoxycholic acid can be considered one of the less expensive, best tested and safest of the drugs currently available. This editorial is aimed to provide a brief review of the principal non-invasive non-surgical medical treatments for cholesterol gallstones. Based on the literature and on our experimental and clinical works we try to summarize the recent developments in ursodeoxycholic acid use, current indications for its use and the more recent advances in understanding its effects in terms of an anti-inflammatory drug. For these reasons, the story would not appear to end herewith but deserves further attention and investigation.

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INTRODUCTION

Gallstone disease still represents a relevant issue for the healthcare system and one of the most common and costly of all digestive diseases if we consider the number of cholecystectomies, which are performed annually all over the world, and the hospital admission rate for complicated gallstone disease[1,2]. A marked variation in overall gallstone prevalence between the different ethnic populations has been reported; native populations from North and South America represent the groups at the highest risk in the world. Symptoms occur in approximately 20% of patients, and this subgroup is at the highest risk of developing serious complications from gallstone disease. These complications can range from simple to severe recurrent biliary colic, ascending cholangitis and/or pancreatitis[3].

Gallstone disease is a complex disorder where both

environmental and genetic factors contribute to the susceptibility to the disease. Risk factors include age, gender, race, parity, dietary factors. A family history of gallstones has also been identified as a risk factor suggesting that genetics play a role in gallstone formation. Genetic factors seems to be responsible for at least 30% of symptomatic gallstone disease[4]. Furthermore, as in atherosclerosis, the risk of cholesterol gallstone disease increases with obesity, type 2 diabetes, insulin resistance and dyslipidaemia, conditions associated with the metabolic syndrome[1,5].

Gallstones are classified as cholesterol and pigment stones. More than 90% of gallstones consist mainly of cholesterol and are formed within the gallbladder[3].

TREATMENT OF GALLSTONE DISEASE

A physician of the Byzantine Empire first described calculi in the human liver, but the earliest evidence of human gallstones is represented by the finding of 30 stones in the intact gallbladder of a mummified Egyptian priestess from around 1500 BC. In the past, a multiplicity of treatments have been used to attempt gallstone dissolution, including prayer, magic, herbs and potions[6].

The modern medical therapeutic management of gallstone disease depends primarily upon the clinical stage: asymptomatic, symptomatic (typical biliary colic pain), and complicated disease.

Asymptomatic gallstones rarely warrant treatment, since they generally have a benign natural course; the progression to symptomatic disease is relatively low, ranging from 10% to 25%. The majority of patients rarely develop gallstone-related complications without having at least one episode of biliary pain. In the pre-laparoscopy era, cholecystectomy was generally performed for symptomatic disease. The minimally invasive laparoscopic cholecystectomy refuelled the controversies regarding the optimal management of asymptomatic or silent gallstones, but most experts agree that the majority of patients should be managed by observation alone (expectant management)[7]. According to the National Institutes of Health Consensus Conference report "the availability of laparoscopic cholecystectomy should not expand the indications for gallbladder removal"[8]. Moreover, follow-up studies on a total of 279 patients with silent gallstone disease reported that the natural history of asymptomatic gallstones is benign and only 20% of these patients developed pain or complications within 24 years[9].

Symptomatic gallstone disease or acute cholecystitis are the primary indications for cholecystectomy that is currently considered the "gold standard" for the treatment of gallstone disease. Cholecystectomy is one of the most commonly performed abdominal surgical procedures, the first carried out in 1882 by Carl von Langenbuch[6]. The credit of establishing surgery of the gallbladder on a firm footing belongs to Langenbuch. The safety and success of this operation was soon established. Laparoscopic cholecystectomy is a minimally invasive surgical technique that was first performed in France, in 1987, and, in the United States, in 1988. This technique has now replaced open cholecystectomy as first-choice treatment for selected types of patients and represents one of the safer surgical procedures[8].

Non-surgical management of gallstones has been widely investigated over the last few decades, including gallstone dissolution both by mechanical and biochemical means[10].

Since its introduction, in 1985, in Germany, extracorporeal shockwave lithotripsy (ESWL) had been shown to be useful for fragmentation of bile duct stones that were not extractable endoscopically and its efficacy was soon established for selected patients at high surgical risk (> 70 years old, high morbidity and mortality rates) presenting gallstone disease (solitary radiolucent calculi < 2 cm in diameter)[11]. ESWL adopts focused shock waves produced by electromagnetic or ultrasound sources to fragment gallstones, but its efficacy depends upon the amount of energy delivered to the stone as well as the emptying and fasting volumes of the gallbladder[6]. Since its introduction in gastroenterology, ESWL had been considered as an adjuvant of oral bile acid in the treatment of gallstones, since it increases the surface for bile salt action fragmenting the stones into smaller particles. The major disadvantage of ESWL is the high post-dissolution recurrence rate (being 11%-26% for a 24-mo period), which had always raised the issue of cost-effectiveness[12]. For this reason, at present, even if advances have been made in lithotripsy technology (*i.e.*, the introduction of pulverization), none of the ESWL machines have been approved by the Food and Drug Administration (FDA) for routine clinical use in the United States, therefore this technique is no longer widely used, except in some European countries[8]. In the early period of the first use of ESWL, much interest was aroused by the application of contact dissolution agents, even if considerably less experience had been recorded. It involved direct entry of a potent cholesterol solvent (such as methyl tertiary-butyl ether, MTBE), either instilled directly into the gallbladder or into the bile duct following endoscopic intubation. Cholesterol prevalent stones could be cleared within hours to days. Interest in this method was soon lost due to the potential side-effects and was therefore limited to patients that were at high surgical risk[13] .

The principal non-invasive non-surgical medical treatment for cholesterol gallstones is still represented by oral litholysis with bile acids[14]. The first successful and documented dissolution of cholesterol gallstones was achieved in 1972 by oral administration of chenodeoxycholic acid (CDCA), a primary trihydroxy bile acid[15]. The use of CDCA due to a dose-dependent increase in aminotransferases, to an increase in serum low-density lipoprotein cholesterol and the development of bile salt-induced diarrhoea, raised concerns[15]. Since the more hydrophilic UDCA appeared to be as effective in gallstone dissolution but practically devoid of side-effects, it rapidly replaced CDCA and represents the most widely recorded experience in the literature[16].

Recently some studies have suggested the possibility of using, as therapeutic agents for gallstone disease, cholesterol-lowering agents such as statins and ezetimibe that inhibit hepatic cholesterol synthesis or reduce the absorption of cholesterol in the small intestine, alone or in combination with other forms of treatment[17-21]. Despite some promising initial data in the literature, there are still some conflicting results, thus suggesting that UDCA is the most suitable of medical treatments for gallstone disease.

URSODEOXYCHOLIC ACID

The use of UDCA in the treatment of liver diseases dates back to the traditional Chinese medicine during the Tang Dynasty. For centuries, the Chinese drug "*shorea spp.*", derived from the bile of adult black bears, has been used to cure various hepatobiliary disorders. Only at the beginning of the 20th century, was UDCA identified from polar bear bile by Hammarsten[22], a swedish research worker, who named this uncharacterized bile acid as ursocholeinic acid. The bile acid he identified was actually CDCA. It is anecdotally said that he ran out of the sample during the course of purification and abandoned its crystallization. Twenty years later, in 1927, Shoda, from Okayama University, isolated UDCA from bear bile imported from China, succeeded in crystallizing it and then called it by its present name, *i.e.*, Urso-deoxycholic ("*urso*", bear in Latin), being the predominant bile acid in bears[22].

Until Makino *et al*[22] clearly demonstrated that treatment with UDCA resulted in dissolution of cholesterol gallstones, UDCA was predominantly used in Japan as a liver tonic being administered in doses that were too small to have any significant therapeutic effect. Thereafter its use spread worldwide following further confirmation of its effectiveness and safety[23].

From the time of marketing to the present day, a large number of investigators all over the world have been involved in biochemical. At present, and clinical studies on UDCA, demonstrating its extreme versatility. UDCA can be used as a therapeutic tool in cholestatic liver diseases, being currently considered the only medical treatment officially approved by the United States FDA, to treat primary biliary cirrhosis. It can also be a therapeutic tool for non-cholestatic diseases and even for non-hepatobiliary ones[24]. For example, it appears to exert an anti-proliferative effect in terms of colon cancer prophylaxis and adenoma recurrence, an immunomodulating effect in patients affected by AIDS and it would appear to play a protective role in idiopathic recurrent pancreatitis[25]. Finally, UDCA, thanks to its biochemical structure, can penetrate the blood-brain barrier, so in the future it may be found an application of UDCA as a cell membrane stabilizer in central nervous system disorders[25].

Despite the extensive evidence accumulated regarding the possible use of UDCA in various types of diseases, the largest amount of evidence still remains the beneficial effect of UDCA in dissolution of cholesterol gallstones.

UDCA IN GALLSTONE DISEASE

UDCA, in pharmacological doses, markedly decreases biliary cholesterol saturation by 40%-60%, by inhibition of cholesterol absorption in the intestine, and cholesterol secretion into bile as indicated by a decrease in the cholesterol fraction of biliary lipids[24]. Moreover, it is well known that UDCA decreases toxicity of bile acids which can damage cell membranes and cause cholestasis, through different means of action: by inhibition of hydrophobic endogenous bile acids absorption from the small intestine, by exerting a choleretic function that induces dilution of endogenous bile salts in the bile ducts and by protecting hepatocytes against toxic bile acids[25,26].

Since Makino *et al*[22] first reported gallstone dissolution with UDCA, it has been used above all in the treatment of gallbladder cholesterol stones as an alternative to cholecystectomy[24,27]. Although gallstones are mainly composed of cholesterol, only a small number of patients (< 10% of total) can be treated with systemic dissolution therapy using UDCA[16]. Candidates for UDCA treatment should have cholesterol-enriched non-calcified gallstones < 20 mm in diameter and a patent cystic duct. The recommended dose of UDCA for gallbladder stones is 8-10 mg/kg per day, larger doses do not offer additional benefits. A dissolution rate of 30%-60% (about 1 mm decrease in stone diameter per mo) has been reported, although the initial gallstone diameter has been shown to be the most important factor affecting the dissolution rate[27-29]. A clinical study demonstrated complete disappearance of small stones (< 5 mm) with UDCA treatment after 6 mo (90% in approximately 90% of cases)[16]. Following complete dissolution, UDCA should be continued for another 3 mo in order to confirm decomposition of microscopic stones that may not be detected by ultrasonography. Absence of, or minimal, change in gallstone diameter within 6 to 12 mo of UDCA treatment represents a poor prognostic sign for dissolution[28]. The chance of reducing, by means of dissolution the size of large (> 20 mm diameter) or multiple stones, is very poor (less than 40%-50% after 1 year of treatment)[16].

Biliary sludge has been considered another therapeutic target of UDCA. Sludge formation in the biliary system can be accelerated for example by rapid weight loss, pregnancy, total parenteral nutrition and solid organ transplantation. The beneficial effect of UDCA in this condition has been shown in a clinical study in which idiopathic acute pancreatitis has been related to microscopic gallstones or biliary sludge. In this study UDCA administration within 3 to 6 mo prevented gallstone recurrence and more episodes of pancreatitis over a follow-up of 44 mo[28].

The greater limit of UDCA therapy for gallstone dissolution can be considered the high recurrence rate. Several studies have reported a recurrence rate of 30%-50% at 5 years and 50%-70% at 12 years, after successful treatment, especially in patients with multiple gallstones[16,28,29].

For these reasons, the therapeutic effect of UDCA in patients with symptomatic gallbladder stones has been controversial over the last few decades but the usefulness of this bile acid, as a therapeutic tool, has been successively reconsidered not only for its dissolution capacity, but also for the anti-inflammatory effect. A long-term follow-up study on UDCA treatment showed a significant decrease in the incidence of gallstone disease complications. In particular, this study showed that UDCA treatment in patients with symptomatic gallstones reduced the incidence of biliary pain and acute cholecystitis compared with no treatment over an 18-year period[30]. Interestingly, this therapeutic effect was independent of gallstone dissolution suggesting that UDCA could achieve these effects by restoring the normal gallbladder environment which more recent studies, on gallstone disease, have clearly shown to be characterized by an inflammatory status. A more recent 3-mo randomized placebo-controlled study showed that UDCA did not exert any beneficial effect on biliary pain or complications[31]. It should be pointed out that, there are significant differences in the recurrence rates of biliary pain and need for cholecystectomy between these two studies. Tomida *et al*[30] reported recurrence rates of < 10% in those patients on UDCA compared to 40% in those on placebo after 4 years. In contrast, in the most recent clinical trial, the need for cholecystectomy after 100 d on UDCA or placebo reached almost 75%[31]. These differences suggest that UDCA may not be effective in patients with more advanced chronic inflammatory gallbladder disease. Our earlier findings showing that UDCA treatment restores gallbladder muscle functions and reduces the biochemical markers of oxidative stress and inflammation may support, and partially explain, the beneficial effects in patients with symptomatic gallbladder stones which were independent of gallstone dissolution[32].

A series of *in vitro* studies have investigated the anti-inflammatory effect of UDCA. Cystic duct ligation in guinea pigs does not to cause acute cholecystitis unless the bile is lithogenic with cholesterol and concentrated bile is injected into the gallbladder[33,34]. Guinea pigs submitted to common bile duct ligation develop acute cholecystitis within 2-3 d together with biochemical and pathologic changes similar to those found in human acute cholecystitis, with or without gallstones[34,35]. Gallbladder muscle cells present increased levels of reactive oxygen species (ROS), lipid peroxidation and prostaglandin E2 (PGE2) levels, their response to cholecystokinin (CCK-8), PGE2 and potassium chloride being impaired, and associated with a significant reduction in receptor binding of these ligands[34]. These abnormalities were reproduced by treating normal human muscle cells with H_2O_2 or with hydrophobic bile acids (tauro-chenodeoxycholic acid, TCDC) and are prevented by pre-treatment with PGE_2 or with the free radical scavenger catalase suggesting that hydrophobic bile acids damage receptors and calcium channels of gallbladder muscle cells by stimulating the generation of ROS[36,37]. Interestingly, *in vitro* studies have shown that muscle cells pre-incubated with UDCA prevent TCDC-induced muscle cell damage and ROS production[36]. This specific beneficial effect of UDCA has been confirmed by the previously mentioned double blind, randomized 4-wk, study, carried out by our group, comparing the effects of UDCA with those of placebo in patients scheduled to undergo cholecystectomy for symptomatic gallbladder stones. In particular, this study revealed that pre-treatment with UDCA restores the normal contraction of gallbladder muscle cells by reducing cholesterol content in the plasma membranes and levels of H_2O_2, lipid per-oxidation, platelet-activating factor-like lipids as well as the production of PGE2 and catalase activity[32]. These results are consistent with data reported in a non-randomized study showing improved gallbladder muscle strip contraction in patients treated with UDCA for 3 wk compared to patients not receiving treatment[38].

These data support the hypothesis that lithogenic bile containing excess cholesterol creates a permissive environment in the gallbladders altering the normal balance between hydrophobic bile acids and gallbladder protective mechanisms. Bile acids stimulate the formation of reactive oxygen species, capable of initiating inflammatory processes and cholecystitis. Thus UDCA, by reducing the excess cholesterol and "neutralizing" the hydrophobic bile acids, restores the balance between aggressive biliary factors and gallbladder protective mechanisms[32].

Hydrophobic bile acids, such as chenodeoxycholic and deoxycholic acid, have also been demonstrated to have a toxic effect on the liver mainly by the generation of reactive oxygen species[39,40]. In particular, hydrophobic bile acids, following hepatic retention, may affect not only the hepatocytes but also the resident macrophages (*i.e.*, Küpffer cells) which generate reactive oxygen species and increase the level of oxidative stress[41]. Therapeutic concentrations of UDCA enrich the bile acid pool with UDCA resulting in a pool profile shifting from hydrophobicity to hydrophilicity[42]. UDCA administration has been shown to prevent and reduce the hydrophobic bile acid damage in the liver; indeed, in addition to displacement of the hydrophobic bile acids, UDCA appears to exert a beneficial effect by preventing hydrophobic bile acid-induced stimulation of macrophage oxidative processes[41].

A study from our group suggests that UDCA appears to exert a prophylactic action on the effects of hydrophobic bile acids on the macrophage oxidative processes in the gallbladder. Data emerging from this study reveal the occurrence, in gallbladders surgically

removed from patients with cholesterol gallstones, of an increased number of macrophages in the muscle layer when compared to the normal gallbladder. Of interest, this double blind randomised 4-wk study comparing the effects of UDCA with those of placebo in patients with symptomatic gallbladder stones, scheduled to undergo cholecystectomy, showed that this hydrophilic bile acid leads to a decrease in the number of activated macrophages in the muscle layer and to the reduced production of PGE2 in the gallbladder muscle[43]. PGs are catalytic products of cyclooxygenase-2 (COX2) and are well-known modulators of gastro-intestinal smooth muscle function[44,45]. In our study, COX2 was mainly expressed in the muscle by macrophages and a direct correlation was found between the number of the COX2 and the CD68 positive cells which represent the macrophages. Although a minor contribution of other cell types, such as mast cells and muscle cells, in which PGE2 production contributes to the mechanisms of cytoprotection[46], cannot be definitely excluded, our findings support the hypothesis that another anti-inflammatory effect of UDCA could result from the decrease in the number of activated macrophages which are the main source of PG production. This finding adds another evidence of the anti-inflammatory effect of this hydrophilic bile acid.

CONCLUSION

The large number of studies concerning the UDCA in gallbladder and liver disease published in the literature, over the last few years, clearly indicates the beneficial effect of this bile acid, supported by the more recent advances in the understanding of its effects in terms of anti-inflammatory drug.

Indeed, as only a small number of patients can benefit from UDCA, in terms of dissolution therapy, its specific beneficial effect is related also to prevention of complications in symptomatic gallstone carriers, which is independent from stone dissolution. In our opinion this hydrophilic bile acid could be an alternative therapeutic approach in high surgical risk patients with symptomatic gallbladder stones.

Furthermore, UDCA is one of the less expensive, best tested and safest drugs currently available. For these reasons, the story would not appear to end herewith but deserves further attention and investigation.

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REVIEW

Portal hypertension and gastrointestinal bleeding: Diagnosis, prevention and management

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Abstract

Bleeding from esophageal varices is a life threatening complication of portal hypertension. Primary prevention of bleeding in patients at risk for a first bleeding episode is therefore a major goal. Medical prophylaxis consists of non-selective beta-blockers like propranolol or carvedilol. Variceal endoscopic band ligation is equally effective but procedure related morbidity is a drawback of the method. Therapy of acute bleeding is based on three strategies: vasopressor drugs like terlipressin, antibiotics and endoscopic therapy. In refractory bleeding, self-expandable stents offer an option for bridging to definite treatments like transjugular intrahepatic portosystemic shunt (TIPS). Treatment of bleeding from gastric varices depends on vasopressor drugs and on injection of varices with cyanoacrylate. Strategies for primary or secondary prevention are based on non-selective beta-blockers but data from large clinical trials is lacking. Therapy of refractory bleeding relies on shunt-procedures like TIPS. Bleeding from ectopic varices, portal hypertensive gastropathy and gastric antral vascular ectasia-syndrome is less common. Possible medical and endoscopic treatment options are discussed.

Key words: Portal hypertension; Esophageal varices; Gastric varices; Portal hypertensive gastropathy; Gastric antral vascular ectasia-syndrome; Variceal bleeding; Endoscopy; Band ligation; Beta-blocker

Core tip: Gastrointestinal bleeding is a life threatening complication of portal hypertension. Primary prevention of bleeding in patients at risk for a first bleeding episode is therefore a major goal. The article gives a concise overview of possible bleeding sites in patients with portal hypertension. The diagnosis, prevention, therapy of acute bleeding and secondary prophylaxis of bleeding from esophageal and gastric varices, portal hypertensive gastropathy gastric antral vascular ectasia and ectopic varices are discussed.

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INTRODUCTION

One of the major complications of portal hypertension is bleeding from esophageal varices. Bleeding from gastric or duodenal varices as well as bleeding from colonic varices or from portal hypertensive gastropathy is less common.

A lot of studies investigating prophylaxis and therapy of bleeding in portal hypertension have been published in the last years. This paper gives a concise overview of the current knowledge.

PRIMARY PROPHYLAXIS OF BLEEDING FROM ESOPHAGEAL VARICES

Definition

Primary prophylaxis of bleeding from esophageal varices

is defined as a therapeutic intervention that aims at the prevention of the first variceal hemorrhage.

Diagnosis

At the time of the first diagnosis, about half of the patients with liver cirrhosis have esophageal varices (Figure 1)[1,2]. During the course of the disease about 90% of the patients develop esophageal varices. Variceal hemorrhage still carries a significant mortality of 7%-15%[3-5]. The identification and prophylactic treatment of patients at risk for esophageal bleeding is therefore mandatory[6].

Risk factors for variceal bleeding are the diameter of the varix, presence of red wale signs and an impaired liver function[7-10]. Hemodynamic studies point at a close association of the hepatic venous pressure gradient (HVPG) and the bleeding risk[9].

Every patient with newly diagnosed liver cirrhosis should underwent upper endoscopy for screening of esophageal and/or gastric varices[6]. In patients with esophageal varices with a diameter of more than 5 mm, prophylactic treatment should be initiated.

Prophylactic treatment is not necessary when only small varices (diameter below 5 mm) are present. Nevertheless, endoscopic follow-up is mandatory[6]. The overall incidence of esophageal varices is 5% per year[11,12]. Esophageal varices tend to increase in size in a linear fashion. One study including 258 patients with small varices and without a history of variceal bleeding found an increase in variceal size in 21%, 45% and 66% of the patients after 1.5, 3 and 4.5 years, respectively[13]. However, it has to be kept in mind that the course of the underlying liver disease is a major determinant of variceal progression[7,13]. The actual recommendation for surveillance in patients with compensated liver disease and small varices at the screening endoscopy is a follow-up examination after 1-2 years[6,14]. If the screening endoscopy showed no varices, a follow-up examination after 2-3 years is sufficient in patients with compensated liver disease[6,13,14].

Prophylaxis/therapy

Non-selective beta-blockers cause vasoconstriction of the splanchnic circulation by β_2-receptor inhibition and decrease cardiac output by β_1-receptor blockade. This leads to a decrease in portal venous inflow and thereby lowers portal pressure.

Beta-blocker therapy is not effective in preventing gastro-esophageal varices in patients with cirrhosis[15]. There is only one study that showed that prophylaxis with a non-selective beta-blocker is effective in preventing the enlargement of small varices[16]. Patients with varices at risk of bleeding (diameter > 5 mm, presence of red-color-signs) should receive prophylactic treatment (see below), since the risk of bleeding is 30%-35% in two years. Effective prophylactic treatment reduces the risk of bleeding by about 50%[17].

A major drawback of beta-blocker therapy is that not all patients respond to beta-blockers with a reduction of the HVPG[18]. Clinical studies have shown that at most

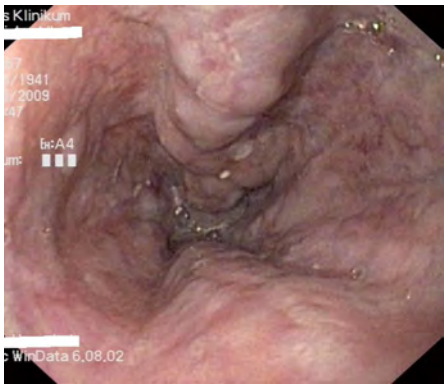

Figure 1 Esophageal varices grade Ⅱ in a patient with liver cirrhosis.

50% of beta-blocker treated patients achieved a reduction of the HVPG below 12 mmHg or > 20% from baseline levels[18]. However, other effects of beta-blocker therapy besides the reduction of HVPG like a decrease in azygos blood flow or a decrease in bacterial translocation from the gut[19] may play a role in the prevention of variceal hemorrhage[20].

Endoscopic sclerotherapy and shunt procedures are obsolete in primary prophylaxis. Standard modalities are drug therapy with non-selective beta-blockers and endoscopic variceal band ligation (VBL) of varices.

Non-selective beta-blockers like propranolol and nadolol were introduced for primary prophylaxis almost 30 years ago[17]. In recent years, the non-cardioselective vasodilating beta-blocker with mild intrinsic anti-$\alpha(1)$-adrenergic activity carvedilol was shown to be at least as effective in lowering HVPG as propranolol[21] or nadolol plus nitrate[22] and to be as effective as VBL for primary prophylaxis of variceal bleeding[23]. A monotherapy with nitrates or a combination of beta-blockers and nitrates compared to beta-blockers alone has no benefit in primary prophylaxis[17,24]. Meta-analysis have shown a reduction of the bleeding risk by a non-selective beta-blocker of about 50%. Around 20% of patients suffer from intolerable side effects that require discontinuation of the drug. After discontinuation, the bleeding risk is not different from an untreated population. That makes an indefinite prophylactic therapy necessary[25]. The most important predictor for variceal bleeding in patients on a therapy with beta-blockers is the dose of the drug[26]. Patients should therefore receive the highest tolerated dose.

An effective alternative treatment for primary prophylaxis is endoscopic VBL[27-30]. One meta-analysis has shown, that compared with untreated controls, prophylactic VBL reduces the risks of variceal bleeding and mortality[31]. Several studies compared endoscopic VBL with propranolol for primary prophylaxis of variceal bleeding[27-30]. Only one study that is controversially discussed because of some methodological flaws found a significant benefit for endoscopic VBL[29]. The other studies found no difference between beta-blockers and VBL concerning prophylaxis of bleeding[27,29,30]. A recently published Cochrane analysis that included 19 ran-

domized trials found a slight beneficial effect for VBL, but that effect was not present when only full published paper articles were analyzed[32]. In terms of efficacy, VBL and non-selective beta-blocker therapy are considered to be equivalent.

Because of the low costs, ease of administration as well as the absence of procedure-related mortality, non-selective beta-blockers are recommended as first-line treatment for the primary prophylaxis of esophageal variceal bleeding[17].

VBL is recommended in patients with serious side effects or intolerability of beta-blocker therapy as well as in patients with contraindications for drug therapy.

ACUTE BLEEDING FROM ESOPHAGEAL VARICES

Definition

Acute variceal bleeding is defined as: (1) active bleeding from esophageal varices at the moment of endoscopy; or (2) non-bleeding varices and blood in the esophagus/stomach are present and no other source of bleeding is found[33]. Recurrent bleeding is defined as rebleeding after 24-h of clinical absence of bleeding.

Therapy

Acute bleeding from esophageal varices is often a dramatic event. Most patients vomit blood but hematochezia and melena might be the only symptoms. Dependent on the amount of lost blood, patients might be hemodynamic instable and present in hemorrhagic shock. Today only 40% of patients die from exsanguinating bleeding. Most deaths are caused by complications of bleeding like liver failure, infections and hepatorenal syndrome[34,35]. Risk factors for an adverse course are the degree of liver dysfunction, creatinine, hypovolemic shock, active bleeding on endoscopy and presence of hepatocellular carcinoma[4,34-37]. Thus, the management of patients with acute variceal bleeding includes not only treatment and control of active bleeding but also the prevention of rebleeding, infections and renal failure[38].

If variceal bleeding is suspected, patients should be hemodynamically stabilized and receive medical treatment with vasopressors and antibiotic treatment[39-43]. In uncomplicated patients antibiotic therapy is done using quinolones[44]. High-risk patients with advanced liver disease (ascites, encephalopathy, jaundice, malnutrition) or previous therapy with quinolones should receive ceftriaxone[41]. Antibiotic treatment of patients with acute variceal bleeding does not only decreases mortality but also decreases the probability of rebleeding[42]. Transfusion of blood should be done with caution with a target hemoglobin level between 7 to 8 g/dL, since higher hemoglobin levels can increase portal pressure[45] and restrictive transfusion strategies are associated with better survival[46]. Patients with massive bleeding and/or patients who are somnolent should undergo endotracheal intubation and mechanical ventilation prior endoscopy to prevent aspiration pneumonia.

Available therapy options include medical and endoscopic treatment, balloon tamponade, placement of fully covered self-expandable metallic stents, transjugular intrahepatic portosystemic shunt (TIPS) and surgical shunts. Nowadays, the initial approach is a combination of vasoactive drugs, antibiotics and endoscopic therapy[47].

Medical therapy

The aim of medical therapy is to reduce splanchnic blood flow and portal pressure. Drugs currently in use are vasopressin, somatostatin and, most important in Europe, terlipressin. Due to its short half-life, vasopressin has to be given as a continuous *iv* infusion. Relevant adverse effects include systemic vasoconstriction with serious implications like mesenteric or myocardial ischemia[48]. Application of vasopressin in combination with nitrates reduces the side effects associated with vasoconstriction[49,50]. Several studies have shown that the vasopressin treatment is effective in terms of bleeding control but does not affect mortality[48,51-53]. Terlipressin is a synthetic vasopressin analogue with a longer half-life and less adverse effects. Several studies have shown that terlipressin is effective in bleeding control and has a positive impact on survival[54-56]. Terlipressin achieves control of bleeding in 75%-80% and 67% of patients at 48 h and at 5 d, respectively[56,57]. It is given at a dose of 2 mg every 4 h for the first 48 h and could be continued for prevention of early rebleeding at a dose of 1 mg every 4 h for up to 5 d[57,58]. A recent study has shown a drop of serum sodium in the range of > 5 mEq/L in 67% of patients and of > 10 mEq/L in 36% of patients treated with terlipressin[59]. Therefore, serum sodium should be monitored in patients receiving terlipressin. Compared to vasopressin, terlipressin is more effective in control of esophageal bleeding[60,61] and compared to vasopressin plus nitrate[62] as well as compared to somatostatin it is comparable effective[63,64].

Somatostatin is given as an initial bolus of 250 μg followed by a 250 to 500 μg/h continuous infusion until a bleed-free period of 24 h is achieved[38]. Octreotide is a synthetic analogue of somatostatin with longer half-life. It is administered as an initial bolus of 25 μg, followed by an infusion of 25 to 50 μg/h[65]. Both, somatostatin and octreotide, have a good safety profile. Possible adverse effects include mild hyperglycemia and abdominal craps. Somatostatin is as effective as vasopressin in control of variceal bleeding; the safety profile is superior to vasopressin[66]. The combination of terlipressin and octreotide is not superior to a monotherapy with terlipressin[67].

In summary, the available data is most convincing for terlipressin, however, the direct comparison of terlipressin and octreotide revealed no superiority of terlipressin[68,69].

Endoscopic therapy

About 80%-90% of acute variceal bleeding episodes are successfully controlled by endoscopic therapy[70]. Nowadays, most important is endoscopic VBL, injec-

tion therapy using sclerosing agents like ethoxysklerol or cyanoacrylate is less commonly used. Ethoxysklerol is injected next to - not into - the varix. It causes local inflammation and scaring and thereby thrombosis and obliteration of the vessel. On the opposite, cyanoacrylate is injected directly into the varix, causing immediate obliteration of the vessel. Endoscopic band ligation is done using a transparent cap that is attached to the tip of the endoscope. By applying suction, the varix is then pulled into the cap and a rubber ring is thrown over the varix causing thrombosis and scaring of the vessel (Figure 2).

Before the introduction of VBL, ethoxysklerol injection was widely used in the treatment of acute esophageal variceal bleeding. Studies have shown that sclerotherapy was at least as effective as balloon tamponade[71,72]. The injection of cyanoacrylate is used as a second line therapy when VBL of variceal bleeding fails.

Endoscopic VBL was first carried out in 1988[73]. The method is now widely available and complications are - compared to sclerotherapy - less common[74]. The most frequent complications are superficial ulcerations and esophageal strictures. Bleeding after the rubber rings have been fallen off is less common. A disadvantage of the method is the impaired sight that is caused by the ligation system. Costs are - compared to sclerotherapy - higher. Mortality rates after VBL are lower as compared to sclerotherapy[75,76].

Balloon tamponade

The use of balloon tamponade for the treatment of acute esophageal variceal bleeding was introduced by Sengstaken *et al*[77] The Minnesota-tube is a modified version with an aspiration channel above the esophageal balloon. For uncontrolled bleeding from gastric varices, the Linton-Nachlas tube is preferred[78]. In the hand of the experienced user the method allows control of bleeding in most patients[79]. A major drawback of the method is the high amount of possible serious complications like necrosis and/or rupture of the esophagus as well as aspiration pneumonia[80]. Deflating of the balloon after six hours reduces the risk of complications. Due to the serious risks, balloon tamponade should only be applied by an experienced physician under fluoroscopic control. After all, balloon tamponade is only a bridging procedure until other, definite therapy options are available.

Self-expandable metal stents

The placement of fully covered self-expandable metal stents (SEMS) is an alternative to balloon tamponade. The SEMS is inserted over an endoscopic placed guidewire using a stent delivery device without the need of fluoroscopy[81]. SEMS controls bleeding by compression of the bleeding varices[81]. The stent can be left in place for up to two weeks and can be easily removed by endoscopy. The effectiveness in the control of refractory esophageal variceal bleeding has been shown in four case series[82-85]. The procedure is save with minor complications like esophageal ulcerations, compression of the bronchial system and stent migration into the stomach being described[82-85]. Like balloon tamponade, the procedure is reserved for patients with bleeding refractory to medical and endoscopic treatment. It does not allow definite treatment of variceal bleeding due to the high percentage of patients with rebleeding after the SEMS has been removed, but has to be considered as an effective and safe bridging procedure that allows stabilization of the patient until definite treatment is possible.

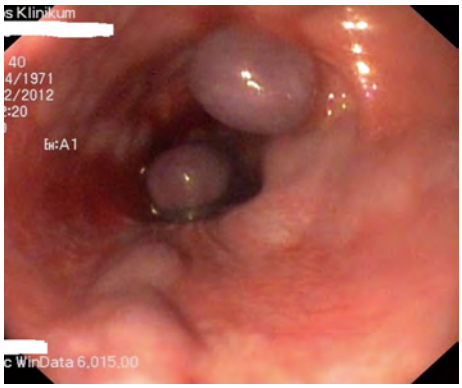

Figure 2 Variceal band ligation of esophageal varices.

Transjugular intrahepatic portosystemic shunt

By TIPS placement a functional portacaval side-to-side shunt is established. TIPS is indicated in patients with refractory acute variceal bleeding that could not be sufficiently controlled by endoscopic and/or medical therapy and in patients with recurrent bleeding despite optimal endoscopic therapy. After TIPS insertion, bleeding is stopped in almost all of the affected patients[86-88]. The rate of recurrent bleeding after one year is 8%-18%[89-91]. However, TIPS insertion is a problem in patients with multi-organ failure and/or in patients with decompensated liver disease. In these patients, the 30-d-mortality rate is as high as 100%[86,88,92]. Disadvantages of the procedure are the risk of hepatic encephalopathy as well as TIPS dysfunction with the risk of recurrent bleeding[93,94]. A major improvement was the introduction of polytetrafluoroethylene (PTFE) covered stents. These stents have higher rates of patency over time and mortality rates are lower[95]. A recently published trial has investigated the role of early TIPS in high-risk patients[96,97]. The multicenter study including 63 patients with esophageal hemorrhage and a high risk of treatment failure (Child B with active bleeding or Child C < 14 points) demonstrated that insertion of a PTFE covered TIPS within 72 h (preferable within 24 h) compared to combined endoscopic and vasoactive drug treatment decreased rebleeding (50% patients without rebleeding in the non-TIPS *vs* 97% in the TIPS group) and 1-year mortality (86% survival in the TIPS *vs* 61% in the non-TIPS group)[96].

Surgery

Surgical procedures in patients with acute or recurrent variceal bleeding are limited to a very small portion of

patients in whom medical and/or endoscopic control of bleeding was not achievable and TIPS was no option because of technical or anatomical problems (*e.g.*, complete thrombosis of the portal vein). Possible procedures are porto-systemic shunt operations[98] or staple transection of the esophagus[99]. Survival of patients who have undergone surgery is dependent on liver function but the mortality rate is as high as 80%.

SECONDARY PROPHYLAXIS OF ESOPHAGEAL VARICEAL BLEEDING

In patients who survive the first episode of esophageal hemorrhage, the risk of recurrent bleeding is as high as 60% with a mortality rate of up to 33%[100]. Prevention of rebleeding is therefore a major goal in patients in whom the initial bleeding episode has been successfully controlled.

Definition

Secondary prophylaxis of variceal bleeding is defined as the prevention of rebleeding from varices.

Medical therapy

Several studies are available that compared the non-selective beta-blockers propranolol or nadolol with no prophylaxis after initial bleeding[101-107]. Most of the studies found a reduction of the rebleeding risk as well as a reduction in mortality. Addition of nitrates further increased this positive effect[108]. Essential is a reduction of the HVPG of at least 20%, even if a reduction below 12 mmHg could not be achieved[26,109-111].

Endoscopic therapy

Several groups studied the effect of sclerotherapy for secondary prophylaxis of variceal bleeding[105,112-114]. The comparison of sclerotherapy to medical therapy with a non-selective beta-blocker found a benefit for patients treated with sclerotherapy in two studies[115,116] and a slight but statistically not significant benefit for beta-blocker therapy[105,117,118]. Three more studies did not find a difference between the two treatment modalities[115,116,119].

For prophylaxis of recurrent bleeding, sclerotherapy is now replaced widely by VBL. Several studies have shown the superiority of VBL over sclerotherapy[74,76,120-124].

Comparing VBL to medical therapy with non-selective beta-blockers in combination with nitrates, two studies found medical therapy to be as effective[110] or more effective[125] than VBL. In contrast, one study found VBL to be advantageous over medical therapy[126]. From the pathophysiological point of view, the combination of VBL and medical therapy is an even more promising approach for secondary prophylaxis. This has been investigated in five studies[127-131]. Whereas two studies found combination therapy to be more effective than VBL alone[127,131] two more recent studies, that compared nadolol plus nitrates with combination treatment of drugs and VBL failed to demonstrate superiority of combination treatment[128,130]. Therefore, it seems that a clear recommendation for medial treatment alone, VBL alone or combination treatment of drugs and VBL cannot be made at the moment. A reasonable approach is to perform VBL alone in patients with contraindications for beta-blocker therapy or in patients who suffer from side effects of beta-blocker therapy. Patients who tolerate drug treatment well should be placed on a combination therapy.

Transjugular intrahepatic portosystemic shunt

TIPS was compared to sclerotherapy[90,132-137] as well as to VBL[89]. In all but two studies[136,138] patients treated with TIPS had lower rates of recurrent bleeding. Three meta-analysis[139-141] summarized the available studies and found a significant lower probability of rebleeding in the TIPS treated patients. The incidence of hepatic encephalopathy was higher in the TIPS-group. A difference in mortality was not evident.

Surgery

Shunt surgery has been shown to be effective in the prophylaxis of rebleeding from esophageal varices. This has been shown for non-selective as well as for selective shunts (*e.g.*, distal spleno-renal shunt) comparing operative shunts with no therapy or endoscopic sclerotherapy[99,142-147]. As in TIPS, the most important side effect was the incidence of hepatic encephalopathy.

One study[148] compared non-covered TIPS with a small diameter prosthetic porta-caval H-shunt. Both shunts led to an adequate reduction in portal pressure, but patency rates of the operative shunts were higher over time. This led to a lower rate of rebleeding as well as to a decrease in mortality in patients with the surgical shunt. A meta-analysis compared different porto-systemic shunts (TIPS, diverse surgical shunts) with endoscopic treatment[149]. All shunts were equally effective in reducing the risk of rebleeding. The incidence of hepatic encephalopathy was higher in patients who received a shunt procedure. TIPS was complicated by a high incidence of shunt dysfunction. Comparing the different shunt procedures, there was no difference in survival.

GASTRIC VARICES AND HYPERTENSIVE GASTROPATHY

In contrast to esophageal variceal bleeding, prevention and treatment of bleeding from gastric varices and from portal gastropathy is less well evaluated in clinical studies.

Definition

According to Sarin *et al*[150] gastric varices (Figure 3) are endoscopically classified as gastro-esophageal varices type I (lesser curvature), gastro-esophageal varices type II (greater curvature), isolated gastric varices type I (located in the gastric fundus) or isolated gastric varices type II (any location in the stomach except the gastric fundus).

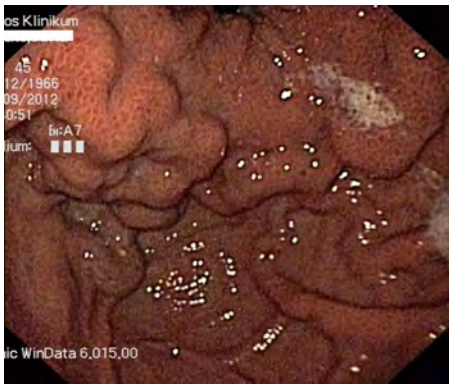

Figure 3 Isolated gastric varices type I and portal hypertensive gastropathy in a patient with liver cirrhosis.

Gastric varices

The diagnosis of gastric varices is made by endoscopy. In case of doubt of the diagnosis, endosonography with Doppler sonography allows further differentiation. If only isolated gastric varices are present, the exclusion of portal or splenic vein thrombosis as the underlying cause is mandatory.

About on fifth of the patients with portal hypertension develop gastric varices[150]. In patients with gastrointestinal bleeding due to portal hypertension, bleeding from gastric varices is the cause in 5%-10% of patients[151]. The risk of the first bleeding from gastric varices is lower than the risk of first bleeding from esophageal varices (4% in one and 9% in three years)[152]. The risk of recurrent bleeding is dependent on the localization of the varix: isolated varices in the gastric fundus (53%) bear the highest risk of recurrent bleeding, followed by varices of the greater curvature (19%) and lesser curvature (6%)[150]. The prophylactic treatment of esophageal varices by VBL does not increase the risk of secondary gastric varices compared to propranolol[153].

Almost no data is available whether medical treatment for the primary prophylaxis of bleeding from gastric varices is effective. Pathophysiological considerations warrant the use of non-selective beta-blockers for this indication[151]. One trial including 27 patients with gastric varices studied the injection of cyanoacrylate for primary prophylaxis of bleeding from large gastric varices and found the injection of cyanoacrylate to be safe and effective in primary prophylaxis[154]. However, before recommending cyanoacrylate injection as prophylactic therapy, more studies are necessary.

Data for the treatment of acute bleeding from gastric varices is sparse. Therapy with terlipressin or somatostatin is recommend although controlled studies are lacking. The endoscopic treatment of choice is injection with cyanoacrylate[155-157]. Control of bleeding is as high as 90% and more effective than sclerotherapy or band ligation in one trial[158], whereas another study found VBL and cyanoacrylate injection equally effective in terms of control of acute bleeding but reported higher rebleeding rates in the VBL group[159]. Known complications of cyanoacrylate injection include mucosal ulcerations as well as thromboembolism. TIPS insertion is highly effective with control of bleeding in more than 90% of patients[160,161] and should be considered in patients in whom endoscopic therapy fails.

The use of non-selective beta-blockers and nitrates for prophylaxis of rebleeding was shown in one study to be not effective[162]. The comparison of cyanoacrylate with propranolol for secondary prophylaxis has shown no difference between the two treatment modalities in terms of rebleeding or mortality but found more complications in the cyanoacrylate group[163]. Another study compared TIPS with cyanoacrylate in patients with bleeding from gastric varices. TIPS was shown to be more effective for prevention of recurrent bleeding, with no difference in mortality[164]. These results are in contrast to a retrospective analysis that found TIPS and cyanoacrylate equally effective in controlling and preventing gastric variceal hemorrhage with no significant differences in survival[165]. Patients who received TIPS experienced significantly more long-term morbidity[165]. Nevertheless, the above mentioned studies have to be interpreted with caution, since they included patients with different types of gastric varices.

PORTAL HYPERTENSIVE GASTROPATHY

The diagnosis of portal hypertensive gastropathy is made by endoscopy. Typical signs are mosaic, also called "snakeskin", pattern of erythema. More severe forms present with red punctuate erythema, diffuse hemorrhagic lesions and/or brown spots that indicate submucosal hemorrhage[166]. Histopathologic features of portal hypertensive gastropathy are vascular ectasia of the mucosal and submucosal veins and capillaries[166]. The exact pathogenesis of portal hypertensive gastropathy is unknown. Important factors in the pathogenesis are the presence of portal hypertension as well as hyperemia of the gastric mucosa. Several authors assumed that the endoscopic treatment of esophageal varices aggravates portal hypertensive gastropathy[167]. The worsening is often transient and portal hypertensive gastropathy shows regression in more than 40% of patients after VBL[168]. The incidence of portal hypertensive gastropathy is around 80% in patients with liver cirrhosis[169]. Acute bleeding from portal hypertensive gastropathy (Figure 4) is a rare event, with an incidence of less than 3% in three years. One study that evaluated the cause of GI-bleeding in 1496 patients found bleeding from portal hypertensive gastropathy the cause in 0.8% of patients, accounting for 8% of non-variceal bleeding in patients with liver disease[170]. The probability of chronic bleeding is around 10%-15% in three years[6].

There is only one small trial that studied the effect of non-selective beta-blockers on portal hypertensive gastropathy[171]. Twenty-four patients with non-bleeding portal hypertensive gastropathy received 160 mg propranolol per day in a double-blind placebo controlled cross-

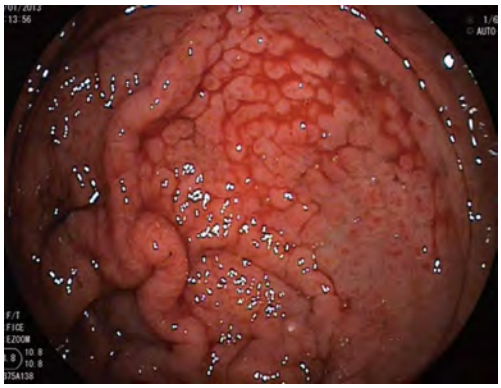

Figure 4 Acute diffuse bleeding from portal hypertensive gastropathy in a patient with decompensated liver cirrhosis.

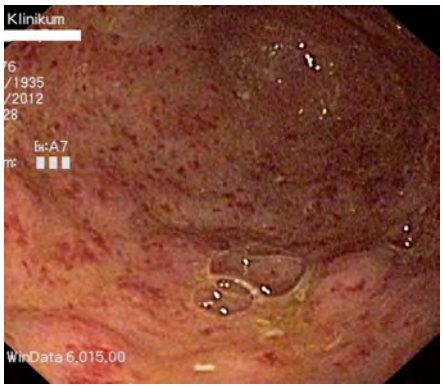

Figure 5 Typical appearance of a watermelon stomach in a patient with gastric antral vascular ectasia-syndrome and compensated liver cirrhosis.

over trial. Endoscopic grading of portal hypertensive gastropathy improved after propranolol in nine patients compared to three after placebo[171].

The therapy of acute bleeding from portal hypertensive gastropathy is mainly based on drugs that decrease portal pressure. In one study, 14 portal hypertensive patients with heavy diffuse bleeding from portal hypertensive gastropathy received propranolol in a dose of 24 to 480 mg per day. Within 3 d, bleeding ceased in 13 (93%) of patients[171]. Since the study did not have a control group of untreated patients, the results have to be interpreted with caution. A small study compared octreotide, vasopressin and omeprazole for therapy of acute bleeding. In this setting, octreotide was more effective than omeprazole or vasopressin[172]. Terlipressin was also shown to be effective in acute bleeding from portal hypertensive gastropathy[173].

No studies that investigated the role of endoscopic treatment using argon-plasma-coagulation in acute or recurrent bleeding from portal hypertensive gastropathy are available. If medical therapy fails, TIPS insertion or surgical shunt are an option[6,174,175].

In the secondary prophylaxis of bleeding from portal hypertensive gastropathy, one study including 54 patients showed that propranolol is effective in the prevention of rebleeding[176]. In the group of the propranolol treated patients 65% were free of rebleeding after one year compared to 38% in the control group. After 30 mo of follow-up, 52% of the patients in the propranolol group were free of rebleeding compared to 7% of the untreated patients[176].

In summary, the risk of bleeding from portal hypertensive gastropathy is low and primary prophylaxis is therefore not necessary. In patients with recurrent bleeding from portal hypertensive gastropathy, propranolol should be considered for secondary prophylaxis.

Gastric antral vascular ectasia-syndrome

Bleeding from gastric antral vascular ectasia (GAVE) is an uncommon but sometimes severe cause of upper gastrointestinal bleeding. It accounts for 4% of non-variceal upper GI-bleeding[177].

Gastric antral vascular ectasia (GAVE-syndrome, also known as "watermelon stomach" or "honeycomb stomach") is endoscopically as well as histologically distinguished form portal hypertensive gastropathy. In most patients, the diagnosis of GAVE is easily made on endoscopy. In case of diagnostic uncertainty, the so called GAVE-score that defines histological changes helps to distinguish the both entities[177]. GAVE-syndrome is most often found in older women and is associated with autoimmune disorders in about 60% of patients[178]. Liver disease is a risk factor for the development of GAVE-syndrome, but only 30% of affected patients suffer from liver cirrhosis[179]. On endoscopy (Figure 5), linear red streaks running longitudinally in the gastric antrum are apparent ("watermelon stomach"). In patients with liver cirrhosis the mucosal pattern is often more diffuse ("honeycomb stomach")[180]. The lesion consists of ectatic vessels of the mucosa with focal thrombosis surrounded by fibromuscular hyperplasia[181]. The pathogenesis of GAVE is not well known. Hypothesis for the pathogenesis include mechanical stress[182], humoural[183] and autoimmune factors[184]. Portal hypertension per se does not seem to be a risk factor for GAVE[185,186].

Different drugs have been used in the treatment of bleeding from GAVE. A small controlled cross-over trial has shown estrogen-progesterone to be highly effective in GAVE related bleeding[187]. Another study confirmed these findings[188]. However, the therapy has to be maintained on a long-term basis since a dose reduction results in recurrent bleeding[189]. Moreover, long-term hormonal treatment is associated with an increased risk for breast and endometrial cancer[190]. One small trial showed octreotide to be effective in bleeding from GAVE[191], but another study failed to confirm the efficacy of octreotide[192].

Treatment consists mainly of endoscopic measures like argon plasma coagulation (APC) (Figure 6), or laser photoablation of the lesions[193,194]. Endoscopic treatment using (Nd: YAG) laser has been shown to be effective in bleeding from GAVE in several studies[195-198]. The treatment is relatively safe, complications like perforation or pyloric stenosis are infrequent[199]. Disadvantages of the method are the high costs and the need for a long

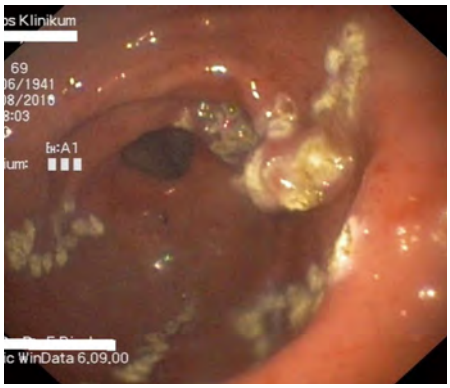

Figure 6 Endoscopic treatment of gastric antral vascular ectasia with argon plasma coagulation therapy.

training period. Argon plasma coagulation has therefore widely replaced laser therapy in the treatment of GAVE related bleeding. The procedure is easy to use, relatively cheap and widely available as well as safe. The efficacy of APC in the treatment of bleeding from GAVE is very high (90%-100% in two studies[194,200]). On average, 2.5 sessions are necessary for successful eradication of the lesions[193,194,201]. Three studies using endoscopic band ligation for the treatment of GAVE related bleeding are available[202-204]. Band ligation was shown to be effective in all trials but a study with sufficient patient numbers comparing band ligation to APC treatment is missing. Lowering portal pressure by TIPS-insertion is not effective in chronic bleeding from GAVE[179,205]. Surgery (antrectomy) is efficient in bleeding from GAVE[206] but bears a significant morbidity and mortality and is therefore reserved for patients with recurrent bleeding despite therapy with argon plasma coagulation.

ECTOPIC VARICES

Definition

Ectopic varices are dilated porto-venous vessels of the gastrointestinal mucosa that are located outside of the esophagus or the stomach.

Ectopic varices have their origin from preexisting small veins of the gastrointestinal mucosa that are porto-systemic collaterals between the portal vein and the inferior vena cava. In the majority of cases, portal hypertension or an extrahepatic obliteration of the portal vein are the cause for the development of ectopic varices.

Diagnosis

Bleeding from ectopic varices is a rare event. It accounts for 1%-5% of all gastrointestinal bleeding episodes in patients with portal hypertension[207,208]. Endoscopy is the most important diagnostic tool. In patients with portal hypertension, acute bleeding and negative findings on upper endoscopy, bleeding from ectopic varices has to be considered. In these patients, accurate examination of the duodenum is mandatory. Examination of the jejunum makes double-balloon enteroscopy necessary.

Colonoscopy is the principal method for the diagnosis of colonic varices. One study found rectal varices *via* endoscopy in 43% and *via* EUS in 75% of patients with portal hypertension, pointing out that rectal varices might be overlooked by conventional endoscopy[209].

In patients in whom bleeding from ectopic varices is assumed but endoscopy was negative, nuclear magnetic resonance (NMR) with NMR-angiography is the diagnostic tool of choice and allows the identification of ectopic varices in most patients.

Therapy

Sclerotherapy/injection therapy: Therapy of ectopic varices is mainly based on sclerotherapy or injection therapy. Controlled studies which method is best are not available but case reports showed that both sclerotherapy with ethoxysklerol as well as injection of the varix with cyanoacrylate are feasible[210-213].

Band ligation may be useful for temporary hemostasis[209,214] in duodenal varices but rebleeding of duodenal varices is a problem with ligation therapy. Additional treatment following band ligation for duodenal varices is therefore mandatory.

Surgery and TIPS: Porta-caval shunts are effective therapy measures in recurrent bleeding from ectopic varices[147,215,216]. Another option in patients without portal vein thrombosis is TIPS-insertion. Several case reports that show that TIPS is an effective option in the treatment of ectopic varices have been published[217-220].

Interventional radiology: Balloon-occluded retrograde transvenous obliteration (B-RTO) was successfully performed for patients with duodenal varices[221,222]. B-RTO can obliterate not only varices but also the afferent and efferent veins and should be considered for treating duodenal varices.

Medical therapy: From a pathophysiological point of view the application of beta-blockers does makes sense in patients with ectopic varices, but no data from controlled trials that investigate the role of non-selective beta-blockers and/or nitrates are available.

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REVIEW

Role of bevacizumab in colorectal cancer growth and its adverse effects: A review

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Abstract

Angiogenesis affects both wound healing and malignant cell growth through nutrients and oxygen. Vascular endothelial growth factor (VEGF) is the most important element involved in this complex process. Inhibition of VEGF influences angiogenesis and may restrict tumor growth and metastatic ability. Modern anti-angiogenic therapy is based on this theory. Bevacizumab is a recombinant humanized monoclonal antibody (immunoglobulin G1) which binds with VEGF-A forming a large molecule. It can not be bound with VEGF tyrosine kinase receptors preventing VEGF-A incorporation; thus its activity is inhibited inducing blockage of VEGF-mediated angiogenesis. Bevacizumab, in combination with chemotherapy or other novel targeted therapeutic agents, is currently used more frequently in clinical practice, mainly for managing advanced colorectal cancer. It is also used for managing other malignancies, such as breast cancer, pancreatic cancer, prostate cancer, non small-cell lung cancer, metastatic renal carcinoma and ovarian tumors. Although it is generally considered a safe treatment, there are reports of some rare side effects which should be taken into account. Recent experiments in rats and mice show promising results with a wider therapeutic range.

Key words: Angiogenesis; Vascular endothelial growth factor; Anti-angiogenic agents; Bevacizumab; Avastin; Cancer targeted therapy; Colorectal cancer

Core tip: Modern targeted therapy with anti-angiogenic agents is based on inhibition of angiogenesis, as the formation of new vessels is crucial for the growth and metastasis of malignant cells. Recent studies on the biological agent, bevacizumab, a humanized monoclonal antibody against vascular endothelial growth factor activity, have shown improved outcome in advanced colorectal cancer. The combination of irinotecan, capecitabine and bevacizumab is currently the most frequently used regime in the treatment of metastatic colorectal cancer with improved response rates. However, the rare side-effects of bevacizumab should always be considered.

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INTRODUCTION

Angiogenesis is a complex process responsible for the formation of new vessels originating from pre-existing vessels. It is necessary for the proliferation and growth of normal cells and tissues during the fetal and neonatal period, but also for the proliferation and growth of cancer cells. Its physiological role in adult life is limited in wound healing and the reproductive cycle of females. The development of such vessel networks, or even col-

lateral circulation, aim to supply the tissues with oxygen and nutrients, remove carbon dioxide and waste products of cell metabolism and transfer hormones. A variety of factors are involved in the regulation of angiogenesis. Vascular endothelial growth factor (VEGF) is one of the main growth factors involved in vessel formation[1,2].

The current targeted therapy of cancer with anti-angiogenic agents is based on angiogenesis inhibition and restriction of tumour spread, as neo-angiogenesis has a crucial effect on the growth and invasion of malignant cells[3-7].

The topic of this study has attracted much interest in clinical oncology and experimental research. VEGF by promoting angiogenesis favours tumor growth, while its inhibition results in tumor limitation. The novel anti-angiogenic agent, bevacizumab, is a recombinant humanized monoclonal antibody against VEGF activity. This targeted therapy is currently combined with chemotherapy and used mainly in the treatment of metastatic colorectal cancer.

ANGIOGENESIS AND ITS INHIBITION

VEGF and its receptor (VEGFr) play important roles in the neo-angiogenesis process in physiological growth and healing as well as in pathological states such as malignancy. VEGF levels are known to be increased, particularly in the most malignant tumors, such as colorectal cancer, and are associated with an increased ability of the malignancy to spread and with poorer prognosis. Thus, inhibition of angiogenesis results in growth restriction or even a reduction in malignant cells[8]. A variety of events and factors at the molecular level have been evaluated for application in novel anti-cancer drugs. VEGF is one of these factors. Targeting VEGF with bevacizumab, a humanized monoclonal immunoglobulin G (IgG) antibody, in combination with adjuvant chemotherapy has been proved to effectively manage advanced colorectal cancer[5,6,8].

Malignant tumors require nutrients for growth, and tumors more than 1-2 mm^3 in size ensure independent blood flow for continuing growth. These new vessels develop *via* angiogenesis. Inadequate blood flow leads to hypoxia, the main stimulus for angiogenesis initiation. Proteins such as hypoxia inducible factor are activated resulting in over-expression of pro-angiogenic factors including VEGF and fibroblastic growth factors. The number of cancer cells is reduced in parallel with the expression of anti-angiogenic factors, such as thrombospondin I. Through the over-expression of pro-angiogenic factors, as opposed to anti-angiogenic factors, endothelial cells are activated, thus triggering the initiation of angiogenesis[8].

In spite of the similarities in the angiogenesis process between wound healing and malignancy, there are differences in the structure of new vessels.

Several angiogenic factors derived from platelets and inflammatory cells are involved in the stages of wound healing through various mechanisms. They include phosphorylation of tyrosine kinase receptors, activation and proliferation of epithelial cells, migration and creation of tubular formations and finally new vessel formation. VEGF initiates angiogenesis by abruption of cell walls and protein lysis of vessel walls, proliferation and migration of endothelial cells and formation of new vessels. This vessel network is derived from endothelial tip cells, which have phenotypic and functional differentiation from other endothelial stalk cells[3,4].

Six subtypes of VEGF have been reported, *i.e.*, VEGF-A, VEGF-B, VEGF-C, VEGF-D, virus VEGF-E and placental VEGF (PIGF). VEGF-A increases vascular permeability, degeneration of the extracellular matrix and cell aggravation. VEGF-B and PIGF are involved mainly in the normal angiogenesis process. However, an increase in PIGF levels promotes angiogenesis in pathological conditions, such as tumors and inflammation. VEGF-C and VEGF-D have a predominant role in lymphatic angiogenesis; VEGF (PIGF) regulates placental angiogenesis[8].

Four isomers of VEGF-A have been reported in humans ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{184}$, $VEGF_{206}$). The isomer $VEGF_{165}$ is over-expressed in the majority of human malignancies. This over-expression enhances growth, invasiveness and metastatic ability.

VEGF is derived from malignant cells and promotes the growth of colorectal cancer[9]. However, a recent study has shown that the expression of EGFR and VEGF are not prognostic factors in the survival of patients with colorectal cancer and the expression of EGFR does not determine lymphatic metastasis; however, this issue remains controversial[10]. It is the over-expression of VEGF and not the density of microvasculature or vein invasion that plays the important role; it is also responsible for hematogenous dissemination after curative resection for gastric cancer[11].

VEGFr is a receptor of tyrosine kinase and has three forms, VEGFr-1, 2, 3. They are expressed in vessel endothelial cells as well as in cancer cells (VEGFr-1 and 2). VEGFr-1 is also found in monocytes and macrophages. VEGFr-3 is found in endothelial cells of the lymphatic system. VEGF-A correlates with receptors VEGFr-1 and 2, VEGF-B and PIGF with receptor VEGFr-1, and VEGF-C and D correlate with receptor VEGFr-3. VEGFr-2 plays an important role in the angiogenesis process in physiological as well as in pathological conditions. VEGFr-2 stimulation promotes cell growth and migration, the creation of tubular formations (endothelial cells) and the increase in vascular permeability[1,2].

The role of VEGF in other diseases such as allergic and immune-mediated diseases has been well-established[12,13]. The potential positive effect of other biological drugs (specific immunotherapy) such as tumor necrosis factor-α inhibitors on the mechanisms of action of VEGF has also been debated[14].

BEVACIZUMAB-ACTION MECHANISM

As mentioned above, angiogenesis plays a pivotal role in cell proliferation and tumor growth. Malignant cells

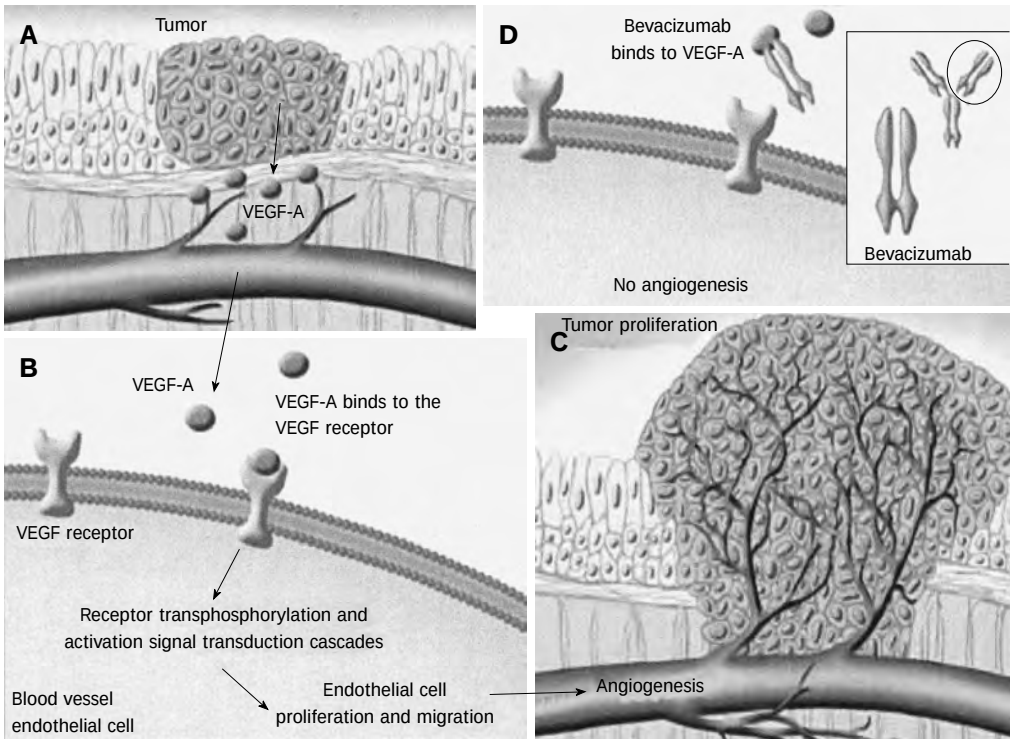

Figure 1 The process of angiogenesis and the mechanism of action of bevacizumab. A: The malignant cells secrete vascular endothelial growth factor (VEGF)-A; B: It is incorporated with its tyrosine kinase receptors (VEGFRs), promoting endothelial cell proliferation and migration; C: It leads to increased angiogenesis inducing tumor growth; D: Bevacizumab is combined with VEGF-A forming a new large molecule that lacks the ability to bind with its receptors; thus avoiding its incorporation and action, it then inhibits angiogenesis. Taken from Shord *et al*[15].

secrete VEGF-A, a growth factor responsible for neoangiogenesis. This action is accomplished by incorporation of its tyrosine kinase receptors, VEGFrs, which are located on the surface of epithelial cells. An increase in angiogenesis facilitates blood flow to malignant cells permitting their growth and spread by ensuring a supply of oxygen and nutrients. Bevacizumab, a recombinant humanized monoclonal antibody, combines with VEGF-A forming a new molecule that lacks the ability to bind with its receptors, VEGFrs, thus avoiding its incorporation and action. This restriction of VEGF-A receptors activity induces a reduction in small vessel growth, inhibits new vessel formation and restores normal tumor blood supply[15].

Bevacizumab is an IgG1 that inhibits the activity of VEGF and its isomers. This monoclonal antibody has been derived from murine antihuman VEGF and is 93% human and 7% murine[16]. The absence of VEGF influences epithelial cells resulting in destruction of neoplastic capillaries. Although it has been reported that malignant cells continue to grow despite the absence of VEGF, they exhibit reduced invasion ability resulting in reduced metastatic activity. Furthermore, their reduced intracellular pressure makes them more vulnerable to chemotherapy and radiotherapy.

The half-life time of bevacizumab ranges from 11 to 50 d (mean half-life time 20 d). As a result, even small doses of the drug (0.3 mg/kg *bw*) may be bound with VEGF preventing incorporation with its receptors, and thus inactivating VEGF efficiency. Bearing in mind that the acceptable dose is 5 mg/kg *bw* every 2 wk, it has been suggested that active levels of the drug may be detected for 12 wk[8] (Figure 1).

CLINICAL APPLICATION

Bevacizumab in colorectal cancer

The current data on the management of colorectal cancer indicate that angiogenesis and its inhibition are key factors. Bevacizumab remains the most important and well-studied drug among the known anti-angiogenic agents. The use of bevacizumab (Avastin, Roche Pharma AG) has been widely accepted as first-line therapy in the management of advanced colorectal cancer in combination with other classic chemotherapy agents such as 5-fluorouracil (5-FU) or novel agents[17-22]. This combination improves the response rates to treatment, progression-free survival and overall survival, in patients with advanced disease, as opposed to chemotherapy alone[23-25]. Its licence was granted in 2004 in the United States and in 2005 in Europe[26]. Currently, the combination of the novel targeted therapy agents irinotecan, capecitabine and bevacizumab is the most widely used in metastatic colorectal cancer resulting in increased response rates[23,24,27,28].

Bevacizumab is the first agent to affect survival in patients with metastatic colorectal cancer, improving survival by 30%[16]. Furthermore, it has been established as the first- and second-line therapy for this cancer, due to its advantages compared with routine chemotherapy,

which include less resistance and toxicity[23]. Its beneficial effect has been proved in phases Ⅱ and Ⅲ clinical trials[25].

Conclusions have been drawn from a variety of trials investigating its safety and efficacy. It has been suggested that surgery should be performed at least 6-8 wk after drug cessation to minimize complications; post-operatively, re-initiation should be after 28 d and/or complete wound healing[29].

The usual dose of bevacizumab is 5 mg/kg *bw* every two weeks in combination with other chemotherapeutic agents such as irinotecan and 5-fluorouracil/leucovorin (LV). It is administered by intravenous (IV) injection which must last 90 min initially and is gradually reduced to 60 min and 30 min; IV bolus injection is contraindicated[16].

Bevacizumab has been used postoperatively 6 wk after colorectal cancer resection for the management of synchronous liver metastasis at a dose of 5 mg/kg *bw* every 2 wk or 7.5 mg/kg *bw* every 3 wk[30].

The usual dose of bevacizumab is 5 mg/kg *bw* every 2 wk for 5 cycles and even the uncommon dose of 10 mg/kg *bw* has been combined with 5-FU/LV or capecitabine in advanced colorectal cancer[31-34].

Recent trials have confirmed the effectiveness of bevacizumab in combination with other chemotherapeutic agents in metastatic colorectal cancer showing its increasing application in clinical practice. A large randomized multi-center controlled trial showed that the addition of bevacizumab to capecitabine plus or minus mitomycin significantly improved progression-free survival (PFS) without inducing further major toxicity; only expected modest adverse events including proteinuria, hypertension, arterial thromboembolism and hemolytic uremic syndrome were observed. However, it did not improve response rate or overall survival (OS), and overall quality of life was similar. Furthermore, there were 11 treatment-related deaths: one in the capecitabine group (sepsis); seven in the capecitabine-bevacizumab group (hemorrhage, myocarditis, bowel perforation, sepsis); and three in the capecitabine-bevacizumab-mitomycin group (hemorrhage, pulmonary embolism, neutropenic colitis)[35]. A meta-analysis of 5 randomized controlled trials showed that the addition of bevacizumab to first-line chemotherapy significantly increased both the PFS and OS. Females and patients with primary rectal tumors seemed to benefit most[36].

Based on a pivotal study, the United States Food and Drug Administration (FDA) in February 2004 approved bevacizumab for the first-line treatment of patients with metastatic carcinoma of the colon and rectum. In this study, 833 patients were randomly allocated to irinotecan, 5-FU, and LV either alone (the IFL regimen) or with bevacizumab (5 mg/kg every 2 wk). In the group treated with bevacizumab, OS was significantly longer (median, 20.3 mo *vs* 15.6 mo) as were PFS and response rate[24]. Subsequently on June 20, 2006, the FDA approved bevacizumab administered in combination with 5-fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) as a second-line treatment for metastatic carcinoma of the colon or rectum. This was based on the Eastern Cooperative Oncology Group open-label, multicenter, randomized, three-arm, active-controlled trial. In this study, 829 patients with recurrence following prior chemotherapy were randomly allocated to bevacizumab (10 mg/kg, as a 90-min *iv* infusion on day 1, every 2 wk) with FOLFOX4, or FOLFOX4 alone. In the group treated with bevacizumab, there was a statistically significant and clinically meaningful improvement in OS (13.0 mo *vs* 10.8 mo) in patients whose disease had progressed after adjuvant chemotherapy with 5-FU and irinotecan and in patients with advanced or metastatic disease who had received prior 5-FU and irinotecan. The administration of bevacizumab was beneficial in these sub groups, well tolerated and with no impact on quality of life[37].

In a recent phase Ⅱ study, bevacizumab was added to capecitabine plus irinotecan (XELIRI) as first-line treatment for metastatic colorectal cancer and acceptable tolerability and improved outcome were observed[38].

An updated meta-analysis and systematic review of 10 randomized controlled trials including 1366 patients with metastatic colorectal cancer identified the additional benefits of bevacizumab to cytotoxic chemotherapy regarding OS and PFS[39].

However, there was controversy regarding the aforementioned findings in a large phase Ⅲ trial of 2672 patients with stage Ⅱ to Ⅲ colon cancer. The addition of bevacizumab to modified FOLFOX6 (mFOLFOX6; *i.e.*, infusional/bolus fluorouracil, leucovorin, and oxaliplatin) as adjuvant treatment for 1 year, did not significantly prolong disease free survival[40].

The development of bevacizumab-induced hypertension as a biomarker did not predict radiological response or survival in patients with poor-risk colorectal liver-only metastases unsuitable for upfront resection[41].

Overall survival, disease-free survival, and local control showed favourable trends in patients with stage Ⅱ/Ⅲ rectal cancer treated with neo-adjuvant bevacizumab with chemoradiotherapy followed by surgery[42]. Another study of neo-adjuvant oxaliplatin, bevacizumab, continuous infusion 5-FU, and radiation in rectal cancer was terminated early because of significant gastrointestinal toxicity[43].

Bevacizumab has been used as first-line treatment early in advanced cancer and in patients with stage Ⅲ unresectable or stage Ⅳ adenocarcinoma of the colon or rectum[44,45].

A retrospective analysis of a large United States managed database estimated that the cost of treatment containing bevacizumab was lower than that containing cetuximab[46].

BEVACIZUMAB IN OTHER MALIGNANCIES

Several clinical trials have confirmed the effectiveness

of bevacizumab in other malignancies, *i.e.*, breast cancer, pancreatic cancer, prostate cancer, non-small cell lung cancer, metastatic renal carcinoma, and ovarian tumors.

Recently, targeted therapy with various anti-angiogenic agents including sunitinib, sorafenib, temsirolimus, everolimus and bevacizumab has been used as first-line systemic therapy with impressive success in patients with metastatic renal cell carcinoma, which otherwise has a poor prognosis[47].

The combination with another anti-angiogenic agent enhances activity and decreases toxicity[48].

Bevacizumab has been accepted in combination with taxanes for the treatment of metastatic breast cancer in unselected patients[49]. Its combination with paclitaxel showed a statistically significant difference in outcome compared to treatment with paclitaxel alone.

Results of trial E2100 led to the initial approval of bevacizumab as first-line therapy for patients with metastatic breast cancer in the United States in February 2008. However, based on results from subsequent trials, the United States FDA Oncologic Drugs Advisory Committee revoked its approval in July 2010[50-52]. The drug costs about $90000 (£58000; €68000). Bevacizumab has not been shown to be safe and effective in metastatic breast cancer, as several studies showed no influence on overall survival or benefits in overcoming the drug's serious and potentially life-threatening side effects.

Despite the FDA decision, it was not withdrawn in Europe by the European Medicines Agency, however, the prescribing practice has been reduced[50]. A recent survey highlighted the discord between the opinion of oncologists and the FDA's recent decision[53]; similarly there is controversy over the FDA decision[54].

Bevacizumab has also been used in primary and metastatic brain tumors, mainly in glioblastomas[55]. It has been extensively studied in patients with primary malignant gliomas and has been approved as second-line chemotherapy alone or in combination with irinotecan following first or second recurrence after radiotherapy and temozolomide[56-58]. Furthermore, the efficacy and safety of combining bevacizumab with standard-of-care therapy in patients with newly diagnosed glioblastoma multiforme is currently being studied by the AVAGLIO phase Ⅲ randomized trial[59].

Bevacizumab has also been proved to be effective as mono-therapy in recurrent ovarian stromal tumors[60].

Chemotherapy plus targeted therapy with bevacizumab had better efficacy than chemotherapy alone in patients with non-small cell lung cancer, which otherwise has a poor prognosis[61]. The combination of paclitaxel/carboplatin with bevacizumab showed increased efficacy (27% *vs* 10% with chemotherapy alone) and raised overall survival to 12.5 mo *vs* 10.2 mo, respectively.

Bevacizumab is currently being used more frequently in the management of breast, ovarian and cervical cancer[62-64]. It has also been used in advanced pancreatic cancer in phase Ⅱ clinical trials alone or combined with other therapeutic agents, but without improved outcome[21,65,66].

BEVACIZUMAB SIDE EFFECTS AND REPORTED COMPLICATIONS

Despite the documented benefits of bevacizumab use in the treatment of colorectal cancer, there have been reports of rare side effects, *i.e.*, thrombosis, arterial hypertension, proteinuria, perforation of the gastrointestinal tract or nasal septum, wound healing abnormalities, irreversible leuco-encephalopathy syndrome, allergic skin rash and hypersensitivity reactions[15,67,68]. Wound healing abnormalities include wound dehiscence, ecchymosis, bleeding and wound infection. Hypersensitivity reactions include flashing, pruritus, arterial hypertension, rigors, broncho-constriction, chest pain, and sweats. The risk of postoperative bleeding is statistically significant[25,29] as well as the risk of thromboembolic events, *i.e.*, deep vein thrombosis, pulmonary embolism, transient ischemic attack, and acute mesenteric ischemia[69-71]. Due to the aforementioned side effects, continuous monitoring of patients receiving bevacizumab treatment is mandatory to achieve the best outcome[72].

The contraindications of bevacizumab use include hypersensitivity to its active components or to recombinant monoclonal antibodies, pregnancy, lactation, brain metastasis without treatment due to bleeding risk, gastrointestinal tract perforation, wound healing complications, persistent arterial hypertension, proteinuria, arterial thromboembolic episodes, hemorrhage and congestive heart failure or cardiomyopathy[16].

The reported wound healing complications include bowel perforation, external abdominal fistula, anastomotic dehiscence, intraperitoneal bleeding, gastrointestinal hemorrhage and cellulitis. In oncoplastic surgery for advanced breast cancer, failure of free flaps due to increased thrombotic risk as well as bleeding episodes increase the morbidity and mortality rate[67].

The risks of GI-tract perforation including free perforation, fistula formation and intra-abdominal abscess are rare, but these are serious complications, which may be fatal[73,74]. These risks depend on the drug dose and increases in cancer patients. The use of non-steroidal or other anti-inflammatory drugs, peptic ulcer and colon diverticular disease are also risk factors. It should be stressed that there have been isolated reports of spontaneous delayed (several months or even one year after operation) leakage from previous colon or rectal anastomosis after treatment with bevacizumab[75-78].

An interesting case reported skin flap necrosis in a female undergoing preoperative bevacizumab and paclitaxel plus 5-FU, epirubicin, and cyclophosphamide treatment for locally advanced breast cancer[78]; we should also mention the case of Fournier's gangrene in a male during bevacizumab treatment 4 mo after chemotherapy with 5-FU/LV/oxaliplatin for advanced colorectal cancer[79].

However, in a recent study of 57 cancer patients who received bevacizumab and underwent immediate insertion of a central venous access port, there were no side-effects such as delayed wound healing, bleeding, infection

or ulceration[80].

The reported long-term anastomotic complications attributed to the use of the anti-angiogenic agent refer to 18 cases[81]. They occurred more than a year or even 78 mo following bevacizumab treatment. The risk factors included low anterior recto-sigmoid resection for rectal cancer, perioperative radiotherapy and healed early anastomotic leakage.

For the aforementioned reasons, it has been recommended that a period of 6 wk should elapse following drug cessation before hepatectomy; post-operatively, a 4-wk period is required before therapy is re-initiated[82]. However, there has recently been a debate based on experimental findings[83,84] and clinical data. The safety and effectiveness of bevacizumab were proved in a large meta-analysis of randomized controlled trials, which found no statistically significant difference in wound healing[85].

BEVACIZUMAB EXPERIMENTAL USE-PERSPECTIVES

Bevacizumab at an IV dose of 5 mg/kg *bw* has been used in combination with irinotecan in an experimental model of implanted colon cancer cells in rats[86].

Intraperitoneal administration of bevacizumab in combination with other novel targeted agents has been proven to be effective in reducing tumor size in an experimental cancer model (colon, renal) in mice[87].

Bevacizumab at an IV dose of 10 mg/kg *bw* per week was effective in reducing tumor size and vasculature in an experimental model of breast cancer with bone metastasis in rats using volumetric computed tomography and magnetic resonance imaging (MRI)[88]. Also, the effectiveness of intraperitoneal administration of bevacizumab at different doses has been documented in an experimental model of implanted breast cancer cells in rats using MRI[89,90].

Intraperitoneal administration of bevacizumab has been used with encouraging results in several experimental tumor models in mice, *i.e.*, tuberous sclerosis[91], glioblastoma[92], medullary thyroid carcinoma[93], gastric cancer[94-96], malignant fibrous histiocytoma[97], ovarian cancer[98], and endometrial cancer[99].

Furthermore, it has been used in lung cancer xenografts[100], immune-mediated vascular remodeling[101], tumor angiogenesis assessment using positron emission tomography (PET) imaging in experimental models of colorectal and ovarian cancer in mice[102], an experimental model of schwannoma[103] and in a mouse model of hepatocellular carcinoma with promising results[104]. PET imaging and VEGF bio-distribution with radio-labeled bevacizumab in colorectal cancer xenografts has been performed[105].

These experimental data on the use of bevacizumab or other novel anti-angiogenic agents in cancer models using rats or mice open new horizons broadening its targeted therapeutic application with promising results.

CONCLUSION

The promotion of angiogenesis by VEGF favors tumor growth. Bevacizumab, which is a recombinant humanized monoclonal antibody against VEGF activity, inhibits angiogenesis restricting the growth of malignant cells and thus prevents tumor spread. It has recently been used as targeted therapy in combination with chemotherapy, mainly in advanced colorectal cancer with hepatic or other metastasis, and in breast cancer despite the debate surroundings its use for this disease, and occasionally in pancreatic cancer (but without proven efficiency), ovarian tumors, small-cell lung cancer, renal cancer and prostate cancer. A number of experimental studies have also attracted great interest on its use in other advanced malignancies. This novel biological agent is generally safe and well-tolerated. However, there are rare, although serious side effects and complications that should be considered.

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MINIREVIEWS

Eosinophilic gastroenteritis: An unusual type of gastroenteritis

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Abstract

Eosinophilic gastroenteritis (EGE) is a rare disorder characterized by eosinophilic infiltration of the bowel wall with various gastrointestinal manifestations. Till date only 280 cases have been described in the literature. A high index of suspicion, by excluding other causes of peripheral eosinophilia, is a pre requisite for accurate diagnosis. EGE is an uncommon gastrointestinal disease affecting both children and adults. It was first described by Kaijser in 1937. Presentation may vary depending on location as well as depth and extent of bowel wall involvement and usually runs a chronic relapsing course. This condition can respond to low dose steroid therapy, thereby preventing grave complications like ascites and intestinal obstruction that might need surgical intervention. The natural history of EGE has not been well documented. Eosinophilic gastroenteritis is a chronic, waxing and waning condition. Mild and sporadic symptoms can be managed with reassurance and observation, whereas disabling gastrointestinal (GI) symptom flare-ups can often be controlled with oral corticosteroids. When the disease manifests in infancy and specific food sensitization can be identified, the likelihood of disease remission by late childhood is high. GI obstruction is the most common complication. Fatal outcomes are rare.

Key words: Eosinophilic gastroenteritis; Unusual type; Review of literature

Core tip: Eosinophilic gastroenteritis is a rare disorder characterised by eosinophilic infiltration of the bowel wall and various gastrointestinal manifestations. Diagnosis requires a high index of suspicion and exclusion of various disorders that are associated with peripheral eosinophilia. Corticosteroids are the mainstay of therapy with a 90% response rate.

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INTRODUCTION

Eosinophilic gastroenteritis is a rare disorder that can present with various gastrointestinal manifestations depending on the specific site and specific layer of the gastrointestinal tract involved. Majority of the cases involve stomach and proximal small bowel. The diagnostic criteria include demonstration of eosinophilic infiltration of bowel wall, lack of evidence of extra intestinal disease and exclusion of other causes of peripheral eosinophilia[1-4].

Eosinophilic gastroenteritis is characterized by the presence of abnormal gastrointestinal (GI) symptoms, most often abdominal pain, eosinophilic infiltration in one or more areas of the GI tract, defined as 50 or more eosinophils per high-power field, the absence of an identified cause of eosinophilia and the exclusion of eosinophilic involvement in organs other than the GI tract.

It can be classified into mucosal, muscular and serosal types based on the depth of involvement[5,6]. The

stomach is the organ most commonly affected, followed by small intestine and colon[7,8]. The anatomical locations of eosinophilic infiltrates and the depth of GI involvement determine clinical symptoms. The therapeutic role of steroids and antihelminthic drugs in the treatment of eosinophilic gastroenteritis is not established. In a few cases, steroids have produced symptomatic improvement in controlling malabsorption syndrome[1,9].

EPIDEMIOLOGY

Eosinophilic gastroenteritis occurs over a wide age range from infancy through the seventh decade, but most commonly between third to fifth decades of life[10,11]. A slight male preponderance has been reported[12].

Although cases have been reported worldwide, the exact incidence of eosinophilic gastroenteritis is unclear. After first described by Kaijser[10], a little less than 300 cases have been reported in the literature. Kim *et al*[2] reported 31 new cases of eosinophilic gastroenteritis in Seoul, Korea, between January 1970 and July 2003.

Venkataraman *et al*[5] reported 7 cases of eosinophilic gastroenteritis over a 10-year period in India[5]. Chen *et al*[3] reported 15 patients including 2 children, with eosinophilic gastroenteritis in 2003. In eosinophilic enteritis the morbidity is mainly due to combination of chronic nonspecific GI symptoms which include abdominal pain, nausea, vomiting, diarrhea, weight loss, and abdominal distension and more serious complications like intestinal obstruction and perforation[13,14].

PATHOPHYSIOLOGY

Eosinophilic gastroenteritis can involve any part of gastrointestinal tract from esophagus down to the rectum. The stomach and duodenum are the most common sites of involvement[1,13-17]. The etiology and pathogenesis is not well understood. There is evidence to suggest that a hypersensitivity reaction may play a role. The clinical presentations of eosinophilic gastroenteritis vary according to the site and depth of eosinophilic intestinal infiltration. The presence of peripheral eosinophilia, abundant eosinophils in the gastrointestinal tract and dramatic response to steroids provide some support that the disease is mediated by a hypersensitivity reaction[1,18]. Moreover, a study at Mayo clinic showed that 50% of patients with eosinophilic gastroenteritis give history of allergy such as asthma, rhinitis, drug allergy and eczema[1]. Peripheral blood eosinophilia and elevated serum immunoglobulin E (IgE) are usual but not universal. The damage to the gastrointestinal tract wall is caused by eosinophilic infiltration and degranulation[19]. Eosinophils are normally present in gastrointestinal mucosa as a part of host defense mechanism, though the finding in deeper tissue is almost always pathologic[20]. In eosinophilic gastroenteritis (EGE) cytokines interleukin (IL)-3, IL-5 and granulocyte macrophage colony stimulating factor may be responsible for the recruitment and activation of eosinophils and hence the pathogenesis. They have been observed immunohistochemically in diseased intestinal wall[21]. In addition eotaxin has been shown to have an integral role in regulating the homing of eosinophils into the lamina propria of stomach and small intestine[22]. Indeed, many patients have history of food allergy and other atopic conditions like eczema, asthma etc. In this allergic subtype of disease, it is thought that food allergens cross the intestinal mucosa and trigger an inflammatory response that includes mast cell degranulation and recruitment of eosinophils[23,24].

CLINICAL PRESENTATIONS

The clinical presentations of eosinophilic gastroenteritis vary according to the site and depth of inflammatory involvement of different layers of the intestinal wall. Approximately 80% have symptoms for several years[25]. Occasionally, the disease may manifest itself as an acute abdomen or bowel obstruction[13,14]. Children and adolescents can present with growth retardation, failure to thrive, delayed puberty or amenorrhea. Adults have abdominal pain, diarrhea or dysphagia. Mucosal disease is the commonest variety that presents with features of protein losing enteropathy, bleeding or malabsorption. Failure to thrive and anaemia may also be present. Lower gastrointestinal bleeding may imply colonic involvement[1,26,27]. Involvement of muscle layer may cause bowel wall thickening and intestinal obstruction. Cramping and abdominal pain associated with nausea and vomiting occurs frequently. It can also present as an obstructing caecal mass or intussusception. The subserosal form, which is least common but can cause more morbidity, usually presents as eosinophilic ascites, which is usually an exudate, with abundant peripheral eosinophilia. Serosal and visceral peritoneal inflammation leads to leakage of fluids but has a more favourable response to corticosteroids. In literature features like cholangitis, pancreatitis[28], eosinophilic splenitis, acute appendicitis and giant refractory duodenal ulcer are also mentioned.

DIAGNOSTIC EVALUATION

Four criteria are required for the diagnosis of eosinophilic gastroenteritis namely-presence of gastrointestinal symptoms, eosinophilic infiltration of gastrointestinal tract, exclusion of parasitic disease and absence of other systemic involvement. The presence of peripheral eosinophilia is not a universal phenomenon[1,29].

A thorough evaluation of the patient is necessary, starting with laboratory evaluation.

After a detailed history and physical examination, a complete blood count plays an important role. Peripheral blood eosinophilia is found in 20%-80% of cases. Average count is 2000 eosinophils (eos)/μL in patients with mucosal layer involvement, 1000 eos/μL in patients with muscle layer involvement, and 8000 eos/μL in patients with serosal involvement. Iron-deficiency anemia may be evident on mean corpuscular volume. Serum albumin may

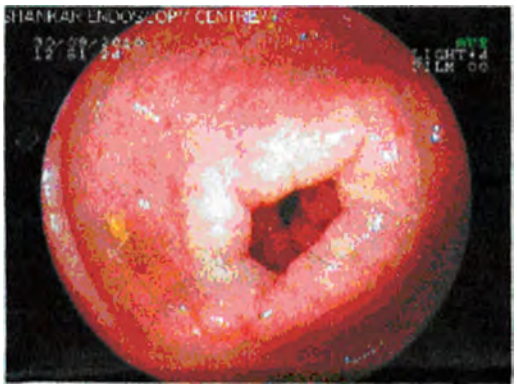

Figure 1 Endoscopy showing small superficial ulcers in stomach.

be low, especially in patients with mucosal involvement.

Fecal protein loss can be assessed by measuring alpha1-antitrypsin in a 24-h feces collection. It is used to identify the inability to digest and absorb proteins in the GI tract. The normal value is 0-54 mg/dL. Patients with eosinophilic gastroenteritis have elevated alpha1-antitrypsin in their feces. Protein loss can also result in low levels of total immunoglobulins, but serum IgE could be elevated, which then strongly supports the diagnosis of eosinophilic gastroenteritis in conjunction with other findings. The erythrocyte sedimentation rate can be elevated in few cases.

Stool examination should be performed to rule out parasitic infestation. Mild-to-moderate steatorrhea is present in approximately 30% of patients. This can be measured by qualitative and quantitative stool tests. Skin prick tests help to identify sensitization to specific ingestant and/or inhalant allergens.

Computed tomography (CT) scan may show nodular and irregular thickening of the folds in the distal stomach and proximal small bowel, but these findings can also be present in other conditions like Crohn's disease and lymphoma. On ultrasonography ascitic fluid is usually detected in patients with serosal involvement.

Radiographic changes are variable, nonspecific, and/or absent in at least 40% of patients. Gastric folds can be enlarged, with or without nodular filling defects. In extensive disease strictures, ulceration or polypoid lesions may occur and valvulae conniventes may be thickened and flattened. In eosinophilic gastroenteritis involving the muscle layer, localized involvement of the antrum and pylorus may occur, causing narrowing of the distal antrum and gastric retention. The small intestine may be dilated, with an increase in the thickness of the mucosal folds. Prominent mucosal folds may be observed in the colon. Other tests like exploratory laparotomy may be indicated in patients with serosal eosinophilic gastroenteritis.

The endoscopic appearance is nonspecific. It includes erythematous, friable, nodular, and occasional ulcerative changes[3] (Figure 1). Sometimes diffuse inflammation results in complete loss of villi, involvement of multiple layers, submucosal oedema and fibrosis[30,31]. When performing endoscopy, it is necessary to obtain at least 6

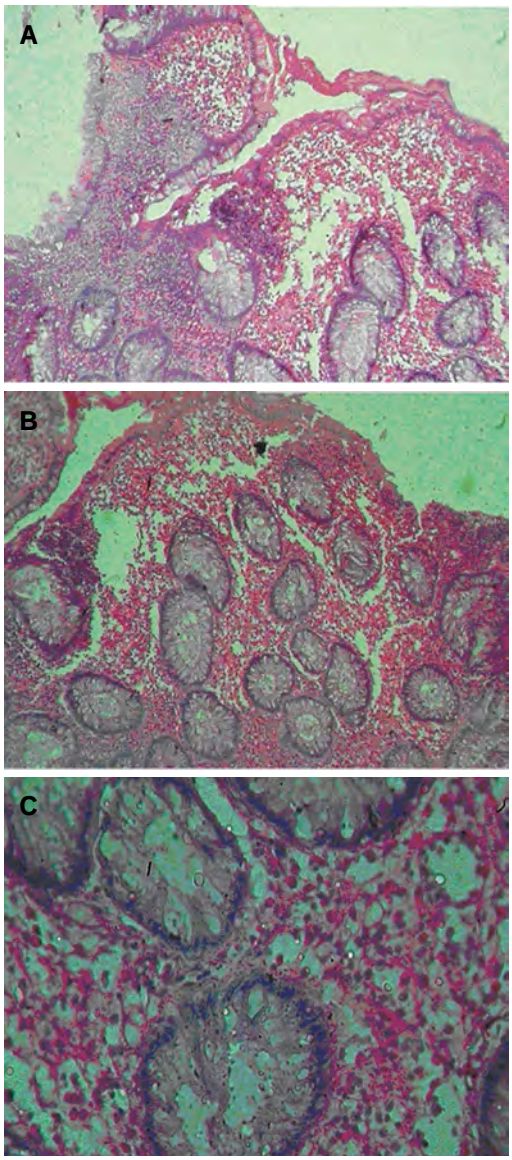

Figure 2 Large numbers of eosinophils are often present in the muscularis and serosa. A, B: Showing dense eosinophilic infiltrates in the lamina propria and mucosa (× 10); C: Showing dense eosinophilic infiltrates in the lamina propria and mucosa (× 40).

biopsy specimens from normal and abnormal areas of the bowel to exclude the possibility of sampling error. In patients with esophageal or colonic symptoms, additional biopsy specimens may be obtained from the relevant sites to aid the diagnosis.

Patients with serosal disease present with ascites. Abdominal paracentesis demonstrates a sterile fluid with a high eosinophil count. Pleural effusion also may be present.

The diagnosis can be confirmed on histopathological examination of gastric and duodenal biopsies. The gross appearance of eosinophilic gastroenteritis upon endoscopy shows erythematous, friable, nodular, and often ulcerated mucosa. Microscopy demonstrates increased numbers of eosinophils (often > 50 eos per high-power field) in the lamina propria. Large numbers of eosinophils are often present in the muscularis and serosa (Figure 2). Localized eosinophilic infiltrates may cause crypt

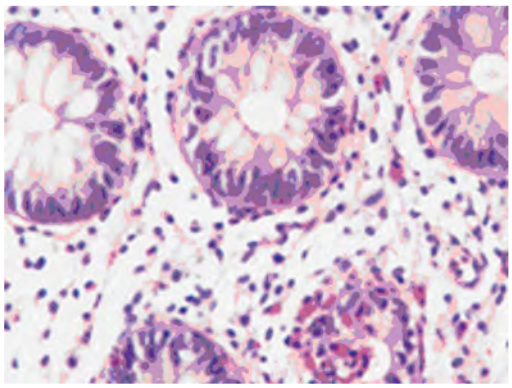

Figure 3 Post treatment (low dose steroid) biopsy showing resolution of disease.

hyperplasia, epithelial cell necrosis, and villous atrophy. Diffuse enteritis with complete loss of villi, submucosal edema, infiltration of the GI wall, and fibrosis may be apparent. Mast cell infiltrates and hyperplastic mesenteric lymph nodes infiltrated with eosinophils may be present[1,27,31,32]. Infiltration is often patchy, can be missed and laparoscopic full thickness biopsy may be required.

Histologic analysis of the small intestine reveals increased deposition of extracellular major basic proteins and eosinophilic cationic proteins.

Radio isotope scan using technetium (^{99m}Tc) exametazime-labeled leukocyte single-photon emission CT may be useful in assessing the extent of disease and response to treatment but has little value in diagnosis, as the scan does not help differentiating EGE from other causes of inflammation[33,34].

When eosinophilic gastroenteritis is observed in association with eosinophilic infiltration of other organ systems, the diagnosis of idiopathic hypereosinophilic syndrome should be considered[35].

Differential diagnosis

The main differential diagnoses are: (1) eosinophilic esophagitis; (2) eosinophilic ascites; (3) coeliac disease; (4) protein losing enteropathy from intolerance to cow milk protein; (5) infantile formula protein intolerance; and (6) idiopathic hypereosinophilic syndrome.

A diagnosis of idiopathic hypereosinophilic syndrome can be ruled out when there is absence of eosinophilic infiltration in all other organs except the bowel[35].

In celiac disease, biopsy of small bowel shows blunting of villi, crypt hyperplasia, and predominantly lymphocyte infiltration of crypts. Coeliac disease is caused by a reaction to gliadin, a prolamin (glutenprotein) found in wheat, and similar proteins found in other grains[36].

In eosinophilic esophagitis only the eosophagus is involved and not the whole bowel. A minimum of 15 eosinophils per high power field is required to make the diagnosis. Typically, eosinophils can be found in superficial clusters near the surface of the epithelium. An expansion of the basal layer is also seen in response to the inflammatory damage to the epithelium. At the time of endoscopy, ridges or furrows may be seen in the esophageal mucosa. Presence of white exudates in esophagus is also suggestive of the diagnosis[37,38].

Treatment

The role of steroids and antihelminthic drugs is not well established. However, in a few cases, steroids have been reported to produce symptomatic improvement in controlling diarrhea and protein losing enteropathy[9].

Corticosteroids are the mainstay of therapy with a 90% response rate in some studies (Figure 3). Appropriate duration of steroid treatment is unknown and relapse often necessitates long term treatment. Various steroid sparing agents, *e.g.*, sodium cromoglycate (a stabilizer of mast cell membranes), ketotifen (an antihistamine), and montelukast (a selective, competitive leukotriene receptor antagonist) have been proposed, centering around an allergic hypothesis, with mixed results[24,39,40].

Corticosteroids

Fluticasone inhaled (Flovent): Decreases recruitment of inflammatory cells including eosinophils and decreases the release of eotaxins and other inflammatory mediators. Dosage required is higher than that used in asthma.

Prednisolone (AK-Pred, Delta-Cortef): Decreases inflammation by suppressing migration of polymorphonuclear leukocytes and reducing capillary permeability. Equivalent dosages of prednisone or methylprednisolone may be used.

Budesonide (Pulmicort Respule) oral viscous suspension: Decreases inflammation, reduces capillary permeability[6].

MAST CELL STABILIZERS

Cromolyn (Intal, Gastrocrom): Inhibits release of histamine, leukotrienes, and other mediators from sensitized mast cells. It also inhibits the influx of neutrophils, as well as the formation of the active form of NADPH oxidase, which in turn prevents tissue damage caused by oxygen radicals.

Leukotriene receptor antagonists

Prevent or reverse some of the pathologic features associated with the inflammatory process mediated by leukotrienes C4, D4 and E4. Successful treatment of eosinophilic gastroenteritis has been reported in few cases, mainly with Montelukast (Singulair) which is a potent and selective antagonist of leukotriene D4 at the cysteinyl leukotriene receptor, CysLT1[41].

Role of surgical care

Surgery is avoided, except when it is necessary to relieve persistent pyloric or small bowel obstruction. Most patients respond to conservative measures and oral glucocorticosteroids. Recurrence is possible, even after surgical excision.

Prognosis

The natural history of EGE has not been well documented. Eosinophilic gastroenteritis is a chronic, waxing and waning condition. Mild and sporadic symptoms can be managed with reassurance and observation, whereas disabling GI symptom flare-ups can often be controlled with oral corticosteroids. When the disease manifests in infancy and specific food sensitization can be identified, the likelihood of disease remission by late childhood is high. GI obstruction is the most common complication. Fatal outcomes are rare.

Preventive and diet therapy

The strong association of eosinophilic gastroenteritis with food allergies has prompted the use of restrictive or elemental diets. Initially, a trial elimination diet that excludes milk, eggs, wheat and/or gluten, soy, and beef may be helpful. Skin testing can identify food hypersensitivity. If a prohibitive number of food reactions are found, an amino-acid-based diet or elemental diet may be considered. Educate patients to avoid foods that they cannot tolerate and to seek medical care when needed.

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ORIGINAL ARTICLE

Serum proteins in chronic hepatitis B patients treated with peginterferon alfa-2b

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Abstract

AIM: To study the differential protein profile in serum of hepatitis B patients.

METHODS: Serum samples were obtained from patients with chronic hepatitis B who were receiving peginterferon alfa-2b. The serum samples were subjected to albumin depletion and analyzed by two-dimensional gel electrophoresis (2-DE). Differentially expressed protein spots were identified by electrospray ionization-quadrupole time-of-flight mass spectrometry. Alpha-2-HS-glycoprotein, complement component C3c and CD5 antigen were further analyzed by an enzyme-linked immunosorbent assay and immunonephelometry.

RESULTS: Nineteen patients with HBeAg-positive chronic hepatitis B (CHB) were studied. These patients were followed for at least 1 year after treatment and were classified according to their treatment response: responders (n = 9) and non-responders (n = 10). 2-DE and MS/MS analysis were performed to compare the serum proteins before initiating peginterferon alfa-2b. From the quantitative analysis of the 2-D gel, 7 proteins were detected between the two groups at different levels before treatment. Among these potential candidates, serum levels of alpha-2-HS-glycoprotein, complement component C3c and CD5 antigen-like precursor were further analyzed. In the validation phase, 23 subjects, 9 sustained responders and 14 non-responders, were recruited. Interestingly, the levels of alpha-2-HS-glycoprotein and complement component C3c were elevated in the serum of the non-responders compared to the responders.

CONCLUSION: Serum alpha-2-HS-glycoprotein and

complement component C3c may be potential serum biomarkers in predicting the treatment response of peginterferon alfa-2b in patients with CHB prior to treatment.

Key words: Proteomics; Peginterferon alfa-2b; Chronic hepatitis B; Alpha-2-HS-glycoprotein; Serum

Core tip: Serum proteins serve as non-invasive biomarkers for several diseases. This is the first report on the potential use of common protein levels in the serum of chronic hepatitis B (CHB) patients to predict treatment responsiveness to peginterferon alfa-2b. We identified 2 potential serum biomarkers, alpha-2-HS-glycoprotein and complement component C3c, that can be used to predict treatment outcome in patients with CHB receiving peginterferon alfa-2b. The identification of these biomarkers prior to treatment is preferable in order to avoid systemic side effects due to interferon therapy.

Kuakarn S, SomParn P, Tangkijvanich P, Mahachai V, Thongboonkerd V, Hirankarn N. Serum proteins in chronic hepatitis B patients treated with peginterferon alfa-2b. *World J Gastroenterol* 2013; 19(31): 5067-5075 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i31/5067.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i31.5067

INTRODUCTION

One of the most common health care problems encountered worldwide is hepatitis B virus (HBV) infection which can progress to liver fibrosis, liver cirrhosis and liver cancer (also known as hepatocellular carcinoma). Treatment for chronic hepatitis B includes immunomodulatory agents and antiviral drugs such as nucleoside or nucleotide analogs (NAs). NAs inhibit the replication process of the virus by inhibiting its DNA polymerase[1], whereas immunomodulating therapy mainly includes treatment with interferon-α and pegylated interferon-α. The treatment duration, eradication and lack of drug resistance strains make type 1 interferons ideal for the treatment of chronic HBV[2]. Unfortunately, only 30% of patients will respond to treatment with interferon type 1[3]. The reason for this is because other factors, including the virus and the host, can significantly influence the treatment outcome. Viral factors such as the level of HBV DNA, HBV genotype, levels of hepatitis B surface antigen and hepatitis B core antigen (HBeAg), and HBV viral mutants can affect the outcome of therapy[4,5]. Other factors such as low levels of viral HBV DNA, higher levels of alanine aminotransferase (ALT), older age, being female, and naive to interferon therapy have been shown to be significantly associated with sustainable virological response among HBeAg-positive chronic hepatitis B (CHB) patients[6]. In HBeAg-negative patients, younger age, being female, having high levels of ALT and low levels of viral HBV DNA have been associated with sustainable virological response[7]. In addition, genetic host factors such as human leukocyte antigen (HLA) class Ⅱ (HLA-DRB1*14 allele), presence of polymorphism A (MxA)-88, levels of interleukin-10 and interleukin-12 have been proposed to predict the patient's treatment response after therapy[8-10]. The use of biomarkers is invaluable in predicting treatment response as well as being cost-effective in managing patients with CHB.

Various biomarkers for hepatocellular carcinoma (HCC)[11-13], HBV inflammation, HBV liver cirrhosis[14,15], and hepatitis C virus treatment response[16] have been investigated using proteomics, however, there are no predictive data for the treatment of CHB. One report from MA Hui and colleagues identified a potential serum biomarker for detecting changes after treatment, but was not able to predict the treatment outcome prior to treatment[17]. In the present study, albumin and immunoglobulin G (IgG) depleted serum was subjected to 2-dimensional gel electrophoresis and mass spectrometry. We identified 2 potential serum biomarkers, alpha-2-HS-glycoprotein and complement component C3c, and found that they can be used to predict treatment outcome in patients with CHB receiving peginterferon alfa-2b.

MATERIALS AND METHODS

Serum samples

Serum samples were obtained from patients with CHB who were followed at the King Chulalongkorn Memorial Hospital. All patients received peginterferon alfa-2b (1.5 mg/kg per week) subcutaneously for 48 wk and their responses to this treatment were assessed. These patients were followed for at least 1 year after treatment and were classified as sustained responders or non-responders. Sustained virological response among HBeAg-positive patients was characterized by undetectable HBeAg, detectable anti-HBe (HBeAg seroconversion) and HBV viral load < 2000 IU/mL 48 wk after treatment[18]. Patients without sustained virological response were classified as non-responders. Serum samples were obtained before initiating peginterferon alfa-2b treatment and at 24 wk after treatment.

Study population used to screen for biomarkers

Nineteen patients with HBeAg-positive CHB (9 sustained responders and 10 non-responders) were included in the proteomic study before initiating treatment. After 24 wk of treatment, 6 patients (3 sustained responders and 3 non-responders) were included.

Study population used to validate the system

Another 23 subjects, 9 sustained responders and 14 non-responders, were enrolled in the validation phase of the proteomic study using an enzyme-linked immunosorbent assay (ELISA) and immunonephelometry.

All studies were approved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University,

Bangkok, Thailand. Informed consent forms were collected from all patients from both phases of the study before any of the procedures were initiated.

Optimization of 2D-gel electrophoresis for pretreatment serum

Albumin and IgG were removed from the patient's serum using the ProteoPrep Blue Albumin Depletion Kit (Sigma: PROTBA) according to the company's protocol. Protein concentrations were measured by the Bio-Rad Bradford total protein assay kit (Biorad Laboratories, Inc., Redmond, WA, United States)[19] using bovine serum albumin (BSA) as the standard curve.

Two-dimensional gel electrophoresis and image analysis

The Immobiline Dry strip (pH 4-7, length 7 cm, Amersham Biosciences, Uppsala, Sweden) was rehydrated with 150 μg protein in 125 μL rehydration buffer containing 9 mol urea, 2% CHAPS, 0.002% w/v bromophenol blue, 0.8% (w/v) DTT, 1% IPG buffer for 14 h at room temperature. Iso-electric focusing (IEF) was performed by IPG ph or IEF apparatus (Amersham Biosciences, Uppsala, Sweden) with a total of 8000 Vhrs. The strip was then equilibrated in equilibration buffer containing 6 mol/L urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue and 50 mmol/L Tris-HCl (pH = 8.8) with 135 mmol/L DTT for 15 min followed by incubation, but replacing with 130 mmol/L iodoacetamide for 15 min. Next, the equilibrated strips were placed on the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) composed of 12.5% acrylamide and sealed with 0.5% (w/v) agarose. The SDS-PAGE was run on constant electric field, 15 mA per gel, using the SE 260 Mini-Vertical Units (GE Healthcare, Uppsala, Sweden) until the bromophenol blue tracking dye reached the bottom of the gel. Protein spots were stained with Coomassie Brilliant Blue G-250 stain[20]. The stained gels were scanned with an ImageMaster scanner (GE Healthcare; Uppsala, Sweden). Intensity analysis was carried out using the software, Image Master 2D Platinum (GE Healthcare, Uppsala, Sweden).

Tryptic digestion of the gels

Differentially expressed protein spots were excised from the 2-DE gels and subjected to in-gel tryptic digestion according to the method modified from Katayama *et al*[21]. The gel pieces were destained with 50% methanol and 50 mmol ammonium bicarbonate, and dehydrated with 100% acetonitrile (ACN). The gel pieces were reduced and alkylated in 10 mmol/L of DTT and 100 mmol/L iodoacetamide at room temperature for 1 h. They were then dehydrated twice with 100% ACN for 5 min after alkylation. The gel pieces were subsequently digested in 10 μL trypsin (modified porcine trypsin, sequencing grade, Promega, Madison, WI, United States) solution (20 ng in 10 mmol/L ammonium bicarbonate in 50% ACN) and incubated at room temperature overnight. The peptides were extracted twice by adding 30 μL of solution containing 50% ACN and 0.1% formic acid. The extracted solutions were dried in a heat box at 40 ℃ and kept at -80 ℃ for further analysis by mass spectrometry. Prior to mass spectrometry analysis, the peptide mixtures were reconstituted in 10 μL of 0.1 % formic acid.

Protein identification by LC/MS/MS analysis

Peptide mixtures were analyzed by ultra-performance liquid chromatography (UPLC) (Ultimate 3000, Dionex, united states) coupled to the micrOTOF-Q Ⅱ ™ ESI-Qq-TOF mass spectrometer (Bruker Daltonics, Germany) equipped with an online nanoESI source. The peptide mixture was injected onto a μ-precolumn cartridge (C18 PepMap; 300 μmol/L × 5 mm; 5 μmol/L particle size) composed of peptides, concentrated and then directly separated using a PepMap100 C18 analytical column (5 μm particle size, with 100 Å pore size). The mobile phase was run for each sample using a linear gradient of 10%-55% of 80% ACN in high performance liquid chromatography (HPLC) water for 30 min, with a hold of 15 min at 90% of 80% ACN in HPLC water, followed by a step to 10% of 80% ACN in HPLC water, hold of 20 min. The Q-TOF instrument was operated in positive ionization mode to switch automatically between MS and MS/MS acquisition. The precursor ion (MS) and fragmentation ion (MS/MS) with a mass range were 400-1600 m/z and 50-3000 m/z, respectively. The source parameters were as follows: capillary 2.0 kV, dry gas 0.3 L/min and dry temperature at 150 ℃ . The MS and MS/MS spectrometry data were processed using data analysis software (Bruker Daltonics, Germany) and searched against the NCBInr database using the MASCOT search engine. The parameters were identified using the following set up: species-homo sapiens; enzyme-trypsin; allowed up to 1 missed cleavage; fixed modification-carbamidomethylation on cystenine; variable modification-oxidation on methionines. The peptide mass tolerance and fragment mass tolerance were set at 1.2 Da and 0.6 Da, respectively[15]. A probability-based Mowse score of more than 43 was considered significant ($P < 0.05$).

Validation of the proteomic data by ELISA and immunonephelometry

Validation of the proteomic study was performed in a different population ($n = 23$) composed of 9 sustained responders and 14 non-responders. ELISA was performed according to the company's protocol using the Alpha 2 HS Glycoprotein Human ELISA kit (Abcam, Cambridge, United Kingdom) and human CD5 antigen like (CD5L) ELISA kit (Cusabio Biotech., Ltd., China). Complement component C3c was further validated using immunonephelometry and the BN ProSpec system (Siemens Healthcare Diagnostics Products GmbH, Germany).

Statistical analysis

SPSS version 17.0 (SPSS Inc., Chicago, IL, United States) was used for all statistical analyses. The values of the intensities of the spots are shown as the mean ± SE. In-

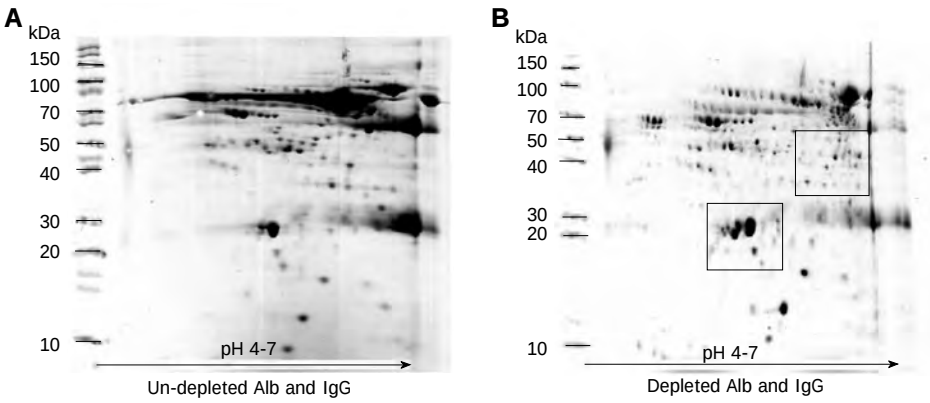

Figure 1 Serum samples from chronic hepatitis B virus-infected patients were run on two-dimensional gels (linear immobilized pH gradients; pH 4-7; 7 cm length). The pictures of the gels show the results before (A) and after (B) treatment using the ProteoPrep Blue Albumin Depletion kit. IgG: Immunoglobulin G; Alb: Albumin.

Table 1 Baseline characteristics of the patients used for screening the biomarkers before initiating chronic hepatitis B therapy (mean ± SE)

	Sustained virological responders (n = 9)	Non-responders (n = 10)	P value
Age (yr)	29.67 ± 8.29	36.90 ± 6.40	0.047
Sex (male:female)	7:2	8:2	0.912
ALT level (U/L)	103.88 ± 105.04	130.50 ± 75.77	0.609
HBV DNA (copies/mL)	(11.07 ± 8.52) × 10^6	(14.35 ± 8.44) × 10^6	0.511
HBeAg	Positive	Positive	NS

HBV: Hepatitis B virus; ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen; NS: Not significant.

dependent sample t test was used to evaluate the baseline characteristics of the patients and compare the intensity data of each matched protein spot between the sustained responders and non-responders; the P value cut-off for the independent sample t test was 0.05. Mann-Whitney U test was used to evaluate the different protein expressions between the two groups 24 wk after treatment. For the Mann-Whitney U test, any proteins identified with $P <$ 0.05 were considered significant. The independent sample t test was performed during the validation phase to compare the different levels of alpha-2-HS-glycoprotein. The Mann-Whitney U test was also performed in the validation phase to compare the different levels of complement component C3c and CD5 antigen like proteins. The Pearson correlation was carried out on age and each protein expression value to determine if age had an influence on the expression of the proteins.

RESULTS

Characteristics of the study population in the screening phase

Basic characteristics of the patients are shown in Table 1. The number of men and women, levels of serum ALT and HBV DNA, and presence of HBeAg were comparable between the two groups. However, non-responders were older than the sustained responders.

Optimization of 2D-gel electrophoresis for pretreatment serum

Before performing electrophoresis on the collected serum, the efficiency of the ProteoPrep Blue Albumin Depletion Kit was determined. Figure 1 shows the two representative maps of the serum samples (chronic HBV infection) before and after treatment with the ProteoPrep Blue Albumin Depletion Kit. In the untreated sample, levels of albumin and IgG in serum were approximately 60%-70% and 10%-20%, respectively (Figure 1A). When an equal quantity of protein was pre-treated with ProteoPrep Blue Albumin Depletion Kit, the resolution of the 2D-gels dramatically improved and several spots of other less abundant proteins became visible (Figure 1B).

Comparisons of the expressed proteins between the sustained responders and non-responders before initiating peginterferon alfa-2b treatment

The results of the protein separation by 2D-gel electrophoresis, gel digestion and protein identification by LC/MS/MS are shown in Figure 2. Seven protein spots were detected with various intensities in the patients (Table 2 and Figure 2). Four proteins were significantly detected among the sustained responders: (1) chain A, alpha-1-antitrypsin; (2) albumin, isoform CRA-b; (3) CD5 antigen-like precursor; and (4) albumin. Three proteins were significantly detected among the non-responders: (1) chain A, crystal structure of lipid-free human apolipoprotein A-I; (2) chain C, human complement component C3c; and (3) alpha-2-HS-glycoprotein. Since the sustained responders and non-responders showed significant age differences, the Pearson correlation was used for age and each protein expression value. No significant correlation between age and protein expression was observed; therefore, it is unlikely that age affected protein expression in this study. The identified proteins have the following functions: alpha-1-antitrypsin is a protease inhibitor, serum albumin is a transport and binding protein, alpha-2-HS-glycoprotein is an acute phase response protein, CD5 antigen precursor and complement component C3c are immune protection proteins, and the human apolipoprotein A-I has a role in lipid metabolism

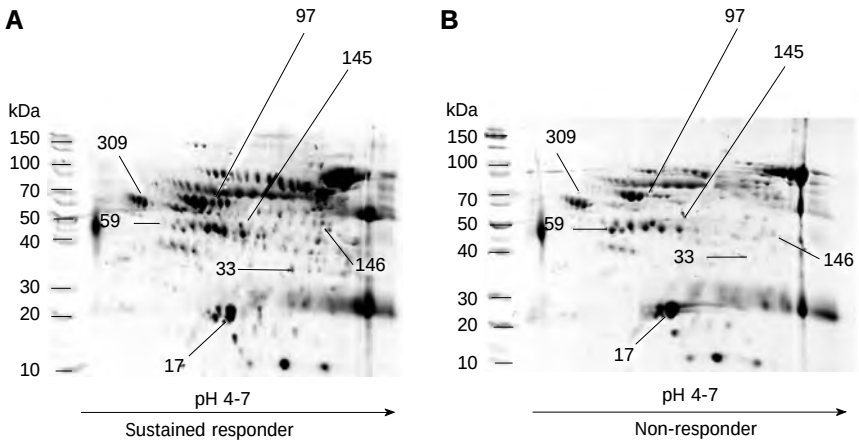

Figure 2 Serum protein spots from the 2-DE gels were significantly different. A: Sustained responders; B: Non-responders before chronic hepatitis B treatment.

Table 2 Proteins found to be significantly different between the sustained responders and non-responders before initiating peginterferon-Interferon alfa-2b treatment

Spot	Protein	NCBI ID	MS score	%cov	pI	MW	Relative intensity (mean ± SE)		SVR/NR	*P* value
							SVR	NR		
Protease inhibitor										
97	Chain A, alpha-1-antitrypsin	Gi|157831596	399	64	5.37	44.28	0.2764 ± 0.0327	0.4275 ± 0.0609	2.34	0.038
Transport protein and protein binding										
33	Albumin, isoform	Gi|119626065	539	38	6.96	61.12	0.0330 ± 0.0121	0.0764 ± 0.0144	2.31	0.033
146	Albumin	Gi|332356380	243	41	5.73	68.48	0.0602 ± 0.0182	0.1516 ± 0.0333	2.52	0.024
Acute phase protein										
309	Alpha-2-HS-glycoprotein	Gi|112910	507	40	5.43	40.09	0.7165 ± 0.0238	0.4782 ± 0.0851	0.67	0.012
Immunity protection										
59	Chain C, human complement C3c	Gi|78101271	513	70	4.79	40.20	0.3969 ± 0.0391	0.2675 ± 0.0403	0.67	0.034
145	CD5 antigen-like precursor	Gi|5174411	443	67	5.28	39.60	0.1086 ± 0.0192	0.1903 ± 0.0197	1.75	0.009
Lipid metabolism										
17	Chain A, crystal structure of lipid-free human apoliprotein A-I	Gi|90108664	1347	78	5.27	28.06	9.4993 ± 0.5044	6.0364 ± 1.0047	0.64	0.005

SVR: Sustained virological response; NR: Non-responder.

Characteristics of the patients from the validation phase of the proteomic study and immunonephelometry

In the validation phase, nine and 14 patients with sustained virological response and nonresponders were enrolled, respectively. The sex ratio, levels of serum and HBV DNA, and HBeAg were not significantly different, but there was a significant difference in age between the groups (Table 3).

Validation of proteins associated with the immune response

We selected 3 proteins (alpha-2-HS-glycoprotein**,** complement component C3c, and CD5 antigen-like proteins) that were significantly different between the 2 groups, and have functions related to immune response for further validation (Figure 3). According to the ELISA results, the serum levels of alpha-2-HS-glycoprotein were significantly elevated among the non-responders when compared to the sustained responders at baseline (Figure 4A). Similarly, the immunonephelometry results showed that serum levels of complement component C3c were significantly elevated in the non-responders when compared to the sustained responders at baseline (Figure 4B), however, the serum levels of CD5 antigen like proteins were comparable between the two groups (Figure 4C). No significant correlation between age and protein expression was observed.

Comparison of the identified proteins after 24 wk of peginterferon alfa-2b treatment

A total of 6 patients, divided into 2 groups, were included in the analysis 24 wk post-treatment. The clinical data were not significantly different between the 2 groups (Table 3). The samples were separated by 2D-gel electrophoresis, and identified by LC/MS/MS. Thirteen protein spots were significantly changed at 24 wk after treatment among the patients in the sustained response group, whereas 6 were found in the non-responders (Table 4). Interestingly, all 13 proteins in the sustained response group which were increased at 24 wk were composed of cholesterol metabolites, proteins from the acute phase response, protease inhibitors, transport proteins and immune protection. Of these, alpha-2-HS-glycoprotein was higher than the baseline level in the responder group. In the non-responder group, levels of proapoliprotein, chain A of human antithrombin Ⅲ complex and alpha-1-B-glycoprotein were higher than the baseline level, whereas

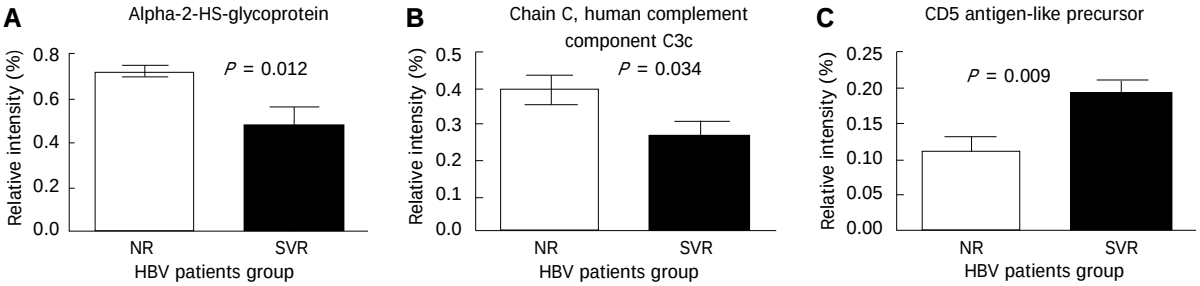

Figure 3 Histograms of the proteomic analysis at different levels. A: Alpha-2-HS-glycoprotein; B: Complement component C3c; C: CD5 antigen-like proteins in sustained responders and non-responders before chronic hepatitis B treatment. The intensity data are presented as mean ± SE (n = 19 gels from all patients and groups). SVR: Sustained virological response; NR: Non-responder.

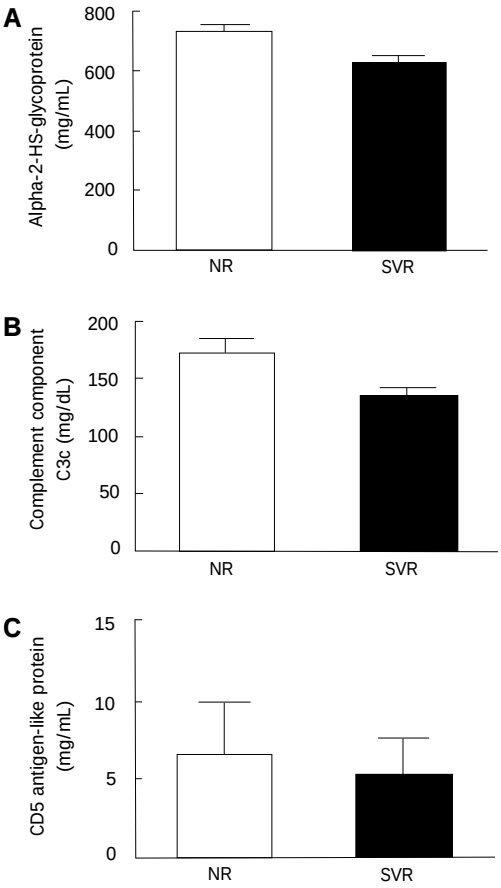

Figure 4 Validation of proteins associated with the immune response. A: Validation by enzyme-linked immunosorbent assay showed that there were elevated levels of alpha-HS-glycoprotein in non-responders before chronic hepatitis B treatment. n = 19 gels from all patients and groups; B: Validation by immunonephelometry showed elevated levels of complement component C3c in non-responders before chronic hepatitis B treatment; C: Validation by immunonephelometry showed elevated levels of CD5 antigen-like proteins in non-responders before chronic hepatitis B treatment. SVR: Sustained virological response; NR: Non-responder.

levels of albumin and alpha-2-HS-glycoprotein decreased 24 wk post-treatment.

DISCUSSION

The proteomic approach is usually used to analyze protein expression. It can be used with various specimens such as tissue, serum, plasma or body fluids. For this study, serum samples were used to identify potential biomarkers that can be further applied to predict the outcome of CHB therapy. Serum was selected because the collection process is non-invasive and proteins from the liver are secreted into the serum. Therefore, serum is an ideal specimen to screen for new proteins or biomarkers. In addition, serum proteomics can be used to detect post-translational modified proteins. 2-DE was used to separate and identify the proteins between 10-200 kDa. The high sensitivity and high throughput of mass spectrometry has resulted in the detection of several new biomarkers in ovarian cancer, prostate cancer, breast cancer and hepatocellular carcinoma. However, it should be noted that 2-DE does have limitations. 2-DE cannot detect low abundant proteins because high abundant proteins such as albumin and IgG can suppress the detection of low abundant proteins. To overcome this obstacle, albumin and IgG were removed from the serum samples before electrophoresis using the ProteoPrep Blue Albumin Depletion Kit. Albumin (-45 mg/mL) and IgG (-10 mg/mL) are the two major protein components of serum, representing 60%-70% and 10%-20% of the total serum protein, respectively[22]. When the high abundant

Table 3 Baseline characteristics of the patients in the validation phase and at 24 wk of treatment (mean ± SE)

	Sustained virological responders	Non-responders	*P* value
Validation phase	(n = 9)	(n = 14)	
Age (yr)	29.56 ± 2.78	37.71 ± 1.97	0.023
Sex (male:female)	7:2	11:3	0.966
ALT level (U/L)	106.88 ± 36.26	130.50 ± 30.93	0.644
HBV DNA (copies/mL)	(11.06 ± 2.84) × 10^6	(13.12 ± 2.45) × 10^6	0.593
HBeAg	Positive	Positive	NS
24 wk	(n = 3)	(n = 3)	
Age (yr)	29.33 ± 6.89	35.67 ± 6.06	NS
Sex (male:female)	Male	Male	NS
ALT level (U/L)	52.33 ± 13.78	253.67 ± 187.74	NS
HBV DNA (copies/mL)	(6.70 ± 6.65) × 10^6	(13.65 ± 6.35) × 10^6	NS
HBeAg	Positive	Positive	NS

HBV: Hepatitis B virus; ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen; NS: Not significant.

Table 4 Serum levels of proteins after 24 wk of treatment in the sustained virological responders and non-responders

Spot	Protein Name	NCBI ID	MW/pI	No. of match peptide	MS score	Fold change	Δ relative intensity	Biological function
Sustained virological response								
38	Proapolipoprotein	Gi|178775	28.94/5.50	63	798	↑ 5.17	0.1525[a]	Cholesterolmetabolism
26	Chain A, the structure of pentameric human serum amyloid P	Gi|576259	23.36/6.12	12	174	↑ 6.65	0.0783[a]	Acute phase protein
86	Alpha-2-HS-glycoprotein	Gi|112910	40.098/5.43	32	494	↑ 2.39	0.3773[a]	
309	Alpha-2-HS-glycoprotein	Gi|112910	40.09/5.43	29	507	↑ 2.04	0.4987[a]	
171	Chain A, the intact and cleaved Ⅲ complex as a model for serpin-proteinase interaction	Gi|999513	49.35/5.95	37	396	↑ 9.66	0.1481[b]	
173	Chain A, the intact and cleaved Ⅲ complex as a model for serpin-proteinase interaction	Gi|999513	49.35/5.95	26	210	↑ 8.01	0.0694[a]	Protease inhibitor
149	Serotransferin precursor	Gi|4557871	79.28/6.81	58	650	↑ 3.43	0.1165[a]	
201	PRO2619	Gi|11493459	58.51/5.96	36	278	↑ 4.95	0.0594[a]	Transport protein and proteinbinding
207	Albumin	Gi|332356380	68.48/5.73	48	781	↑ 6.97	0.1653[a]	
280	Albumin	Gi|332356380	68.48/5.73	31	201	↑ 7.33	0.1206[a]	
202	CD5 antigen-like precursor	Gi|5174411	39.60/5.28	11	70	↑ absent at baseline	0.0348[a]	
229	Ig J-chain	Gi|532598	16.04/4.62	3	42	↑ absent at baseline	0.0923[b]	Immunity protection
299	Immunoglobulin light	Gi|218783338	24.16/5.95	26	747	↑ absent at base line	0.1709[b]	
Non-responder								
20	Proapolipoprotein	Gi|178775	28.94/5.45	63	798	↑ absent at base line	0.2383[b]	Cholesterol metabolism
171	Chain A, the intact and cleaved Ⅲ complex as a model for serpine-proteinase interaction	Gi|999513	49.35/5.95	37	396	↑ 2.39	0.0896[a]	Acute phase protein
4	Albumin isoform	Gi|119626066	27.67/6.39	20	660	↓ 0.04	-0.2832[a]	Transport protein and protein binding
264	Serum albumin	Gi|62113341	71.09/5.85	21	115	↓ absent at 24 wk	-0.1196[a]	
123	Alpha-1-B-glycoprotein	Gi|69990	52.47/5.69	27	247	↑ 1.38	0.1610[a]	Serum protein
310	Alpha-2-HS-glycoprotein	Gi|112910	40.09/5.43	30	475	↓ 0.46	-0.4119[a]	Acute phase protein

Relative intensity, [(volume of spot/volume of all the spots in the gel) × 100]. [a]$P < 0.05$, [b]$P < 0.01$ *vs* relative intensity.

proteins were depleted from the serum, this allowed the low abundant protein spots to become visible. However, it is also possible to miss certain low abundant proteins when the high abundant proteins are removed[23]. The reason for this is that low abundant proteins sometimes bind themselves to high abundant proteins. Hence, only albumin and IgG were removed in order to prevent the loss of other important proteins[24]. As expected, in the untreated sample, albumin dominated the gel, obscuring signals from other less abundant proteins. When an equal quantity of protein was pre-treated with the ProteoPrep Blue Albumin Depletion Kit, the resolution of the 2D-gels significantly improved. This process cannot completely eliminate all albumin, but can eliminate enough to allow several protein spots to be clearly visible. Using these techniques, a total of seven protein spots were found to be differentially expressed in the serum of CHB patients, 9 sustained responders and 10 non-responders, before starting peginterferon alfa-2b treatment. Interestingly, four of the proteins were higher in the sustained responders. Of these proteins, only 3 were involved in immune responses: alpha-2-HS-glycoprotein, complement component C3c and CD5 antigen-like proteins. We further validated the proteomic results using the sensitive ELISA and nephelometry assay. In the validation phase of the study, the serum levels of alpha-2-HS-glycoprotein and complement component C3c were significantly higher in the non-responders when compared to the sustained responders. However, the level of CD5 antigen-like protein was not statistically different between the two groups. Since the band intensity of this protein was lower than the other proteins and the level was detected only in the validation phase of the study when the abundant proteins were not depleted, it is possible that the level of CD5 antigen-like protein may have altered when albumin and IgG were removed.

Based on these findings, the authors believe that alpha-2-HS-glycoprotein and complement component C3c are potential serum pre-treatment biomarkers in predicting sustained virological response in CHB patients treated with peginterferon alfa-2b. In this study, the levels of serum alpha-2-HS-glycoprotein were elevated in the non-responders before treatment initiation, whereas the levels were much lower in the sustained responders. In addition, we performed a subsequent serum proteomic analysis in a subset of samples at 24 wk after peginterferon alfa-2b treatment. Serum alpha-2-HS-glycoprotein was also up-regulated in the sustained virological responders, but downregulated in the non-responders. This finding is consistent with the results reported earlier by Ma *et al*[17]. However, a previous study only detected changes after treatment, but was not able to predict the treatment out-

come prior to treatment. The identification of biomarkers prior to treatment is preferable in this case to avoid systemic side effects due to interferon therapy.

Alpha-2-HS-glycoprotein is a high abundant protein produced by the liver and osteoblasts and is usually concentrated in the mineralized tissues. This protein belongs to the cystatin super family[25]. Several studies have reported various levels of alpha-2-HS-glycoprotein in patients with liver diseases[26-28]. Some have found low levels of serum alpha-2-HS-glycoprotein in patients with acute drug-induced hepatitis, alcoholic hepatitis, chronic autoimmune hepatitis, primary biliary cirrhosis, fatty liver and HCC[26,28]. Aside from its multiple functions and ability to affect metabolic diseases, tumor and sepsis[29,30], Dai *et al*[31] reported that it was an independent marker for liver injury and a prognostic marker for CHB. They suggested that the protein may decrease liver inflammation by inhibiting the release of inflammatory factors from activated peripheral blood mononuclear cells[31]. Similarly, Patel *et al*[32] found reduced levels of alpha-2-HS-glycoprotein in chronic hepatitis C patients identified as sustained virological responders before initiating treatment. The reduced levels of alpha-2-HS-glycoprotein in chronic hepatitis C[32] and B patients identified as sustained virological responders before treatment suggest that this protein may potentially be used as a biomarker in predicting the outcome of peginterferon alfa-2b treatment.

Another protein detected in this study was complement component C3c which is the degradation product of complement C3. This protein is important for both the acquired and innate immune systems, especially against microbial infection as it switches the cellular responses from cell death to opsonization[33]. 80%-90% of complement is produced by hepatocytes and is associated with the pathogenesis of many chronic human diseases such as autoimmune diseases, complement-mediated hemolytic anemia, vascular and liver diseases[33,34]. Interestingly, low levels of C3 fragments were detected in HBV and HCC patients compared to normal, healthy controls[11]. According to the results obtained from the screening and validation phases of this study, the expression and levels of complement component C3c were elevated in non-responders before treatment. Thus, complement component C3c may be a possible biomarker in predicting the outcome of peginterferon alfa-2b treatment in patients with CHB.

In conclusion, alpha-2-HS-glycoprotein and complement component C3c proteins were elevated in non-responders indicating that these proteins may be potential biomarkers in predicting the response to peginterferon alfa-2b treatment in patients with CHB prior to treatment. However, external validation is needed to assess the clinical applicability of these two proteins as predictors of anti-HBV treatment outcome.

COMMENTS

Background

The treatment duration, eradication and lack of drug resistance strains make type 1 interferons ideal for the treatment of chronic hepatitis B virus. Unfortunately, only 30% of patients will respond to treatment with interferon type 1. To date, there is no effective predictor of interferon responsiveness.

Research frontiers

The authors identified 2 potential serum biomarkers, alpha-2-HS-glycoprotein and complement component C3c, and determined that they can be used to predict treatment outcome in patients with chronic hepatitis B (CHB) receiving peginterferon alfa-2b.

Innovations and breakthroughs

Serum proteins serve as non-invasive biomarkers for several diseases. This is the first report of the potential use of common protein levels in the serum of CHB patients to predict responsiveness to peginterferon alfa before treatment. The identification of biomarkers prior to treatment is preferable in this case to avoid systemic side effects due to interferon therapy.

Applications

These 2 serum proteins can be added to the list of potential biomarkers to predict responsiveness to peginterferon alfa. However, these findings require further validated using a larger sample size and other independent studies.

Peer review

The authors examined the differential protein profile in serum of hepatitis B patients before treatment with peginteferon-2b. From the quantitative analysis of the 2D-gel and validation step using Enzyme-linked immunosorbent assay and immunonephelometry, serum levels of alpha-2-HS-glycoprotein and complement component C3c were elevated in the serum of the non-responders compared to the responders. This indicated that these 2 serum proteins may be potential serum biomarkers in predicting the treatment response in CHB patients on peginterferon alfa-2b therapy.

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ORIGINAL ARTICLE

Impact of mesocaval shunt on safe minimal liver remnant: Porcine model

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Abstract

AIM: To investigate the capacity of shunts to relieve portal hypertension and decrease the safe minimal liver remnant in pigs.

METHODS: A subtotal hepatectomy with < 60 mL blood loss and without hepatic pedicle occlusion was performed. The mesenteric venous inflow was diverted through a mesocaval shunt (MCS) constructed using the prepared left renal vein with an end-to-side running suture of 5-0 proline. All 21 animals that underwent subtotal hepatectomy and/or MCS were divided into three groups. In the 15% group, the residual volume was 14%-19% of total liver volume (TLV); in the 15%+ S group, the residual volume was also 14%-19% of TLV with a mesocaval shunt (MCS); and in the 10%+ S group, the residual volume was 8%-13% of TLV with an MCS. In the three groups, the intraoperative portal vein pressure (PVP) and portal vein flow (PVF) were monitored and compared at laparotomy and 1 h post-hepatectomy. The survival rate, sinusoidal endothelial damage, tissue analysis, and serum analysis were investigated among the three groups.

RESULTS: The percentage residual liver volume was 15.9%, 16.1% and 11.8% in the 15%, 15%+ S, 10%+ S groups, respectively. After hepatectomy, PVF and portal-to-arterial flow ratio in the 15%+ S group significantly decreased and hepatic artery flow (HAF) per unit volume significantly increased, compared to those in the 15% group. The PVP in the 15%+ S group and 10%+ S group increased slightly from that measured at laparotomy; however, in the 15% group, the PVP increased immediately and significantly above that observed in the other two groups. The 14-d survival rates were 28.5%, 85.6%, and 14.2% in the 15%, 15%+ S, and 10%+ S groups, respectively. In the 15%+ S group, the shunts effectively attenuated injury to the sinusoidal endothelium, and the changes in the serum and tissue analysis results were significantly reduced compared to those in the 15% and 10%+ S groups.

CONCLUSION: MCS can decompress the portal vein and so attenuate liver injury from hyperperfusion, and make extreme or marginal hepatectomy safer.

Key words: Hepatectomy; Safe minimal remnant volume; Mesocaval shunt; Pigs

Core tip: When the residual liver volume is extremely small after extended hepatectomy or living-donor liver transplantation, postoperative hepatic failure (PHF) or small-for-size syndrome (SFSS) may result from portal hypertension or hyperperfusion. We demonstrated that mesocaval shunt attenuated portal overflow injury, however, it is unknown how much the shunt can decrease, and whether the shunt can do the same for small liver remnants following subtotal hepatectomy. We showed that the residual volume was the determinant factor of

PHF or SFSS after subtotal hepatectomy, and the shunt attenuated injury from hyperperfusion, and made marginal hepatectomy safer.

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INTRODUCTION

Currently, there is no definitive answer to the question "How much liver excision is too much?"[1-4]. When the residual liver volume or graft is extremely small after extended hepatectomy or living-donor liver transplantation (LDLT), postoperative hepatic failure (PHF) or small-for-size syndrome (SFSS) may ensue, and portal hypertension or hyperperfusion is regarded as the determinant factor of liver failure or SFSS. It has been demonstrated the portal decompression, such as portacaval or mesocaval shunt (PCS/MCS), splenic artery ligation or splenectomy, can attenuate portal overflow injury, and result in smaller graft or liver remnant generated successfully in animal experiments and clinical studies[5-8]. However, it is unknown how much the shunt can decrease portal overflow, and whether the shunt can do the same for small liver remnants following subtotal hepatectomy[7]. Large-animal models provide a clinically relevant means of investigating the pathophysiology of a disease process that can be more readily applied in the human setting[9-11]. We investigated the capacity of MCS to relieve sinusoidal microcirculatory injury and to decrease the safe minimal liver remnant (MLR) value in massive hepatectomy.

MATERIALS AND METHODS

Animals and husbandry

Twenty-five male Bama miniature pigs (15-20 kg) were obtained from the Pig and Poultry Production Institute (Guangxi Province, China). The pigs were raised from a closed herd and kept under strict quarantine. The study was approved by the Chinese PLA General Hospital Clinic Committee on Ethics in Animal Experimentation. All animals in this study were treated humanely and in accordance with institutional and national guidelines for ethical animal experimentation.

Anesthesia

The pigs were food-deprived for 8 h before the operation. All pigs were anesthetized in the following way: initial sedation was obtained with a deep intramuscular injection of ketamine (15-20 mg/kg) and chlorpromazine (6-8 mg/kg) 15 min after atropine (0.01 mg/kg). Oxygen saturation and heart rate were monitored throughout the operation. A size 4 laryngeal mask airway was inserted, and anesthesia was maintained using 1.5% halothane in oxygen titrated to provide anesthesia. Central venous access was established with a tunneled catheter from the right femoral vein. Intraoperatively, 1 L of normal saline and 500 mL 5% dextrose were administered intravenously. No attempt was made to lower central venous pressure.

Surgical technique

An upper-midline incision with right or bilateral subcostal extensions (inverse "L" shape or Mercedes incision) was performed. A subtotal hepatectomy with < 60 mL blood loss and without hepatic pedicle occlusion was performed as previously described described[12-14]. A 16-gauge catheter was inserted into the main portal vein via the gastroduodenal vein to measure the portal vein pressure (PVP). Another catheter was advanced into the suprahepatic inferior vena cava (IVC) through one of the phrenic veins to monitor the pressure in the IVC. Ultrasonic flow probes were connected to a flow meter (TS420; Transonic Systems, Ithaca, NY, United States) to measure hepatic artery flow (HAF) and portal vein flow (PVF).

MCS

The mesenteric venous inflow was diverted through an MCS constructed using the prepared left renal vein with an end-to-side running suture of 5-0 proline (Qiangsheng, Shanghai, China), while the mesenteric vein was partly occluded (Figure 1). After the shunt, its patency was examined, and the size of shunt was adjusted to preserve PVF.

Postoperative management

After the operation, the pigs were monitored for 14 d: every 2 h in the first day and every 24 h thereafter, and one dose of 375 mg penicillin/375 mg streptomycin was given intramuscularly to all pigs. This dose was repeated daily every morning until euthanasia. They were given free access to water. Food and water intake and serum glucose levels were evaluated at each postoperative assessment, and animals that had limited or no intake per os and/or low serum glucose levels (< 70 mg/dL) were administered 50 g intravenous glucose (500 mL 10% glucose solution). Every dead or euthanized pig was necropsied to examine the patency of the shunt.

Experimental protocols

Twenty-five pigs were included and four were excluded for the obliteration of MCS or other surgery-related complications. Based on previous studies[13,14], the remaining 21 animals, which were submitted to massive hepatectomy with different liver mass removed, were divided into three groups: 15% group (n = 7), which was submitted to massive hepatectomy with a residual volume of approximately 15% (range 14%-19%, median: 15.9%) of TLV (Table 1); 15%+ S group (n = 7), which was subjected to MCS (Figure 1) and then massive hepatectomy with a residual volume of approximately 15% (range 14%-19%,

Table 1 Study characteristics and evolution of hemodynamic parameters

	15% group	15%+ S group	10%+ S group	[1]*P* value	[2]*P* value
Body weight (kg)	19.6 ± 2.9	18.4 ± 3.0	18.9 ± 3.9	NS	NS
Left-trilobes (g)	347.51 ± 18.2	334.6 ± 16.4	340.7 ± 17.2	NS	NS
ETL (g)	434.4 ± 22.5	418.1 ± 21.4	425.8 ± 21.2	NS	NS
WRL (g)	362.1 ± 17.3	355.0 ± 16.6	368.1 ± 16.9	-	-
ERL (g)	69.3 ± 4.5	67.8 ± 4.8	47.7 ± 3.1	-	-
Rate of RL (%)	15.9	16.1	11.8	-	-
PVF, mL/min per 100 g					
BAS	61.3 ± 7.1	62.9 ± 5.9	59.1 ± 4.3	NS	NS
PH	312.4 ± 24.1	215.4 ± 20.3	231.4 ± 31.2	0.001	NS
HAF, mL/min per 100 g					
BAS	21.1 ± 4.6	19.4 ± 4.5	18.6 ± 3.4	NS	NS
PH	8.3 ± 3.4	15.5 ± 4.1	14.1 ± 3.4	0.001	NS
P/A					
BAS	2.9 ± 0.3	3.2 ± 0.4	3.3 ± 0.3	NS	NS
PH	36.3 ± 4.1	14.1 ± 2.6	16.4 ± 3.6	0.000	NS

All flow values are reported in mL/min per 100 g hepatic tissue. Data expressed as mean ± SD. Estimated total liver weight = (weight of left trilobes) × 100/80. [1]Indicating difference between 15%+ S and 15% groups; [2]Indicating difference between 15%+ S and 10%+ S groups. ETL: Estimated total liver weight; RL: Residual liver volume; ERL: Estimated residual liver volume; WRL: Weight of resected liver; NS: Not significant; BAS: Baseline; PH: Post-hepatectomy; HAF: Hepatic artery flow; PVF: Portal vein flow; P/A: Portal-to-arterial flow ratio.

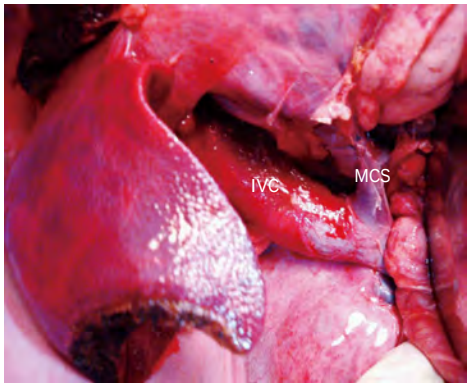

Figure 1 Photograph of the vascular anastomosis with the renal vein in the experimental group. IVC: Inferior vena cava; MCS: Mesocaval shunt.

median: 16.1%) of TLV; 10%+ S group (n = 7), with the same surgical procedure as the 15%+ S group, but with a residual volume of approximately 10% (range 8%-13%, median: 11.2%) of TLV (Table 1). In the 15%+ S group and 10%+ S group, there was a portal inflow of 3.0-3.5 times baseline per unit volume, which was regarded as a optimum flow for liver regeneration based on our previous study and other studies[15]. This was maintained through regulating the size of the MCS after hepatectomy. In the three groups, the intraoperative PVP and PVF were monitored and compared at laparotomy and 1 h post-hepatectomy (PH). The survival rate and tissue and serum analysis among the three groups were also investigated.

Blood and serum analysis

Blood sampling was performed preoperatively, and 1 h PH, then daily for 7 d or until death. During the follow-up period, levels of alanine aminotransferase (ALT) and total bilirubin (TB) and the international normalized ratio (INR) were determined. Hyaluronic acid (HA) is a polysaccharide synthesized by mesenchymal cells and eliminated chiefly by receptor-mediated endocytosis in the hepatic sinusoidal endothelium, and increased serum HA levels reflect sinusoidal endothelial damage[16]. HA was measured by a radiometric assay with the Pharmacia HA test (Yihua BioScience, Shanghai, China) in pre-reperfusion and post-reperfusion serum samples. The arterial ketone body ratio (acetoacetate/β-hydroxybutyrate, AKBR) is a useful tool for the estimation of liver functional reserve. Liver mitochondrial redox state (liver mitochondrial free NAD+/NADH ratio), which indicates hepatic energy charge, is known to reflect the ketone body ratio (acetoacetate/3-hydroxybutyrate) in liver tissue[15,17]. Ozawa *et al*[17] first demonstrated that the AKBR was correlated with the ketone body ratio in liver tissue, and it has been reported as a useful tool for the estimation of liver functional reserve in hepatic surgery. The AKBR was measured preoperatively and at 2 h and 48 h PH.

Tissue analysis

Hepatic tissue specimens were obtained from the edges of the liver at laparotomy and from the edges of the remnant liver at 2 h PH, and then divided into two sections. One was preserved in 10% formaldehyde for subsequent fixation in paraffin, and the other was immediately cut into 1-mm cubes and fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 mol/L sodium cacodylate-HCl buffer, pH 7.4) overnight at 4 ℃ prior to sectioning for transmission electron microscopy to study hepatocyte and sinusoidal ultrastructure. Platelet endothelial cell adhesion molecule-1 (CD31) helps maintain endothelial stability by interacting with other CD31 molecules at the extracellular border of adjacent cells. Sections of hepatic

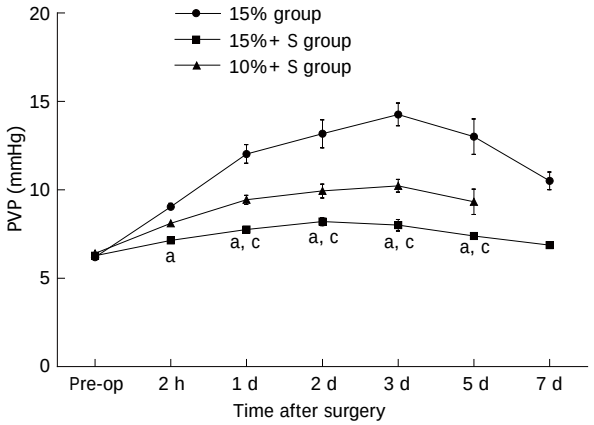

Figure 2 Serial changes in portal vein pressure in the three groups. There was a significant difference in changes in portal vein pressure (PVP) among the three groups. [a]*P* < 0.05 *vs* 15% group; [c]*P* < 0.05 *vs* 10%+ S group.

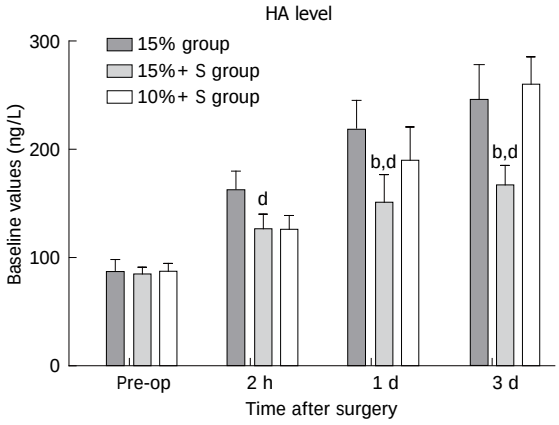

Figure 3 Baseline values of hyaluronic acid among the three groups were not significantly different, but in the 15%+ S group it was significantly decreased at 1 and 3 d post-hepatectomy compared to that in the 15% group. [b]*P* < 0.01 *vs* 10% group; [d]*P* < 0.01 *vs* 15% group. HA: Hyaluronic acid.

tissue were immunostained with porcine anti-CD31 antibody (Serotec, Oxford, United Kingdom) to evaluate the integrity of the endothelial cells in the hepatic sinusoid, as previously described[18].

Lipopolysaccharides and inflammation response

The lipopolysaccharides (LPS) level was quantitated by a limulus amebocyte lysate (LAL) assay based on the methods first introduced by Iwanaga *et al*[19] using the commercially available chromogenic LAL Endpoint Kit (Yihua BioScience, Shanghai, China) following the manufacturer's instructions. A calculated value of 0.1 EU/mL (10 pg/mL) was considered the threshold for LPS positivity. Standards and samples were analyzed in duplicate. Serum levels of tumor necrosis factor (TNF)-α and interleukin (IL)-6 were measured using commercial ELISA kits (Jingmei Biotech Co. Ltd., Shenzhen, China) following the manufacturer's instructions.

Statistical analysis

The survival rates in the three groups were calculated using a generalized Wilcoxon test. The biochemical results were compared by Student's *t* test, comparing mean values among the three groups. Parameters are presented as mean ± SD. Statistical significance was determined by Student's *t* test (SPSS, Chicago, IL, United States). $P < 0.05$ was regarded as significant.

RESULTS

Study characteristics and hemodynamic studies

The characteristics of the study and the evolution of hemodynamic parameters are shown in Table 1. The percentage RLV was 15.9%, 16.1%, and 11.8% in the 15%, 15%+ S, and 10%+ S groups, respectively. After hepatectomy, PVF and portal-to-arterial flow ratio in the 15%+ S group significantly decreased and HAF per unit volume significantly increased, compared to those in the 15% group.

PVP

Serial changes in PVP in the three groups are shown in Figure 2. PVP in the 15%+ S and 10%+ S groups increased slightly from that measured at laparotomy; however, in the 15% group, PVP increased immediately and significantly compared to that observed in the other two groups ($P < 0.05$ for all comparisons).

Hepatic endothelial cell injury

Changes in HA concentration are shown in Figure 3. Two hours after subtotal hepatectomy, serum HA concentration increased in all pigs. In the 15%+ S group, HA level was significantly reduced compared to that in the 15% group. At other time points, the values were significantly lower than those observed in the 15% and 10%+ S groups ($P < 0.01$). The histological changes in tissue samples taken at 1 h PH in the three groups are shown in Figure 4.

Hepatocellular injury

The serial measurements of serum ALT, TB, and INR are shown in Figure 5, in which significant differences were noted. There were significant differences between the 15% and 15%+ S groups, and between the 10%+ S and 15%+ S groups ($P < 0.05$ for two comparisons).

Survival rate

The animals were followed-up for 14 d. An observation period of 14 d was chosen because liver function recovered to normal within 14 d after major hepatectomy[20]. The survival rate was calculated by the Kaplan-Meier method. Survival in the 15%+ S group with a shunt was better than in the 15% and 10%+ S groups (85.7% *vs* 28.5% *vs* 14.3%, $P < 0.01$). In the 15% group without a shunt, all pigs survived for > 4 d, and only two pigs survived until 14 d. In the 10%+ S group, all pigs survived > 3 d, but only one pig survived until 14 d.

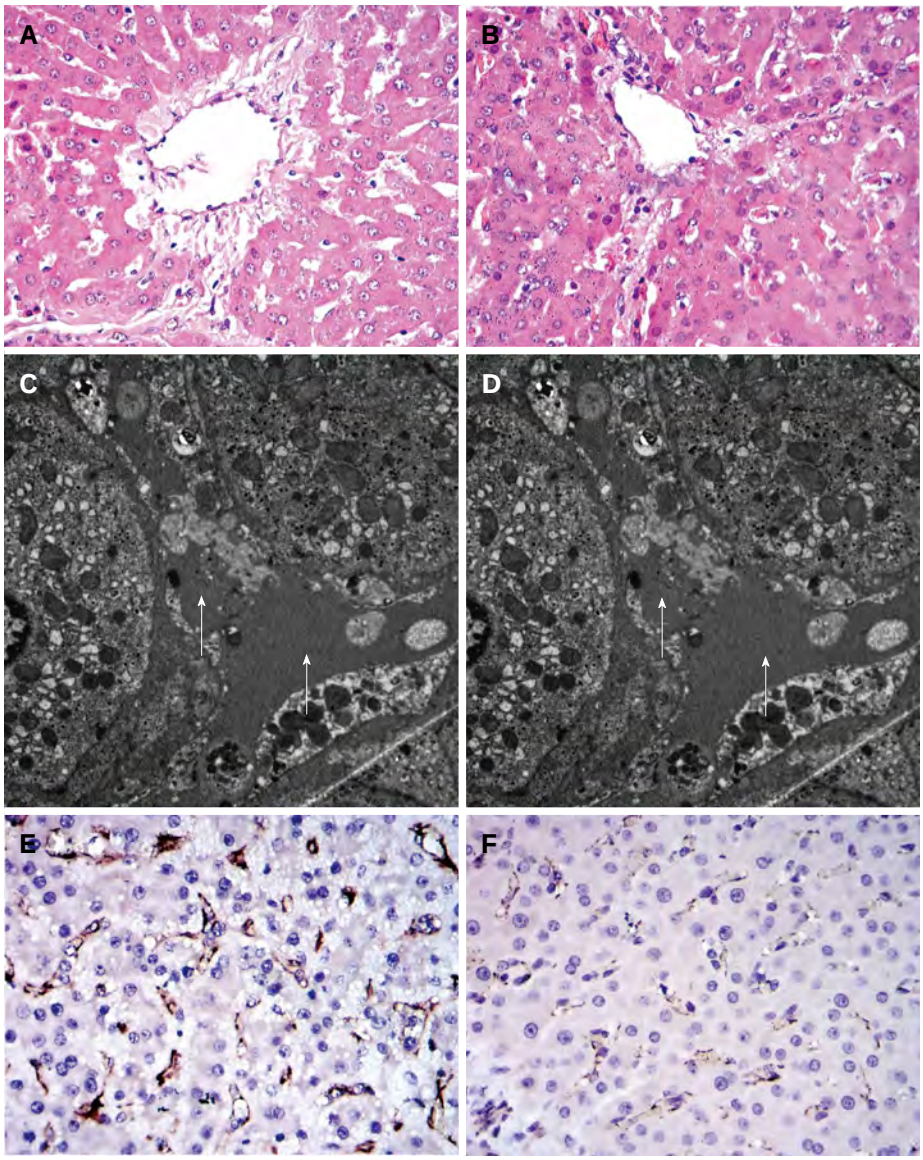

Figure 4 The histological changes in tissue samples were taken at 1 h post-hepatectomy in the three groups. Hematoxylin and eosin (magnification × 400); Transmission electron microscopy (magnification × 6000) and CD31 immunohistochemical staining in the three groups. In the 15% group, there was significant endothelial denudation, sinusoidal dilation, hydropic changes in hepatocytes, and hemorrhage into the perivenular connective tissue (A); the sinusoidal endothelial lining was slightly damaged and detached into the sinusoidal space, with enlargement of the Disse's spaces (C, arrow); CD31 immunostaining also revealed destruction of the endothelial lining (E); whereas in the 15%+ S or 10%+ S group, there was no intraparenchymal hemorrhage present (B); Transmission electron microscopy demonstrated the sinusoidal endothelial cells, and the structure of the endothelial lining can also be seen (arrow) (D); CD31 immunostaining also revealed mild sinusoidal microarchitecture injury (F).

DISCUSSION

In hepatectomy, when the RLV decreases below a certain threshold, the liver vascular bed immediately decreases, and vascular resistance in the residual liver increases. This leads to portal hypertension or hyperperfusion and a steady decrease in liver function; the liver remnant cannot sustain metabolic, synthetic, and detoxifying functions; and SFSS or PLF ensues[1,3]. The portal hypertension or hyperperfusion is regarded as the determinant factor of liver failure or SFSS. Portal decompression, such as PCS/MCS, splenic artery ligation or splenectomy, is often used to improve the prognosis of an SFSS graft in LDLT when the graft-to-recipient weight ratio is < 0.8% or the graft volume/standard liver volume (GV/SLV) is < 30%[5-7,21-23]. PCS/MCS could make a GV/SLV < 30%, or as low as 20%-25% a viable option with a fair prognosis[24-26], indicating shunts can make small grafts successfully regenerate or make them safe. However, it is unknown how much the shunt can decrease portal pressure, and whether the shunt can do the same for small liver remnants following subtotal hepatectomy as it does in LDLT[7].

In our previous study, we showed that the survival rate in pigs with approximately 15% residual liver volume was 24.8%, which was similar to the present study; whereas it was 100% in pigs with approximately 20% RLV. We established that the safe MLR should be >

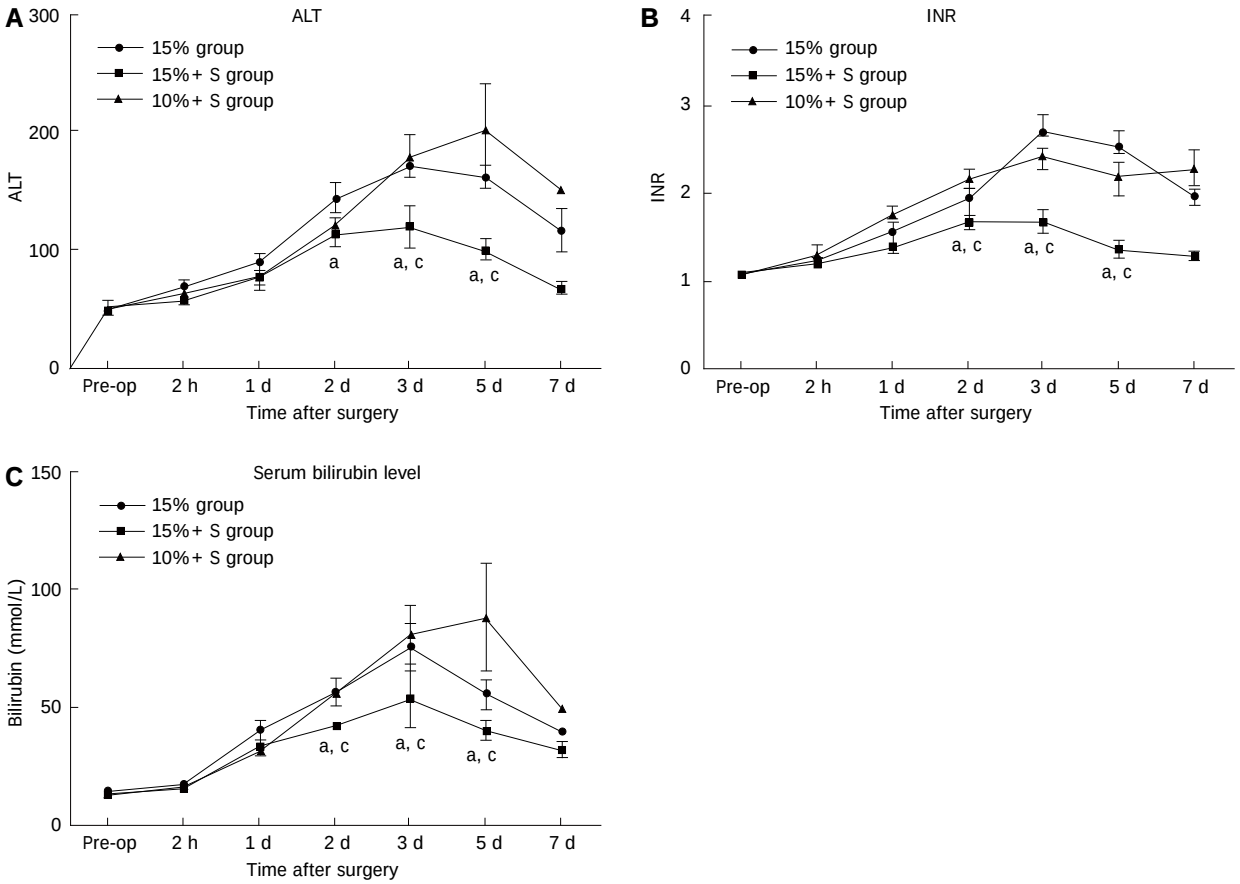

Figure 5 Changes in serum alanine aminotransferase and bilirubin level, and international normalized ratio in the three groups. A: Serum alanine aminotransferase (ALT); B: International normalized ratio (INR); C: Serum bilirubin level. [a]*P* < 0.05 *vs* 15% group; [c]*P* < 0.05 *vs* 10%+ S group.

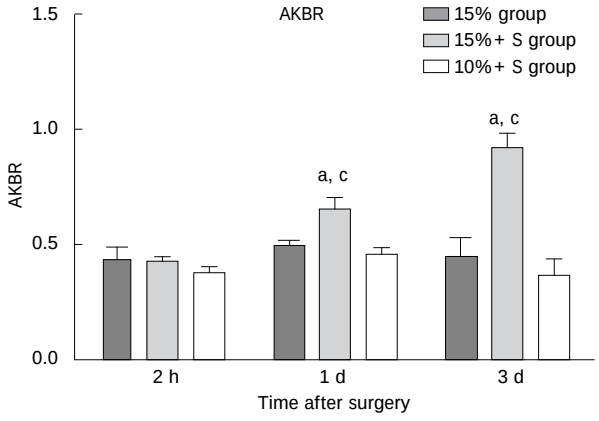

Figure 6 Changes in the arterial ketone body ratio level in the three groups. [a]*P* < 0.05 *vs* 15% group; [c]*P* < 0.05 *vs* 10%+ S group. AKBR: Arterial ketone body ratio.

15% of TLV in a porcine model. However, in the present study, we found that MCS could decrease the degree of sinusoidal injury, protect liver function, regenerate liver with approximately 15% RLV, and increase the survival rate up to 85.7%. Nonetheless, none of the pigs with 10% RLV in the 10%+ S group could sustain metabolism and failed to regenerate even though portal decompression was performed. These data also indicate that portal decompression can make extreme liver resection or marginal size liver remnant (approximately 15% of TLV) safe, but it cannot make LRV < 5% (10% of TLV) viable.

In the normal state, PVF and HAF are linked by the hepatic artery buffer response[27,28], which induces a decrease in hepatic artery diameter and flow if PVF increases, and is synonymous with liver microcirculation failure. In the present study, it also showed the HAF in the 15% group was significantly decreased (Table 1), and this insufficient HAF might be another important contributor to the failure of liver remnant regeneration. However, the MCS prevented injury from excess PVF and significantly decreased PVF, resulting in a significant increase in HAF in the 15%+ S group; successfully regenerated liver in animals with approximately 15% RLV; but it could not regenerate 10% RLV. This is probably due to the liver remnant being too small to sustain the metabolic, synthetic and detoxifying functions, despite the presence of sufficient arterial flow. AKBR is a predictor of liver viability and responds to disorders of energy metabolism in the mitochondria[16]. The present study also demonstrated that there was no significant difference among the three groups at 2 h PH. At other time points, the 15%+ S group showed significant differences from the 15% and 10%+ S groups ($P < 0.05$),

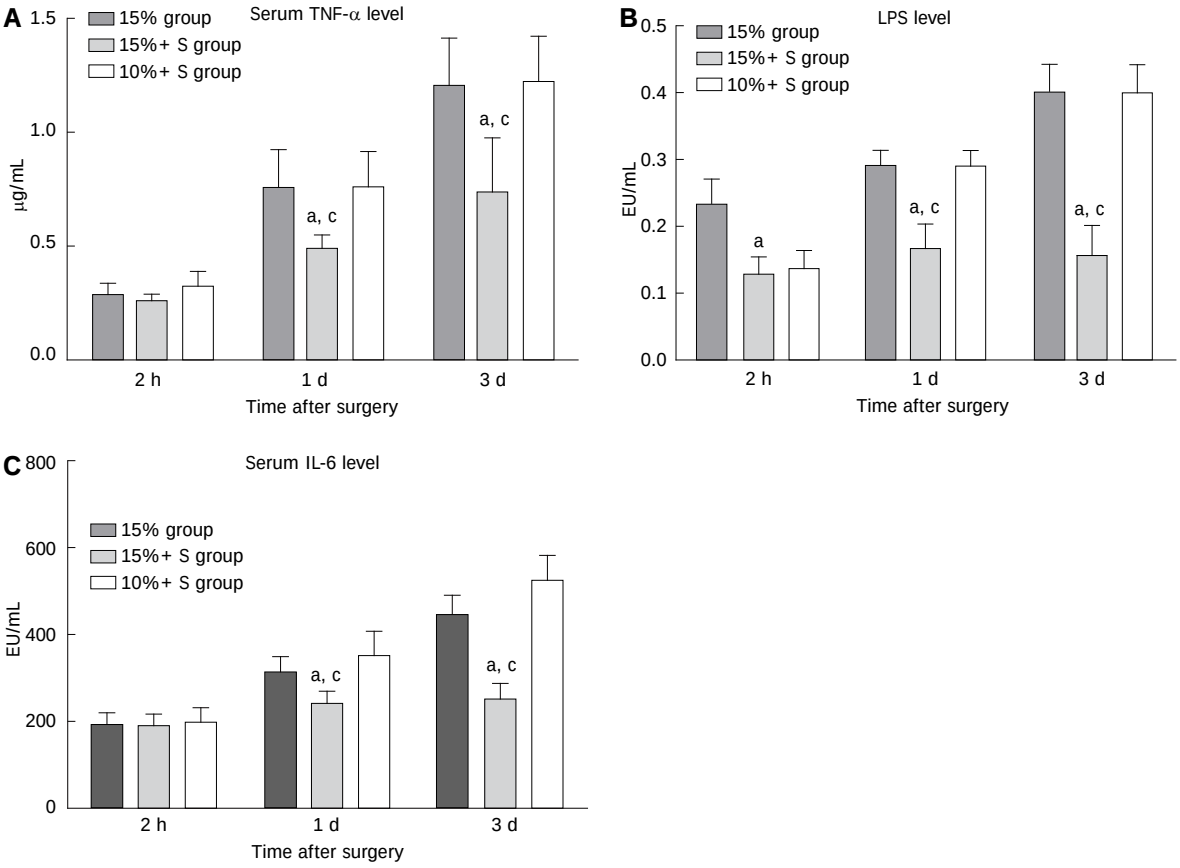

Figure 7 Serial changes in the serum lipopolysaccharides, tumor necrosis factor-α, and interleukin-6 level in three groups, in which significant differences were noted. A: Serum level of tumor necrosis factor-α (TNF-α); B: Serum level of lipopolysaccharides (LPS); C: Serum level of interleukin-6 (IL-6). [a]*P* < 0.05 *vs* 15% group; [c]*P* < 0.05 *vs* 10%+ S group.

indicating the optimum portal inflow and safe MLR in the 15%+ S group were important for recovery of liver energy metabolism (Figure 6).

However, MCS was a "double-edged sword". Excessive diversion of portal flow results in a portal pressure that is insufficient to promote liver regeneration[24,25,29,30]. It is well known that vascular shear stress in the portal vein is a major determinant factor of regeneration[25]. Therefore, diversion by MCS should be controlled. Hessheimer *et al*[29] demonstrated twice-baseline portal inflow was necessary for the functional recovery of a small-for-size liver graft. In the present study, the portal flow was preserved at approximately 3.2 times baseline to avoid portal hypoperfusion and benefit liver remnant regeneration. The portal flow was similar to that in 70% hepatectomy model which was supposed to be the optimum portal flow for liver regeneration[31]. It was also indentified that a portal inflow 3.2 times the baseline value can greatly stimulate the regeneration of the liver remnant without causing hyperperfusion injury.

In addition, the hepatic parenchyma contains an abundance of reticuloendothelial cells; after subtotal resection, the reticuloendothelial function declines, and portal hyperperfusion further promotes endotoxin absorption and bacterial translocation. Bacterial infection and bacteremia are serious complications that are frequently encountered in patients with subtotal hepatectomy. In this study, severe endotoxin or bacterial translocation in the 15% group was significantly elevated compared to the 15%+ S group, and serum TNF-α or IL-1 level was significantly elevated (Figure 7), also indicating that optimum portal decompression relieves portal overflow injury, and decreases the endotoxin/bacterial translocation[32], which play important roles in delaying liver remnant regeneration.

In summary, the decompression of portal vein can decrease the hyper-reperfusion injury, and make the marginal size hepatectomy safer. Therefore, the portal decompression modality should be considered when the risk of PHF or SFSS in hepatectomy is high or one-stage resection is adopted for the small future residual liver volume, for which portal venous embolism or two-stage resection is usually adopted.

COMMENTS

Background

Currently, there is no definitive answer to the question "How much liver excision is too much?". When the residual liver volume or graft is extremely small after extended hepatectomy or living-donor liver transplantation, postoperative hepatic failure (PHF) or small-for-size syndrome (SFSS) may ensue, and portal hypertension or hyperperfusion is regarded as the determinant factor of liver failure or SFSS.

Research frontiers

The authors demonstrated that mesocaval shunt could attenuate portal overflow injury, however, it is unknown how much the shunt can decrease, and whether the shunt can do the same for small liver remnants following subtotal hepatectomy.

Innovations and breakthroughs

The authors showed that portal vein decompression decreased hyper-reperfusion injury, and made "marginal size" hepatectomy safer, but did not reduce the safe value of the minimal residual volume (MRV) to < 5% of TLV.

Applications

The portal decompression modality should be considered when the risk of PHF or SFSS in hepatectomy is high, or one-stage resection is adopted for small future residual liver volume, in which portal venous embolism or two-stage resection is usually adopted.

Peer review

This study demonstrated that portal vein decompression decreased hyper-reperfusion injury, and made marginal size hepatectomy safer, but did not reduce the safe value of the MRV to < 5% of TLV. Therefore, the portal decompression modality should be considered when the risk of PHF or SFSS in hepatectomy is high, or one-stage resection is adopted for small future remnant liver volume.

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ORIGINAL ARTICLE

Effects of radix curcumae-derived diterpenoid C on *Helicobacter pylori*-induced inflammation and nuclear factor kappa B signal pathways

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Abstract

AIM: To study effect of diterpenoid C extracted from radix curcumae on *Helicobacter pylori* (*H. pylori*)-infected inflammation, intestinal metaplasia, and nuclear factor kappa B (NF-κB) signaling pathway *in vitro*.

METHODS: We used I-type *H. pylori* to infect human gastric epithelial gastric epithelium cell line (GES-1) cell lines, and then *H. pylori*-infected GES-1 cells were treated with radix curcumae (RC)-derived diterpenoid C of different concentrations (5, 10, 20 μg/mL) and amoxicillin. The expression of p65, IκB kinase (IKK) α and IKKγ proteins was detected with Western blotting, and the expression of interleukin (IL)-8, IL-6 and IL-4 was determined with enzyme-linked immunosorbent assay method. Data were analyzed using SPSS software ver18.0. For comparisons between groups of more than two unpaired values, one-way analysis of variance (ANOVA) was used. If an ANOVA *F* value was significant, *post hoc* comparisons were performed between groups. If results were not normally distributed, the Mann-Whitney *U* test was used to compare two groups of unpaired values, whereas for comparisons between groups of more than two unpaired values, the Kruskal-Wallis *H* test was used. Statistical significance was established at $P < 0.05$.

RESULTS: The MTT assay results revealed the inhibited rate of GES-1, and indicated that the IC5 of RC-derived diterpenoid C and amoxicillin all were 5 μg/mL for gastric GES-1 cells. The expression of IL-8 was significantly increased, especially at 12 h time point; and the expression of IL-4 was decreased in *H. pylori*-infected GES-1 cells. After *H. pylori*-infected GES-1 cells were treated with RC-derived diterpenoid C of different concentrations and amoxicillin, the expression of IL-8 was decreased at 12, 24, 48, 72 h points ($P < 0.01$), especially in high-concentration diterpenoid C (20 μg/mL) group; and the expression of IL-4 was increased, especially in moderate and high-concentration diterpenoid C (10 and 20 μg/mL) groups. RC-derived diterpenoid C had the inhibitory effects on *H. pylori*-induced p65 translocation from cytoplasm into cell nucleus, *H. pylori*-stimulant IkBα degradation, the phosphorylation of p65 and IkBα, and the expression of IKKα and IKKβ proteins.

CONCLUSION: RC-derived diterpenoid C can block NF-κB signal pathway, effectively reducing the secretion of *H. pylori*-induced proinflammatory cytokine and increasing the secretion of anti-inflammatory cytokine.

Key words: Radix curcumae-derived diterpenoid C; *Helicobacter pylori*; Nuclear factor-κB; Inflammatory cytokine

Core tip: Radix curcumae (RC), a common Chinese crude drug, has a wide range of pharmacological activity including hypolipidemic effect, hepatoprotective effect, anti-tumor, anti-radiation and anti-anaphylaxis. RC-derived diterpenoid C is recently obtained from RC ether extract by us, and its chemical properties and constitution are different from curcumin and β-elemene. Our results showed that RC-derived diterpenoid C can block nuclear factor kappa B signal pathway, effectively reducing the secretion of *Helicobacter pylori*-induced proinflammatory cytokine and increasing the secretion of anti-inflammatory cytokine. RC-derived diterpenoid C may become an effective drug for treatment of chronic gastritis.

Huang X, Lv B, Zhang S, Dai Q, Chen BB, Meng LN. Effects of radix curcumae-derived diterpenoid C on *Helicobacter pylori*-induced inflammation and nuclear factor kappa B signal pathways. *World J Gastroenterol* 2013; 19(31): 5085-5093 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i31/5085.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i31.5085

INTRODUCTION

Gastric carcinogenesis is usually believed to undergo the process including *Helicobacter pylori* (*H. pylori*) infection, chronic gastritis, atrophy, intestinal metaplasia, atypical hyperplasia abd gastric cancer[1]. *H. pylori* infection can bring to inflammation continuing through activating nuclear factor kappa B (NF-κB) signal pathway[2]. As *H. pylori* drug resistance becomes strong, it is difficult to eradicate *H. pylori*. How early to block the progression of chronic gastritis and to reduce gastric carcinogenesis is a main problem for us[3]. At present, there are no effective drugs for treatment of chronic gastritis. Our previous review has indicated that the total effective rate and pathological improvement (atrophy and intestinal metaplasia) are better in Chinese medicine group than in Western medicine group in the treatment of chronic gastritis[4]. But the mechanism of Chinese medicine is still unclear.

Radix curcumae (RC), a common Chinese crude drug, has a wide range of pharmacological activity including hypolipidemic effect, hepatoprotective effect, anti-tumor, anti-radiation and anti-anaphylaxis. RC-derived diterpenoid C is recently obtained from RC ether extract by us, and its chemical properties and constitution are different from curcumin and β-elemene. Our previous experiments have shown that RC-derived diterpenoid C has better anti-tumor activity and RC-derived diterpenoid C of high concentration can induce apoptosis[5,6]. Inflammation is strongly associated with tumor and the activation of some signal pathways occur in both inflammation and tumor[7,8], so we investigated the role of RC-derived diterpenoid C in anti-inflammation. Since biological properties are similar in gastric epithelium cell line (GES-1) cells and normal gastric epithelial cells, GES-1 cells were used in this study. The purpose of this study was to observe the effects of RC-derived diterpenoid C on inflammation, intestinal metaplasia and the expression of NF-κB signal pathway-related proteins in *H. pylori*-treated GES-1 cells.

MATERIALS AND METHODS

Materials

H. pylori strain, (CagA$^+$, VacA$^+$) NCTCl 1637 consistent with international standards, was purchased from China Disease Control and Prevention Center (Beijing, China). Human gastric epithelial GES-1 cells were purchased from the Institute of Cancer Research, Peiking University. RC-derived diterpenoid C (molecular weight: 380; molecular formula: C22H36O5) was provided by the College of Pharmacy, Zhejiang University (Hangzhou, China). Amoxicillin (molecular weight: 365.4) dispersible tablets with the batch number 63-110604 were from Xiansheng (Nanjing, China). Enzyme-linked immunosorbent assay (ELISA) kits was purchased from Nanjing KeyGey Biotech Co., Ltd. Primary antibodies were used. Horseradish peroxidasecoupled secondary antibodies were bought from Promega (Promega). The protein bands were detected employing electrochemi-luminescence chemiluminescence (Thermo Scientific).

Preparation of RC-derived diterpenoid C

Extraction of RC-derived diterpenoid C: RC-dried rhizome (10 kg) was used in extraction with 80 L of 95% ethanol, which was repeated four times to obtain 247 g of crude extract. After dispersion with 500 mL of water, the crude extract was respectively extracted with 500 mL of petroleum ether, dichloromethane and n-butanol to obtain 95.1 g of methylene bichloride. The methylene bichloride underwent silice gel column chromatography with petroleum ether/acetic ether (100:0, 100:10, 100:20, 100:30, 100:40, 100:50, 100:60, 100:70, 100:80 and 100:90), respectively, to obtain fractions A-J. The fraction E underwent chromatograph with acetonitrile/water (7:3) for 0-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70 and 70-80 min, respectively, to obtain subfractions E1-E8. The subfraction E8 underwent RP-HPLC with acetonitrile/water (45:55) as eluant to obtain diterpenoid C (5.0 mg, tR: 43.7 min). Its molecular structure was shown in Figure 1.

Preparation of diterpenoid C of different concentrations: RC-derived diterpenoid C was made into 10 mg/mL of stock solution with dimethyl sulfoxide (DMSO), and then stored at -20 ℃. The stock solution was diluted with fetal calf serum-free Dulbecco's Modification of Eagle's Medium (DMEM) containing high glucose for use in the experiment. DMSO concentration was controlled at 0.1% (volume percentage).

Cell culture

The tube containing frozen cells was placed in 37 ℃

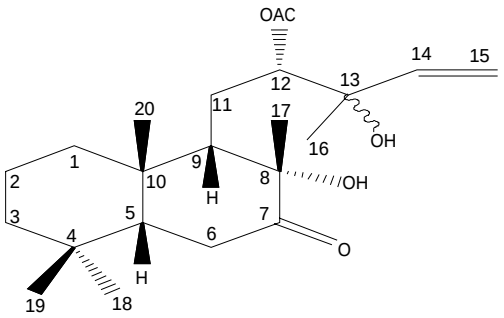

Figure 1 The molecular structure of radix curcumae-derived diterpenoid C. Originated from Huang *et al*[9], with permission.

water bath with constant shaking, and the frozen cells were melted within one minute. The tube was sterilized with 75% alcohol, and then quickly placed on a sterile bench for operation. After the tube was opened, cells were placed in high glucose-DMEM containing 10% fetal calf serum for incubation at 37 ℃ in an atmosphere of 5% CO_2. Next day, the medium was changed. When cells reached 80% confluence, cells were digested with 0.25% trypsin for passage. One passage was performed every 2-3 d and the cells after passage 3 were used in this experiment.

Preparation of viable H. pylori suspensions

NCTCI 1637 was incubated in Bushi-modified selective plating medium containing 10% yolk, 10% fetal calf serum, soluble amylum, vancomycin, trimethoprim, amphotericin and polymyxin B at 37 ℃ in an atmosphere of 85% nitrogen, 5% oxygen and 10% CO_2 for 3 d for future use. *H. pylori* was placed in 0.01 mol/L of PBS followed by quantitation with 752 type-spectrophotometer, and then diluted to 3.2×10^4-2.0×10^7 CFU/mL with RPMI1640 containing 2% fetal calf serum. The assays of Gram's stain, urease, katalase and oxidase were performed to confirm the presence of *H. pylori* before application.

Cell infection and intervention

Gastric epithelial GES-1 cells were cultured in an incubator containing antibiotics-free RPMI1640 with 10% fetal calf serum. Gastric epithelial GES-1 cells in logarithmic phase were digested with 0.25% trypsin for counting, and then were seeded in 96-well plate at 5×10^4/mL-1×10^5/mL. When cells reached 80% confluence, *H. pylori*-negative control group without *H. pylori* was set. After adherence of viable *H. pylori* suspensions, *H. pylori*/GES-1 cells (200:1) were incubated at 37 ℃ in an atmosphere of 5% CO_2 for 2 h, and then RC-derived diterpenoid C of different concentrations were added to incubate for 12, 24, 48 and 72 h, respectively, followed by observation on cell morphology under an electron microscopy. Three wells were set for each group. There were 3 RC-derived diterpenoid C groups with different concentrations, negative control group with 100 μL of RPMI1640 containing GES-1 cells, model group with *H. pylori* and positive control group with amoxicillin.

Inhibitory effects of RC-derived diterpenoid C and amoxicillin on GES-1 cell proliferation (MTT assay)

After GES-1 cells were incubated for 24 h, RC-derived diterpenoid C and amoxicillin (0, 5, 10, 20, 40, 80 ng/mL) were added for 24 h-culture. Three wells were set for each group. MTT (20 μL, 5 mg/mL) was added in each well for 3 h-incubation, and then the supernatant was taken followed by addition of 150 μL of DMSO. At the same time, the blank control group without RC-derived diterpenoid C and amoxicillin was set. Absorbance values were measured with a microplate reader (490 nm) for calculating inhibition rates. The inhibitory concentration 5% (IC_5) was adopted in the following experiments, and inhibitory rate (IR) was calculated as follows: IR = (A of control group - A of experimental group/A of control group) × 100%.

Cell morphology

The status of cell growth was observed under an optical microscope after GES-1 cells were incubated for 12, 24, 48 and 72 h, respectively.

Levels of IL-8 and IL-4 in cell supernatant determined with ELISA

We detect the level of IL-8 and IL-4 with ELISA methods according to the manufacturer's instructions.

Effects of RC-derived diterpenoid C on NF-κB signal pathways in H. pylori-induced GES-1 cell inflammation (Western blotting)

The effects of RC-derived diterpenoid C on the nuclear localization of NF-κB p65 were analyzed with Western blotting. Cells were divided into blank control group, model (*H. pylori*) group in which cells were treated for 60 min, and RC-derived diterpenoid C (20 μg/mL) + *H. pylori* group in which cells were first treated with RC-derived diterpenoid C for 2 h, and then infected with *H. pylori*. After nuclear proteins and cytoplasmic proteins were extracted, p65 protein in them was respectively determined. The effects of RC-derived diterpenoid C on the key proteins of NF-κB signal pathways were analyzed.

Statistical analysis

The experiments were repeated three times independently. Data were presented as the mean ± SD. Data were analyzed using SPSS software ver18.0. If the results were distributed normally, the two independent samples t test was used for comparison. For comparisons between groups of more than two unpaired values, one-way analysis of variance (ANOVA) was used. If an ANOVA F value was significant, *post hoc* comparisons were performed between groups. If results were not normally distributed, the Mann-Whitney U test was used to compare two groups of unpaired values, whereas for comparisons between groups of more than two unpaired values, the Kruskal-Wallis H test was used. Statistical significance was established at $P < 0.05$.

Table 1 Inhibition rates of radix curcumae-derived diterpenoid C on human gastric gastric epithelium cell line cell proliferation (n = 3)

Drug level (μg/mL)	Action time		
	24 h	48 h	72 h
Radix curcumae-derived diterpenoid C			
0 (negative control)	-	-	-
5	4.320% ± 0.056%	5.695% ± 0.657%	9.043% ± 0.121%
10	8.409% ± 0.879%	11.734% ± 0.547%	20.512% ± 1.098%
20	10.537% ± 1.098%	19.96% ± 2.093%	29.841% ± 2.345%
40	13.273% ± 0.897%	28.473% ± 5.093%	45.723% ± 5.876%
80	15.805% ± 0.975%	65.056% ± 6.098%	79.527% ± 6.879%
Amoxicillin			
0 (negative control)	-	-	-
5	6.671% ± 0.987%	7.935% ± 0.567%	10.769% ± 1.087%
10	8.325% ± 0.765%	14.769% ± 0.897%	19.130% ± 1.098%
20	9.731% ± 0.345%	18.530% ± 1.876%	29.154% ± 1.543%
40	12.929% ± 1.098%	25.691% ± 1.786%	31.832% ± 1.346%
80	14.953% ± 1.876%	38.427% ± 2.765%	43.790% ± 2.983%

RESULTS

Effects of RC-derived diterpenoid C and amoxicillin on GES-1 cell proliferation

As shown in Table 1 and Figure 1, RC-derived diterpenoid C and amoxicillin inhibited human gastric GES-1 cell proliferation in time and dose-dependent manners, namely that with the increase in drug concentration and the extension in drug action time, the inhibition rate was increased. The maximum un-cytotoxic concentration (IC_5) was 5 μg/mL. We adopted 5, 10, 20 μg/mL of RC-derived diterpenoid C as low, moderate and high-concentration diterpenoid C groups, and 5 μg/mL of moxicillin as drug-intervention group in the following experiments. The highest inhibition rate was 79.527% ± 6.879% obtained by 80 μg/mL of diterpenoid C with 72 h action time.

Effects of RC-derived diterpenoid C on human gastric GES-1 cell morphology

In bland group, GES-1 cells were polygon-shaped or spindle-shape with pseudopodia and island-like growth. Cells gradually were adherent. With prolonged incubation time, the number and density of cells were increased with a few floating cells (Figure 2A). In the GES-1 cells treated with *H. pylori* for 12 (Figure 2B), 24 (Figure 2C), 48 (Figure 2D) and 72 h (Figure 2E), cells became round; adherent cells were decreased and floating cells were increased; fragments occurred around cells; cell junction was reduced; the boundaries between cell nucleus and cytoplasm were obscure, and nucleus-cytoplasm fusion was seen. In the GES-1 cells treated with RC-derived diterpenoid C (5, 10, 20 μg/mL), adherent cells increased and cell morphology gradually recovered at 24 h (Figure 2F-I, respectively). Amoxicillin had no marked effects on cell morphology.

Effects of RC-derived diterpenoid C on H. pylori-induced human gastric GES-1 cell inflammation

Effects of RC-derived diterpenoid C on the secretion of IL-8: As shown in Figure 3A, after human gastric GES-1 cells were infected with *H. pylori*, IL-8 in the supernatant was significantly increased, especially at 12 h time point. With prolonged time, IL-8 level was gradually decreased. There were statistical differences in IL-8 levels at 12, 24, 48 and 72 h time points (all P = 0.000). After human gastric GES-1 cells were treated with diterpenoid C of different concentrations and amoxicillin, compared with model group, IL-8 level at each time point was significantly decreased with statistical significance.

Effects of RC-derived diterpenoid C on the secretion of IL-4: As shown in Figure 3B, after human gastric GES-1 cells were infected with *H. pylori*, IL-4 in the supernatant was significantly decreased with statistical differences compared with that at each time point of blank control group. After human gastric GES-1 cells were treated with diterpenoid C of low concentration, IL-4 level at each time point was increased, but P values at 12, 24, 48 and 72 h time points were 0.472, 0.550, 0.446 and 0.067, respectively, without statistical differences. After human gastric GES-1 cells were treated with diterpenoid C of moderate and high concentrations, IL-4 level at each time points was increased with statistical differences. After human gastric GES-1 cells were treated with amoxicillin, IL-4 level at each time point was increased, but their P values at 12, 24, 48 and 72 h time points were 0.092, 0.245, 0.446 and 0.053, respectively, without statistical differences. The results above suggest that the diterpenoid C of moderate and high concentrations can promote GES-1 cells to secrete IL-4, while amoxicillin has no the similar effect.

Effects of RC-derived diterpenoid C on NF-κB signal pathway activated by H. pylori in human gastric GES-1 cells

Nucleic localization of NF-κB p65: Our results indicated that 60 min after *H. pylori* infected human gastric GES-1 cells, p65 expression was increased in cell nucleus, but decreased in cytoplasm, suggesting that *H. pylori* can allow p65 translocation from cytoplasm to cell nucleus. In blank control group, there was a lot of p65 expression in cytoplasm. In high-concentration group of RC-derived diterpenoid C, p65 translocation was reduced, demonstrating that RC-derived diterpenoid C can inhibit p65 translocation from cytoplasm into cell nucleus induced by *H. pylori* (Figure 4).

Effects of RC-derived diterpenoid C on IkBα degradation caused by H. pylori

After GES-1 cells were respectively treated with *H. pylori* for 0, 15, 30, 60 and 90 min, cytoplasm was isolated to be used for determination of IkBα degradation with

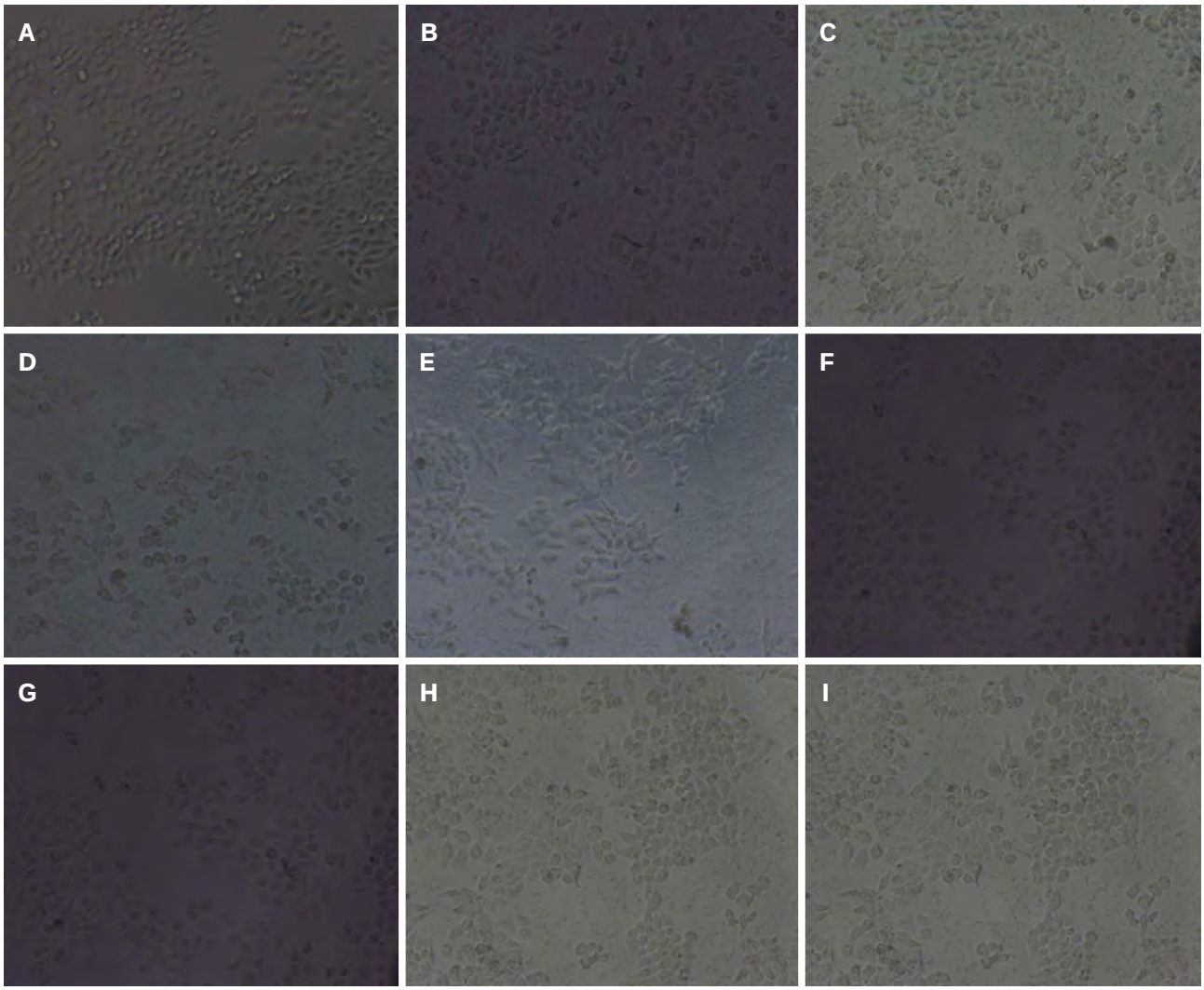

Figure 2 Gastric epithelium cell line cell morphology (× 200). In bland group, gastric epithelium cell line (GES-1) cells were polygon-shaped or spindle-shape with pseudopodia and island-like growth. Cells gradually were adherent. With prolonged incubation time, the number and density of cells were increased with a few floating cells (A). In the GES-1 cells treated with *Helicobacter pylori* for 12 (B), 24 (C), 48 (D) and 72 (E), cells became round; adherent cells were decreased and floating cells were increased; fragments occurred around cells; cell junction was reduced; the boundaries between cell nucleus and cytoplasm were obscure, and nucleus-cytoplasm fusion was seen. In the GES-1 cells treated with radix curcumae-derived diterpenoid C (5, 10, 20 μg/mL), adherent cells increased and cell morphology gradually recovered at 24 h (F-I, respectively). Amoxicillin had no marked effects on cell morphology.

Western blotting. Results indicated that IkBα began reducing at 15 min time point and was the lowest at 30 min time point; 60 min later, the decreased IkBα gradually recovered (Figure 5A and B). These results suggest that *H. pylori* can lead to IkBα degradation. Based on this, we observed the effects of RC-derived diterpenoid C on IkBα degradation caused by *H. pylori*, and foud that IkBα was basically unchanged. This suggests that RG-derived diterpenoid C can inhibit IkBα degradation caused by *H. pylori* (Figure 5C).

Expression of IkBα and p65 phosphorylated proteins, and IκB kinase α, IκB kinase β and p65 proteins

H. pylori rapidly induced phosphorylation of p65 and IkBα proteins. p65 phosphorylation was clearly seen at 5 min time point, and was the most strong between 15 and 30 min, and then gradually weakened. IkBα phosphorylation was seen at 5 min time point, and was the most strong at 15 min time point, and then gradually weakened. In a short time, the expression of p65, IκB kinase (IKK)α and IKKβ proteins was not markedly changed in *H. pylori* group. These results suggest that *H. pylori* is a good activator of NF-κB signal pathways. RC-derived diterpenoid C inhibited *H. pylori*-induced p65 and IkBα phosphorylation, decreased the expression of p65, IKKα and IKKβ proteins (Figure 6). These results indicated that RC-derived diterpenoid C decreased IkBα protein degradation through inhibiting phosphorylation of p65 and IkBα and the expression of IKKα and IKKβ proteins. RC-derived diterpenoid C may be an effective inhibitor of NF-κB.

DISCUSSION

Recent studies indicate that *H. pylori* activates NF-κB through two pathways. One pathway is dependent on Cag

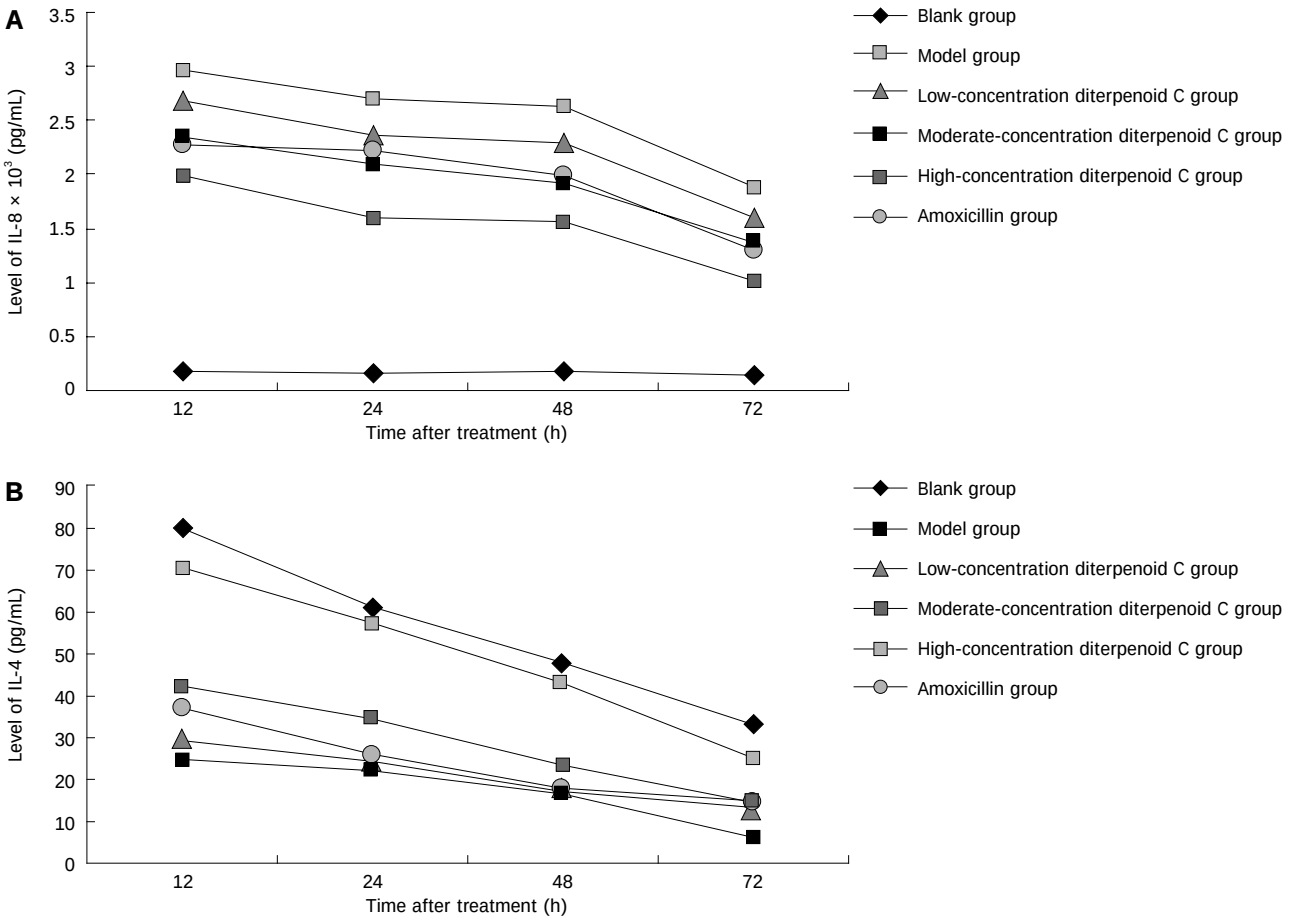

Figure 3 Effects of radix curcumae-derived diterpenoid C on *Helicobacter pylori*-induced human gastric epithelium cell line cell inflammation. A: The changes in the level of interleukin (IL)-8 in cell supernatant; B: The changes in the level of IL-4 in cell supernatant.

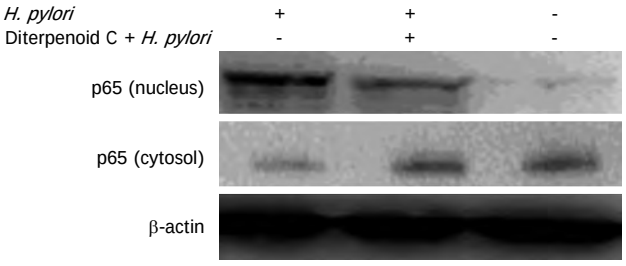

Figure 4 Effects of radix curcumae-derived diterpenoid C on nucleic localization of nuclear factor kappa B p65. *H. pylori*: *Helicobacter pylori*.

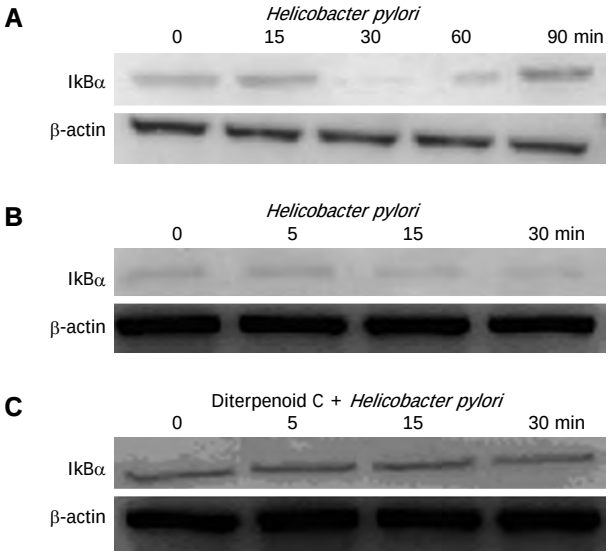

Figure 5 Effects of radix curcumae-derived diterpenoid C on IkBα degradation caused by *Helicobacter pylori*. A: After gastric epithelium cell line cells were respectively treated with *Helicobacter pylori* for 0, 15, 30, 60 and 90 min, cytoplasm was isolated to be used for determination of IkBα degradation with Western blotting; B: *Helicobacter pylori* for 0, 5, 15 and 30 min; C: Diterpenoid C + *Helicobacter pylori* for 0, 5, 15 and 30 min.

pathogenicity island (CagPAI), but independent of CD14 and interleukin-1 receptor-associated kinase. Another pathway is dependent on CD14 and toll-like receptor 4, but independent of CagPAI. *H. pylori* chiefly activates NF-κB classics approach. So it is important to p53 moving nuclear and IkBα degradation in NF-κB classics approach. In addition, *H. pylori* infection induces IkB-β attenuation. In gastric cancer cells, the activities of IkB-α and IkB-β are increase, and the phosphorylation of serine residues of IkB-α and IkB-β induces the degradation of regulatory proteins of NF-κB, activating NF-κB. *H. pylori* infection may induce gastric mucosal inflammatory, and increase the release of PGE2, IL-8 and ROS[10-12], the possible mechanism of which may be related to NF-κB pathways[13].

NF-κB, an important nuclear factor, is involved in cell

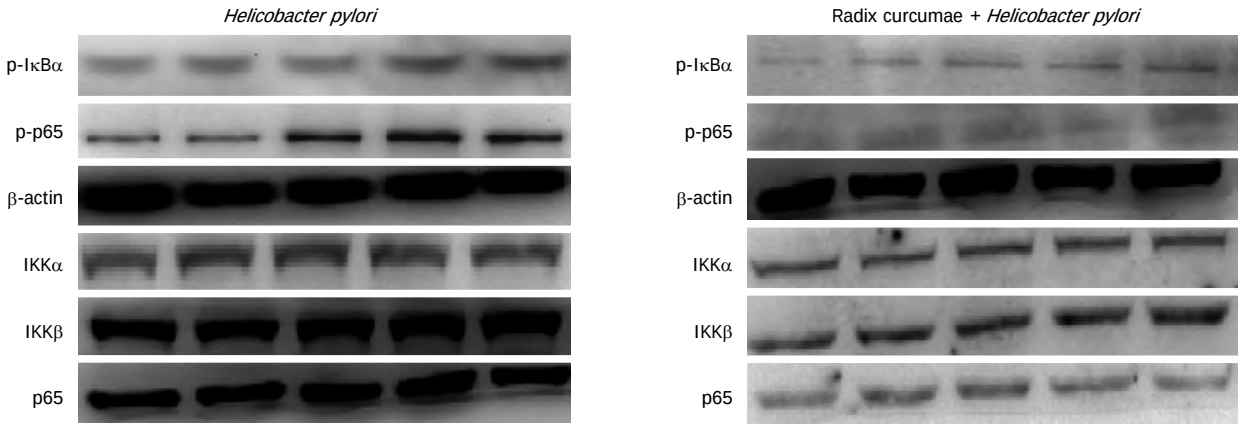

Figure 6 Effects of radix curcumae-derived diterpenoid C on the expression of nuclear factor kappa B proteins. p-IκBα: Phosphorylated IκBα; IKK: IκB kinase.

proliferation[14], immune response[15] and inflammation[16] through regulating the transcription of many genes[17]. In recent years, a great deal of attention has been paid to its role in inflammation and cancer[18,19]. Kim *et al*[20] believes that chronic inflammation is the seventh feature of tumor, chronic inflammation is strongly associated with tumor, and carcinogenesis is from the site of chronic inflammation. In some chronic inflammation-related tumors such as ulcerative colitis and colon cancer, chronic hepatitis and liver cancer, and chronic cervicitis and cervical cancer, NF-κB is found to be super-activated. NF-κB is an important molecule between chronic inflammation and tumor, and is regarded as a bridge between chronic inflammation and tumor.

Many studies have found that the curcumin, a main component of RC-ethanal extract, has highly effective anti-cancer activity with tumor cells[21-24], tumor-associated proteins[25,26], tumor-associated genes[27] and tumor-associated signal transduction pathways[28,29] as targets. It has been classified as the third-generation cancer-chemoprophylactic drug by United States National Cancer Institute. The elemene, a main component of RC-ether extract, can induce cancer apoptosis through down-regulating the expression of Bcl-2 and vascular endothelial growth factor, increasing the levels of cytochrome C and caspase-3 and blocking cell cycle progression[30-32]. Elemene emulsion with β-elemene as the main raw material has been widely used in the treatment of solid tumors, malignant hydrothorax and ascites, and metastasis tumor of brain[33,34]. However, the bioavailability of curcumin is lower, and elemene can produce vein injury, so their clinical application is limited. Therefore, due to this, we successfully obtained a new diterpenoid C from RC-ether extract, and its chemical constitution and properties are different from curcumin and elemene[35,36]. In this study, we explored the inhibitory effects of RC-derived diterpenoid C on *H. pylori*-induced GES-1 cell inflammation.

In this study, in the absence of stimulus, GES-1 cells secrete a tille cytokine. After GES-1 cells were treated with *H. pylori*, the levels of proinflammatory cytokins including IL-8 and IL-6 were significantly increased, and the the level of anti-inflammatory cytokine IL-4 was significantly decreased. RC-derived diterpenoid C was conducive to the balance between proinflammatory cytokines and anti-inflammatory cytokines. The possible mechanism is that RC-derived diterpenoid C has the cascaded inhibitory effects on the expression of IKKα and IKKβ, *H. pylori*-induced IkBα degradation, *H. pylori*-induced p65 translocation from cytoplasm into cell nucleus, the combination of p65 with inflammatory target genes and the release of inflammatory cytokins. Therefore, we infer that RC-derived diterpenoid C is an effective inhibitor of NF-κB.

In summary, RC-derived diterpenoid C, a newly effective anti-inflammatory factor, plays its role in *H. pylori*-infected GES-1 cells possibly through inhibiting NF-κB pathway. In view of the complexity of human life control and cell-signal transduction network, there may be more potential mechanisms about the anti-inflammatory effects of RC-derived diterpenoid C. Exploring RC-derived diterpenoid C to block the combination of NF-κB with its target gene with a reduction or elimination of cytokines has become a new idea to interrupt the progression of chronic gastritis into gastric cancer. This has important values in research and application.

COMMENTS

Background

Gastric carcinogenesis is usually believed to undergo the process including *Helicobacter pylori* (*H. pylori*) infection, chronic gastritis, atrophy, intestinal metaplasia, atypical hyperplasia abd gastric cancer. *H. pylori* infection can bring to inflammation continuing through activating nuclear factor kappa B (NF-κB) signal pathway. As *H. pylori* drug resistance becomes strong, it is difficult to eradicate *H. pylori*. How early to block the progression of chronic gastritis and to reduce gastric carcinogenesis is a main problem for them.

Research frontiers

At present, there are no effective drugs for treatment of chronic gastritis. Their previous experiments have shown that radix curcumae-derived diterpenoid C has better anti-tumor activity and radix curcumae (RC)-derived diterpenoid C of high concentration can induce apoptosis. Inflammation is strongly associated with tumor and the activation of some signal pathways occur in both inflammation and tumor, so the authors investigated the role of RC-derived diterpenoid C in anti-inflammation.

Innovations and breakthroughs

Since biological properties are similar in gastric epithelium cell line (GES-1) cells and normal gastric epithelial cells, GES-1 cells were used in this study. The purpose of this study was to observe the effects of RC-derived diterpenoid

C on inflammation, intestinal metaplasia and the expression of NF-κB signal pathway-related proteins in *H. pylori*-treated GES-1 cells. However, prior study is rare.

Applications

The study demonstrated RC-derived diterpenoid C to block the combination of NF-κB with its target gene with a reduction or elimination of cytokines has become a new idea to interrupt the progression of chronic gastritis into gastric cancer. This has important values in research and application.

Terminology

RC, a common Chinese crude drug, has a wide range of pharmacological activity including hypolipidemic effect, hepatoprotective effect, anti-tumor, anti-radiation and anti-anaphylaxis. RC-derived diterpenoid C is recently obtained from RC ether extract by us, and its chemical properties and constitution are different from curcumin and β-elemene.

Peer review

This paper showed that RC-derived diterpenoid C can block NF-κB signal pathway, effectively reducing the secretion of *H. pylori*-induced proinflammatory cytokine and increasing the secretion of anti-inflammatory cytokine. RC-derived diterpenoid C may become an effective drug for treatment of chronic gastritis.

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BRIEF ARTICLE

Acute effects of rotavirus and malnutrition on intestinal barrier function in neonatal piglets

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Abstract

AIM: To investigate the effect of protein-energy malnutrition on intestinal barrier function during rotavirus enteritis in a piglet model.

METHODS: Newborn piglets were allotted at day 4 of age to the following treatments: (1) full-strength formula (FSF)/noninfected; (2) FSF/rotavirus infected; (3) half-strength formula (HSF)/noninfected; or (4) HSF/rotavirus infected. After one day of adjustment to the feeding rates, pigs were infected with rotavirus and acute effects on growth and diarrhea were monitored for 3 d and jejunal samples were collected for Ussing-chamber analyses.

RESULTS: Piglets that were malnourished or infected had lower body weights on days 2 and 3 post-infection ($P < 0.05$). Three days post-infection, marked diarrhea and weight loss were accompanied by sharp reductions in villus height (59%) and lactase activity (91%) and increased crypt depth (21%) in infected compared with non-infected pigs ($P < 0.05$). Malnutrition also increased crypt depth (21%) compared to full-fed piglets. Villus:crypt ratio was reduced (67%) with viral infection. There was a trend for reduction in transepithelial electrical resistance with rotavirus infection and malnutrition ($P = 0.1$). ^{3}H-mannitol flux was significantly increased (50%; $P < 0.001$) in rotavirus-infected piglets compared to non-infected piglets, but there was no effect of nutritional status. Furthermore, rotavirus infection reduced localization of the tight junction protein, occludin, in the cell membrane and increased localization in the cytosol.

CONCLUSION: Overall, malnutrition had no additive effects to rotavirus infection on intestinal barrier function at day 3 post-infection in a neonatal piglet model.

Key words: Rotavirus gastroenteritis; Kwashiorkor; Occludin; Ussing chamber; Villus

Core tip: We are quite excited about these results which suggest involvement of intestinal tight-junction proteins in the pathology of rotaviral gastroenteritis. The work further examines the interplay of malnutrition superimposed on viral infection. ^{3}H-mannitol flux was significantly increased in rotavirus infected piglets compared to non-infected piglets, but there was no effect of nutri-

tional status. Furthermore, rotavirus infection reduced localization of the tight junction protein, occludin, in the cell membrane and increased localization in the cytosol. This extends work on the molecular mechanisms of rotavirus in the neonatal intestine that we previously published.

Jacobi SK, Moeser AJ, Blikslager AT, Rhoads JM, Corl BA, Harrell RJ, Odle J. Acute effects of rotavirus and malnutrition on intestinal barrier function in neonatal piglets. *World J Gastroenterol* 2013; 19(31): 5094-5102 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i31/5094.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i31.5094

INTRODUCTION

Pediatric diarrheal diseases are the second-leading cause of childhood mortality, and responsible for about 1.34 million deaths each year in children under 5 years of age[1]. Rotaviruses are the most common causes of acute, severe gastroenteritis and dehydrating diarrhea. Furthermore, rotavirus-associated enteritis represents a class of zoonotic diseases that cause major health concerns not only for humans, but also most domestic livestock species[2]. The food animal livestock industry estimated a multi-million dollar annual economic loss due to diarrheal diseases associated with a reduction in weight gain, treatment and death of young animals[2]. In addition to the mortality rates associated with diarrheal disease there is a about 60% increase in pediatric patient mortality rates when diarrheal disease is compounded with malnourishment[3]. In the neonatal piglet model, Zijlstra *et al*[4] demonstrated that malnutrition extends rotavirus infections up to a week longer in malnourished piglets compared with well nourished infected piglets.

Rotaviruses infect the differentiated epithelial cells of the mid- to upper-villus of the small intestine[5]. The infection is associated with cell death, reduced villus surface area, loss of absorptive capacity, osmotic deregulation, and infiltration of the lamina propria by mononuclear cells[6,7]. In pigs, acute viral injury to enterocytes leads to increased epithelial cell loss and intestinal lesions leaving the intestinal epithelial barrier compromised[6]. Rapid restoration of epithelial continuity is important following injury and depends on the migration of uninjured enterocytes from the crypts to cover the compromised barrier. Protein-energy malnutrition (PEM) also decreases intestinal barrier function and integrity, increasing bacterial translocation with subsequent enteritis and diarrhea[8]. Moreover, PEM also inhibits epithelial crypt cell proliferation which delays cellular migration along the crypt-villus axis and results in longer repair periods[9].

Intestinal barrier function is maintained in part by actual physical links between enterocytes by intercellular junction complexes. Tight junctions are located on the uppermost basolateral surface of polarized enterocytes and regulate diffusion between cells. They allow the epithelia to form a cellular barrier separating the luminal content of the intestine from the lamina propria. In cell culture models using Madin-Darby Canine Kidney and Caco-2 cells, studies have demonstrated dysregulation of the paracellular pathways[10,11]. In fact, rotavirus infection in these cells caused alterations in tight junction structure and function related to epithelial cell resistance and permeability. The authors determined that there was a time dependent disruption in localization of tight junction proteins claudin-1, occludin and zonula-occuluden when Caco-2 cells were infected with rotavirus[10,11]. Claudin-1 was the first tight-junction protein to become solubilized in the cytosol of the epithelial cells[11].

Nutritional factors have been shown to impact neonatal intestinal health[12]. In particular, our laboratory has investigated how dietary components impact intestinal health in neonatal piglets with rotavirus infection[13-15]. We have demonstrated supplemental dietary arginine activates mammalian target of rapamycin, mitogen-activate protein kinase, and ribosomal p70S6 kinase signaling in rotavirus infected enterocytes[15]. These cell signaling mechanisms lead to increase jejunal protein synthesis, cell migration and intestinal restitution in rotavirus infected piglets[13,14]. Moreover, we have demonstrated the value of dietary plasma protein because it maintained growth rates, reduced diarrhea, and maintained enzymatic activity in the small intestine of neonatal pigs with rotavirus infection[14]. Additionally, others have shown soy-based infant formula isoflavones are effective in reducing rotavirus infectivity in cell culture models of rotavirus infection[16]. These reports demonstrate the importance of nutritional factors involvement in modulation of host immune response and repair mechanisms associated with rotaviral infection. Therefore, the pathophysiological mechanisms of rotavirus infection and its diarrheal mechanism are the focus of much work toward developing effective vaccines and nutritional treatments for the virus. Understanding the viral interruption of paracellular pathways and the impact of nutritional status on these pathways is critical in the development of adequate medical treatment.

MATERIALS AND METHODS

Animals and experimental design

All protocols were approved by the Institutional Animal Care and Use Committee of North Carolina State University. The full experimental protocol was previously reported in Rhoads *et al*[15]. Briefly, 24 piglets were collected directly from the birth canal, colostrum deprived, cleaned with 70% ethanol and transported to a biosecure rearing facility. Piglets were individually housed and contained in two rooms with a temperature of 32 ℃. Pigs were fed milk diet *via* a gravity flow feeding system, adapted from Oliver *et al*[17]. The formula composition was previously reported by Rhoads *et al*[15]. A liquid colostrum diet (La-Belle Associates, Inc., WA, United States) was fed for the first 24 h to provide passive immunity. Feedings (about 300 mL/kg body weight per day) were offered four times

per day (8:00 am, 1:00 pm, 6:00 pm and 11:00 pm), and non-infected pigs were pair-fed to the level of their infected counterparts.

We compared well-nourished and malnourished piglets (n = 16) in a 2 × 2 factorial design examining effects of malnutrition and viral infection as follows: (1) full-strength formula (FSF) (180 g/L), non-infected (positive control); (2) FSF, rotavirus infected; (3) half-strength formula (HSF, 90 g/L), non-infected; or (4) HSF, rotavirus infected (negative control). Intestinal samples from this study were collected only on day 3 post-infection.

Rotavirus inoculation and clinical measurements

Rotavirus inoculation and clinical measures were previously described by Rhoads *et al*[15]. Briefly, the rotavirus inoculum, initially isolated by Lecce *et al*[18], was passaged through colostrum-deprived pigs and prepared as a bacteria-free intestinal supernatant. Approximately 10^7 particles of rotavirus or sham inoculants were suspended in full strength milk formula, and piglets were gastrically intubated at 10:00 am on day 0.

Piglet weights, feed intakes, and fecal consistency were recorded daily. Feces were given a diarrhea score of 0, 1, 2 or 3, corresponding with firm, soft but formed, runny, and severe watery diarrhea, respectively, by a single individual blinded to treatments. A rectal swab was collected daily from each piglet for the detection of rotavirus shedding (Virogen Rotatest; Wampole Laboratories, Cranbury, NJ, United States).

Intestinal sampling

On day 3 post-infection, pigs were anesthetized with isoflurane and killed by the AVMA approved electrocution followed by exsanguination. Intestinal samples from the mid-jejunum area were collected, snap frozen and stored at -80 ℃ until analysis by Western blotting. Intestinal segments were collected and fixed for histological analysis of intestinal morphology[17]. Intestinal morphology and lactase measurements were performed as previously described[17].

Ussing chamber measurements

Segments of mid-jejunum were harvested from the pigs and the mucosa was stripped from the seromuscular layer in oxygenated (95% O_2/5% CO_2) Ringer's solution. Tissues were mounted in 1.14 cm^2 aperture Ussing chambers, as described previously[19]. Tissues were bathed on the serosal and mucosal sides with 10 mL Ringer's solution. The serosal bathing solution contained 10 mmol/L glucose, which was osmotically balanced on the mucosal side with 10 mmol/L mannitol. Bathing solutions were oxygenated (95% O_2/5% CO_2) and circulated in water-jacketed reservoirs maintained at 37 ℃. The spontaneous potential difference (PD) was measured using Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through Ag-AgCl electrodes using a voltage clamp that corrected for fluid resistance. Transepithelial electrical resistance ($\Omega \cdot cm^2$) was calculated from the spontaneous PD and the short-circuit current (I_{sc}), as previously described[19].

Mucosal permeability was assessed following experimental treatments by adding 0.2 μCi/mL ^{3}H-mannitol on the mucosal side of the Ussing chamber-mounted tissues and measuring the flux to the serosal compartment. Following a 15 min equilibration period samples were collected from the mucosal side of the chamber and following a 60 min flux period samples were collected from the serosal side of the chamber as previously described[20]. The concentration of ^{3}H-mannitol was quantified using a liquid scintillation counter (LKB Wallac, model 1219 Rack Beta, Perkin Elmer Life and Analytical Sciences, Boston, MA, United States). The directional flux of ^{3}H-mannitol from the mucosal to serosal chamber were determined by using the mannitol specific activity added to the mucosal bathing solution and calculating the net appearance of ^{3}H-mannitol over time in the serosal bathing solution.

Protein isolation and Western blotting analysis

Intestinal mucosa scrapings from all animals were snap frozen and stored at -80 ℃ for SDS-PAGE analysis. Triton X-soluble and X-insoluble fractions were prepared as previously described[21]. Briefly, samples were homogenized in Triton X-soluble buffer and allowed to rest on ice for 30 min with intermittent vortexing. Thereafter, the homogenates were centrifuged at 400 g for 10 min at 4 ℃ to remove cell debris. The supernatant was removed to a new tube and centrifuged at 9000 g for 10 min at 4 ℃ to separate the soluble and insoluble protein fractions. The insoluble fraction pellet was dissolved in Triton X-insoluble fraction extraction buffer by heating at 95 ℃ for 5 min with intermittent vortexing. Protein concentrations of tissue extracts were determined using the DC protein assay (Bio-Rad; Hercules, CA, United States). Tissue extracts of equal protein concentrations were mixed with equal volumes of 2 × Laemmli Sample Buffer (Bio-Rad; Hercules, CA, United States) and boiled for 5 min. Protein lysates were loaded on a 12% SDS polyacrylamide gel, and electrophoresis was completed as recommended for Bio-Rad Criterion™ gels. Proteins were transferred to polyvinylidene diflouride membrane (Immobilon-S; Millipore, Billaerica, MA, United States) by Criterion™ Blotter (Bio-Rad; Hercules, CA, United States). Membranes were blocked and incubated with primary and secondary antibodies as previously reported by Moeser *et al*[21].

Statistical analysis

Data were analyzed using the general linear models procedure of SAS (Cary; NC 27513, United States) appropriate for a 2 ×2 factorial design, with feeding level (FSF *vs* HSF) and infection (± rotavirus) as the factors.

RESULTS

Animal observations

Body weight for 1-d-old pigs was 1.4 ± 0.2 kg. The pigs were adapted to the feeding system until day 5 when they were switched to either FSF (well-nourished) or

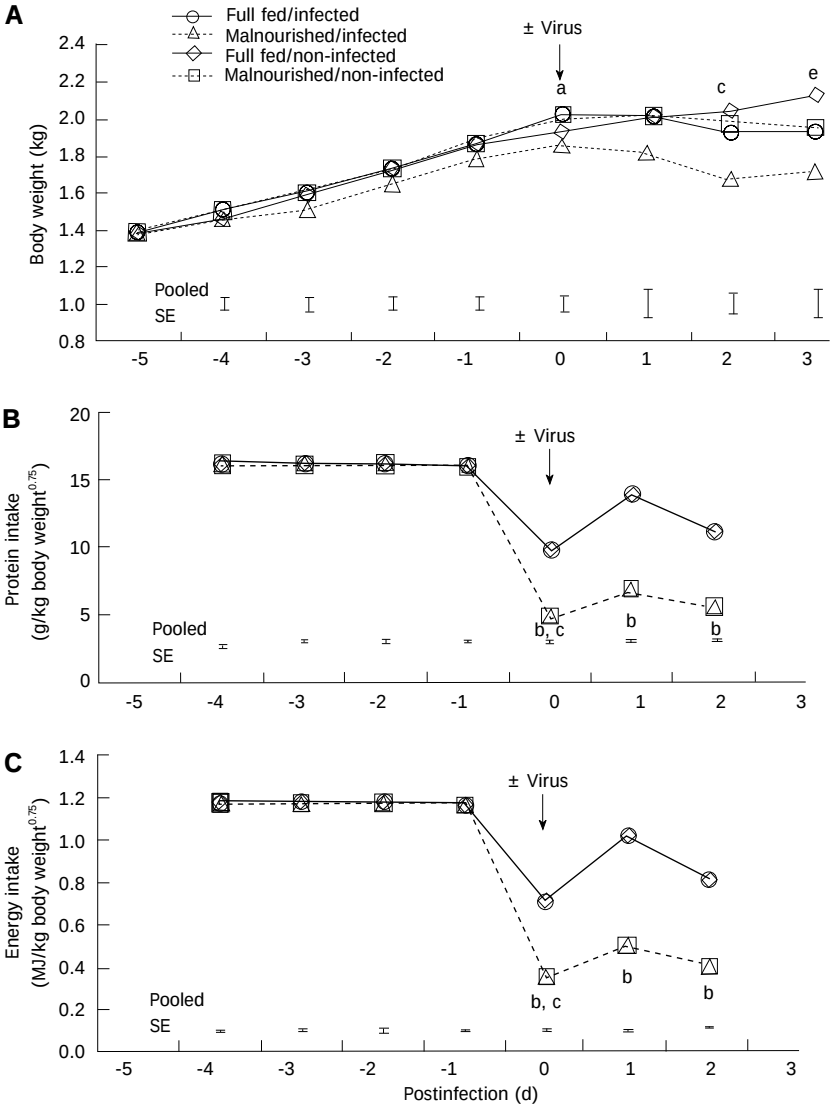

Figure 1 Growth, protein intake and metabolizable energy intake of newborn pigs fed full-strength formula (full fed) or half-strength formula (malnourished) and inoculated with rotavirus or vehicle (non-infected) as indicated. Values are reported as least-square means (n = 6/treatment), and error bars represent ± pooled SE per day. A: Piglet growth curves, calculated using initial body weight as a covariate. [a]$P < 0.05$, feeding-level/rotavirus interaction; [c]$P < 0.05$, feeding-level; [e]$P < 0.05$, rotavirus effect; B, C: Protein intakes (B) and metabolizable energy intakes (C) of newborn pigs over time. Non-infected pigs were pair-fed to their rotavirus-infected counterparts. [b]$P < 0.01$, feeding level effect; [c]$P < 0.05$, rotavirus effect.

HSF (malnourished) diets. Twenty-four hours after pigs were assigned to dietary treatments they were inoculated with rotavirus. There was a feeding level by infection interaction on day 0 (Figure 1A; $P < 0.05$). The interaction was due to the drop in body weight of the HSF/infected piglets compared with pigs in other dietary treatment groups. On days 1-3 post-infection there was no interaction of feeding level and infection. However, on days 2 and 3 post-infection there were main effects of both feeding level and infection. Full-strength formula/non-infected pigs maintained a higher body weight compared HSF/infected pigs ($P < 0.05$). Feed intake of non-infected pigs was pair-fed to the level of their infected counterpart, so there were no major differences in protein and energy intake between the non-infected and infected pigs from the same dietary treatment (Figure 1B and C). Malnourished pigs received a 50% reduction in nutrient intake, but daily intakes of water, sodium, potassium and chloride were similar to full-fed pigs, because electrolyte solution was used for formula dilution. The significant effect of viral infection on body weight on day 3 could be related to multiple factors, however, we have controlled nutrition by pair feeding, and measured growth, so the most likely cause of weight loss was diarrheic water loss. Pigs had no diarrhea prior to rotavirus inoculation (data not shown). However, viral infection resulted in diarrhea scores of 3 (severe, watery diarrhea) for both rotavirus infected groups. On day 3 post-infection there was a main effect of virus and feeding level on diarrhea score ($P < 0.01$ and $P < 0.05$, respectively; Figure 2A); however, there was no additive effect of malnutrition. Additionally, rotavirus-inoculated pigs had a significant increase in viral shedding from days 1 to 3 post-infection (Figure 2B).

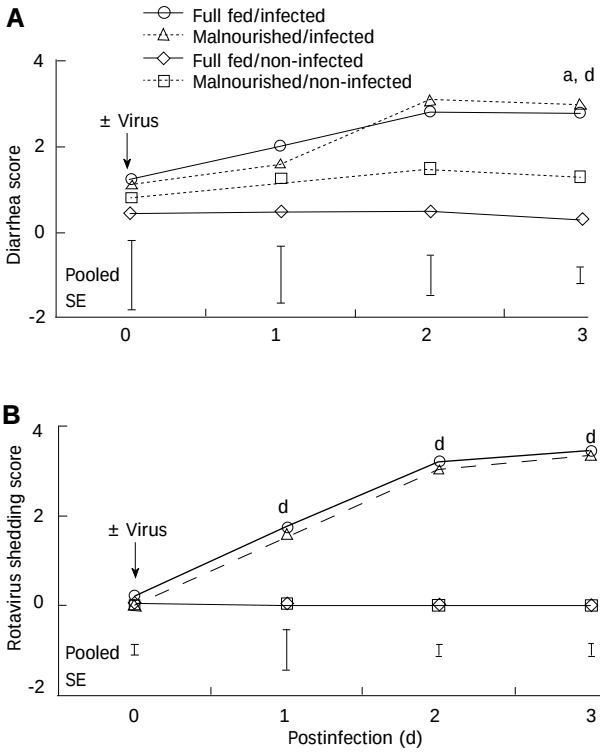

Figure 2 Daily diarrhea (A) and rotavirus shedding scores (B) measured in newborn pigs fed full-strength formula (full fed) or half-strength formula (malnourished) and inoculated with rotavirus or vehicle (non-infected) as indicated. Values are reported as least-square mean (n = 6/treatment), and error bars represent ± pooled SE per day. [a]$P < 0.05$, feeding level effect; [d]$P < 0.01$, rotavirus effect.

Intestinal lactase activity and morphology

Rotavirus infected pigs had significantly reduced lactase activity (Table 1; $P < 0.05$) on day 3 post-infection. In addition, there was a main effect of virus on villus height, crypt depth and villus height:crypt depth ratio (Figure 3 and Table 1; $P < 0.05$). There also was a main effect of feeding level on crypt depth with malnourished pigs having a greater depth than FSF pigs (Figure 3 and Table 1; $P < 0.05$). However, there was not a significant interaction between rotavirus and feeding level on intestinal lactase or morphology.

Intestinal barrier function

Transepithelial electrical resistance (TER) data were recorded on mid-jejunum tissues from pigs on each dietary and viral treatment (Figure 4A). There was no significant interaction of feeding-level by virus and no main effects on TER data. However, there was a trend for FSF/rotavirus infected pigs to have decreased TER compared with FSF/non-infected pigs (Figure 4A; $P = 0.1$). Malnourished animals had similar TER readings regardless of rotavirus infection.

To assess the effect of nutritional status and rotavirus infection on intestinal permeability, ^{3}H-manitol flux was measured on mid-jejunum tissues from pigs. The feeding-level by rotavirus infection interaction was not significant (Figure 4B). However, there was a main effect of rotavirus infection on intestinal permeability ($P < 0.05$). Pigs infected with rotavirus had 50% greater intestinal permeability than non-infected pigs. Conversely, there was no main effect of feeding level on intestinal permeability.

Western blotting for the tight-junction protein, occludin, demonstrated that rotavirus infected pigs had greater quantity of occludin protein in the soluble fraction of protein than in the insoluble fraction (Figure 5). Additionally, non-infected pigs had no occludin protein

Table 1 Jejunal lactase activity and morphology of piglets fed full-strength formula (fully fed) or half-strength formula (malnourished) and infected or non-infected with rotavirus

	Fully fed		Malnourished			
	Non-infected	Infected	Non-infected	Infected	SE	Effect[1]
Lactase activity [mmol/(min·g protein)]	169.8	9.6	160.3	19.7	21.0	V
Intestinal morphology						
Villus height (mm)	0.87	0.37	0.85	0.34	0.13	V
Crypt depth (mm)	0.10	0.13	0.13	0.16	0.01	V, F
Height:depth ratio	8.96	3.12	6.86	2.12	1.24	V

Values are least-square means for n = 6 pigs per group, measured 3 d post-inoculation. [1]V, main effect ($P < 0.05$) of rotavirus infection; F, main effect ($P < 0.05$) of feeding level; Interaction of rotavirus and feeding level (V, F) was not detected ($P > 0.05$).

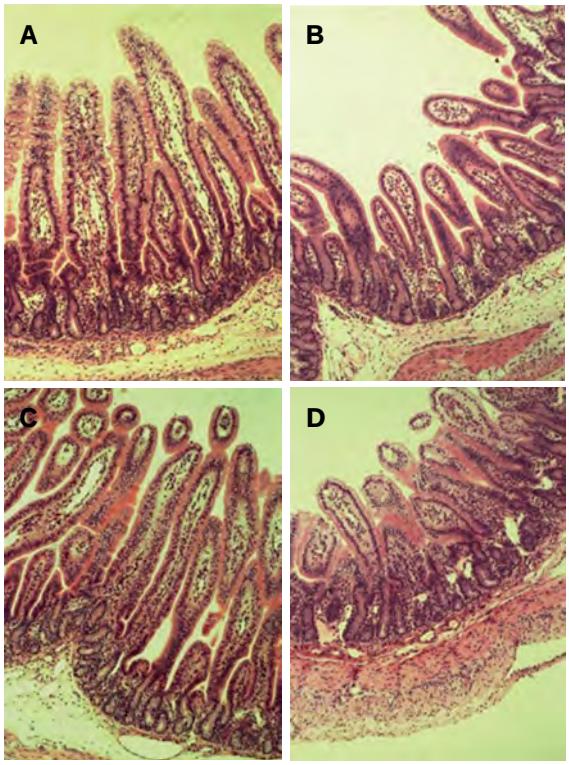

Figure 3 Hematoxylin and eosin stained intestinal sections from newborn pigs fed full-strength formula (full fed; A and B) or half-strength formula (malnourished; C and D) and inoculated with rotavirus (B and D) or vehicle (non-infected; A and C) as indicated (× 10 magnification). Numerical measurements and statistical analysis of the intestinal morphology are reported in Table 1.

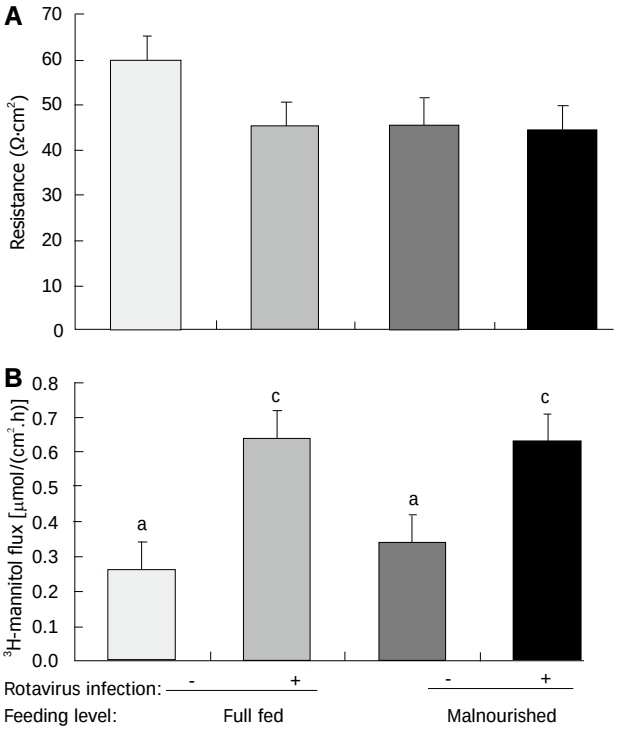

Figure 4 Transepithelial electrical resistance (A) and ^{3}H-mannitol flux (B) in jejunal mucosa from new-born pigs fed full-strength formula (full fed) or half-strength formula (malnourished) and inoculated with rotavirus or vehicle (non-infected) as indicated. Measurements were made 3 d post-inoculation. Values are reported as least-square means (n = 6/treatment), and error bars represent pooled SE. $^{a}P < 0.05$, $^{c}P < 0.05$ between groups.

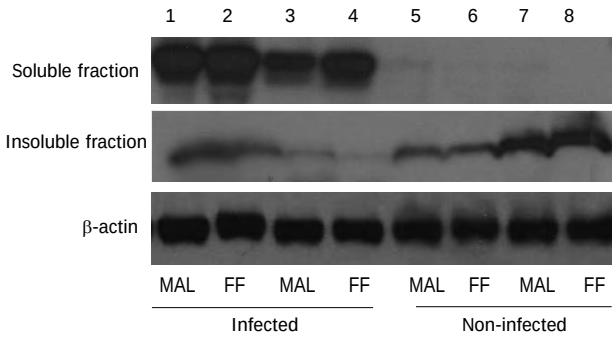

Figure 5 Occludin Western blotting analysis of jejunal tissues from new-born pigs fed full-strength formula or half-strength formula and inoculated with rotavirus or vehicle as indicated. Tissues were collected 3 d post-inoculation. Full-strength formula [full fed (FF)]: Lanes 2, 4, 6, 8; Half-strength formula [malnourished (MAL)]: Lanes 1, 3, 5, 7; Infected rotavirus: Lanes 1-4; Non-infected vehicle: Lanes 5-8; Upper panel: Soluble protein.

in the soluble fraction and higher levels of occludin in the insoluble fraction. As was observed in TER and flux measures of barrier function, feeding level did not alter the cellular localization of the tight-junction protein occludin (Figure 5).

DISCUSSION

Rotavirus gastroenteritis accounts for 30%-40% of pediatric diarrheal deaths worldwide[22], and PEM is also a primary cause of childhood morbidity and mortality especially in developing nations[1,23]. While rotavirus vaccine safety and efficacy has improved over the last 10 years for children in developed countries, the efficacy of rotavirus vaccine for children in poor settings is compromised due to environmental factors associated with reduction in vaccine effectiveness[24]. Our laboratory and others working in neonatal piglet models have sought to determine possible nutritional therapies which could reduce the severity of the infection or enhance the effectiveness of vaccines[13-16]. Nutritional therapies will be affected by previous nutritional status of individuals, and nutritional deprivation is a key component to overcome for treatment of enteric diseases afflicting impoverished children. In mouse models of rotavirus infection and malnutrition there is a significant increase in gut permeability to environmental macromolecules, as well as a significant decrease in minimal infectious dose needed to produce diarrhea[25,26]. Therefore, understanding the mechanisms associated with gut barrier function under normal rotavirus infection as well as rotavirus infection compounded by malnutrition is an important component for developing efficacious treatment and prevention strategies in young children and animals.

The purpose of this study was to examine the effects of PEM and rotavirus infection on intestinal barrier function in neonatal piglets. Diarrhea and malnutrition are two major problems in pediatric patients and a better understanding of the interaction and underlying mechanisms may lead to improved treatment. The study design was a 2 × 2 factorial with two levels of nutrition and either non-infected or infected with rotavirus.

Rotavirus infection caused decreased body weight, reduced protein intake, reduced energy intake, diarrhea, decreased lactase activity, trends for decreased jejunum TER, increased jejunum permeability, and decreased cell membrane localization of occludin. However, malnutrition did not have a significant additive effect to rotavirus infection on intestinal barrier function measured 3 d post-infection. The lack of additive effects on malnutrition is most likely related to the time line of the study. The 3 d post-infection time point may have been too short to evaluate repair of the intestine in this model. It is also possible that if we had applied the nutritional treatment prior to rotavirus infection there might have been a significant effect of PEM in the piglets.

Previously, we have shown that rotavirus infection causes intestinal damage and diarrhea within 2 d post-infection in the neonatal piglet model[4]. Additionally, the effects of the viral infection began to subside nearly 1 wk earlier in full-fed pigs than in the malnourished, infected pigs[4]. Herein, we report similar results of rotavirus infection causing significant weight loss and increased diarrhea by day 2 post-infection. Although there was a significant interaction of feeding level and infection on weight loss on day 0 of inoculation the interaction was not sustained for the next 3 d post-infection. However, the main ef-

fects of rotavirus and feeding level on weight loss were significant on days 2 and 3 post-infection. The reduced weight gain, increased diarrhea scores and increased viral shedding were expected following inoculation. However, we did anticipate there would be additive effects of malnutrition on all three outcomes, which we did not observe. Others have shown that PEM alters physiological and immunological properties of the intestine leaving individuals more susceptible to diarrhea associated illnesses[25-29]. Nevertheless, this bidirectional relationship between PEM and susceptibility to enteric pathogens in pediatric patients is related to the type of pathogen and many environmental factors playing a significant role in susceptibility to infection[30]. We may have underestimated the time needed to detect a plain of nutrition response with rotavirus infection. In fact, in mouse models of malnutrition and intestinal permeability the dietary treatments were applied to the mice a minimum of 5 d before rotavirus inoculation, and the research showed increased ovalbumin absorption in malnourished, infected mice compared to well nourished, infected mice[25].

Dietary nutrients are essential for gastrointestinal growth and health[12]. Malnutrition reduces the integrity of intestinal epithelium, facilitating bacterial translocation with subsequent enteritis and diarrhea[8], and it can also impair epithelial cell proliferation in crypts of the small intestine, resulting in delayed cellular migration along the crypt-villus axis[9]. This impairment is inhibitory to intestinal repair processes associated with gastrointestinal enteritis. We found that PEM did not further reduce lactase activity or villus blunting in the small intestine beyond the reduction seen with rotavirus infection alone. Additionally, crypt depth was increased by PEM and viral infection, but there was no additive effect. Previously, Zijlstra *et al*[4] reported a reduction in crypt depth with infected, malnourished pigs compared to infected, full-fed pigs. However, our data suggest that HSF/infected pigs had increased crypt depth compared to all other treatment groups. This may be related to the exact location of sampling between our study and Zijlstra *et al*[4]. Zijlstra *et al*[4] found the reductions in crypt depth were more distal in the small intestine on day 2 post-infection, and the reduction in crypt depth in the proximal to mid small intestine was not significant until day 9 post-infection. Additionally, in piglet models of transmissible gastroenteritis, increased jejunal crypt depth following infection have been reported[31-33].

Maintenance of migrating crypt cells is essential in maintaining gut barrier function following intestinal insult to seal the basement membrane and close leaky epithelial intercellular spaces and tight junctions. Rotavirus is known to disrupt tight junctions and decrease TER in Caco-2 cells between 8 and 24 h post-infection. We found *in vivo* treatments of rotavirus showed a trend for reduced TER in mid-jejunal tissue from neonatal pigs. TER data showed similar resistance between infected pigs and HSF/uninfected pigs; however there was not an additive effect of malnutrition and rotavirus infection in the neonatal pigs. Serosal to mucosal flux of ^{3}H-mannitol was significantly greater for infected pigs with no effect of PEM on mucosal paracellular permeability. The numerical reduction in TER for all treatment groups except FSF/non-infected piglets does not completely align with the differences in mannitol flux we observed, and this could be explained by recent understandings of tight junction pore and leak pathways[34]. Piglets in the full-fed/infected groups had diarrhea accompanied by alterations in tight junctions, TER, and mannitol flux and we reason that virus infection in the well-nourished state caused tight junction pores to open (allowing electrolytes and water passage) together with a pore/shunt pathway allowing macromolecule passage. In contrast, malnourished piglets, regardless of infection, had numerically reduced TER, but mannitol flux was only increased in malnourished/infected piglets suggesting that malnutrition alone was sufficient to alter passage of small ions across the barrier associated with reductions in TER and diarrhea, but rotavirus infection altered the tight junctions to allow increased macromolecule flux through pore/shunt pathways.

Expression of the tight junction protein, occludin, revealed that although virus infection impacted the proportion of occludin in the membrane (insoluble fraction) versus the cytosolic (soluble) fraction there was no effect of feeding level on occludin localization. Rotavirus infection significantly increased occludin in the soluble fraction of the protein extraction compared to non-infected pigs which had greater occludin in the membrane fraction of the protein extraction. This finding corroborates the effects seen in the TER and flux data showing no additive effects of PEM to the rotavirus infected pigs intestinal barrier function. Other models of starvation and injury in rats suggest an additive effect of malnourishment and intestinal insult on gut barrier permeability[35]. However, our results are consistent with previously reported data showing that PEM did not affect jejunal tissue protein synthesis rates and phosphorylation of $p70^{S6K}$, a key enzyme activated by mammalian target of rapamycin in regulating protein synthesis[15]. Zijlstra *et al*[4] showed that the growth factors, insulin-like growth factor (IGF)- I and IGF-Ⅱ, were not significantly reduced in the malnourished, infected pigs until 9 d post-infection. These growth factors are known trophic factors in the intestine and have been shown to increase jejunal uptake of glucose in the intestine[36]. Because IGF- I and IGF-Ⅱ concentrations were probably not decreased in our pigs by 3 d post-infection the effects of PEM on gut barrier function may not have been detectable at this early time point post-infection.

In conclusion, the present study provides clear evidence that rotavirus infection significantly affects small intestinal TER, and is the first report of increased paracellular permeability in neonatal pigs resulting from altered tight junction protein localization in enterocytes. However, additive effects of PEM on intestinal TER, paracellular permeability, and tight junction protein lo-

calization are not seen by 3 d post-infection in neonatal pigs. Though it is likely that during a more extended timeline wherein metabolic hormone responses decrease following viral infection and PEM there is potential for reduction in crypt cell proliferation[37]. This reduction in proliferation may potentially exacerbate the effect of viral infection in PEM neonates. Further identification of paracellular permeability mechanisms associated with rotavirus and PEM would be useful in developing treatments for pediatric patients facing environments where malnutrition and diarrhea are intertwined.

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COMMENTS

Background

Rotavirus enteritis and malnutrition are common problems for children in the developing countries. Rotavirus infections are common for all children under the age of five. However, children in less than desirable circumstances must deal with rotavirus infection compound by malnourishment. There is limited information on the interactions between the mechanisms of rotavirus and malnutrition on gut barrier function.

Research frontiers

In the present study, investigation of the interactions between rotavirus enteritis and malnutrition on gut barrier function were studied in the neonatal piglet model to determine the mechanisms involved in barrier function failure.

Innovations and breakthroughs

Rotavirus infection significantly blunted villus height in neonatal piglets, increased gut barrier permeability and reduced localization of tight junction proteins in the cell membrane of intestinal enterocytes during acute infection. Determination of the mechanisms of rotavirus and malnutrition interaction could lead to development of new nutritional or pharmacological treatments in high risk children in developing countries.

Applications

The effects of rotavirus and malnutrition in the neonatal piglet experimental model demonstrated the virus significantly effects gut barrier function and could potentially be used to study effective nutritional or pharmacological treatments for rotavirus in children. Further investigation into signaling mechanisms controlling intestinal tight junctions may provide insights into protein targets for development of effective therapies.

Terminology

Protein energy malnutrition is a common problem that compounds gastroenteritis in developing countries and neonatal health.

Peer review

This study is well constructed and it is based on previous work on nutritional impacts on viral infections especially in developing countries. This is an interesting and relevant study which confirms that rotavirus acutely impacts gut barrier function in the neonatal pig making animals more susceptible to flux across intestinal epithelial layer. Moreover, this study suggests that longer term studies should be completed to investigate the interaction of malnutrition with rotavirus infection in the developing neonate.

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BRIEF ARTICLE

Colonic preparation before colonoscopy in constipated and non-constipated patients: A randomized study

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Abstract

AIM: To compare the efficacy of different doses of sodium phosphate (NaP) and polyethylenglicol (PEG) alone or with bisacodyl for colonic cleansing in constipated and non-constipated patients.

METHODS: Three hundred and forty-nine patients, older than 18 years old, with low risk for renal damage and who were scheduled for outpatient colonoscopy were randomized to receive one of the following preparations (prep): 90 mL of NaP (prep 1); 45 mL of NaP + 20 mg of bisacodyl (prep 2); 4 L of PEG (prep 3) or 2 L of PEG + 20 mg of bisacodyl (prep 4). Randomization was stratified by constipation. Patients, endoscopists, endoscopists' assistants and data analysts were blinded. A blinding challenge was performed to endoscopist in order to reassure blinding. The primary outcome was the efficacy of colonic cleansing using a previous reported scale. Secondary outcomes were tolerability, compliance, side effects, endoscopist perception about the necessity to repeat the study due to an inadequate colonic preparation and patient overall perceptions.

RESULTS: Information about the primary outcome was obtained from 324 patients (93%). There were no significant differences regarding the preparation quality among different groups in the overall analysis. Compliance was higher in the NaP preparations being even higher in half-dose with bisacodyl: 94% (prep 1), 100% (prep 2), 81% (prep 3) and 87% (prep 4) (2 *vs* 1, 3 and 4, $P < 0.01$; 1 *vs* 3, 4, $P < 0.05$). The combination of bisacodyl with NaP was associated with insomnia ($P = 0.04$). In non-constipated patients the preparation quality was also similar between different groups, but endoscopist appraisal about the need to repeat the study was more frequent in the half-dose PEG plus bisacodyl than in whole dose NaP preparation: 11% (prep 4) *vs* 2% (prep 1) ($P < 0.05$). Compliance in this group was also higher with the NaP preparations: 95% (prep 1), 100% (prep2) *vs* 80% (prep 3) ($P < 0.05$). Bisacodyl was associated with abdominal pain: 13% (prep 1), 31% (prep 2), 21% (prep 3) and 29% (prep 4), (2, 4 *vs* 1, 2, $P < 0.05$). In constipated patients the combination of NaP plus bisacodyl presented higher rates of satisfactory colonic cleansing than whole those PEG: 95% (prep 2) *vs* 66% (prep 3) ($P = 0.03$). Preparations containing bisacodyl were not associated with adverse effects in constipated patients.

CONCLUSION: In non-constipated patients, compliance is higher with NaP preparations, and bisacodyl is related to adverse effects. In constipated patients NaP plus bisacodyl is the most effective preparation.

Key words: Colonic cleansing; Sodium phosphate; Polyethylenglicol; Bisacodyl constipation; Colonoscopy

Core tip: Colonoscopy has become the standard procedure for the diagnosis and treatment of colon diseases. Adequate bowel cleansing is essential for a high-quality effective and safe colonoscopy. In non-constipated patients, compliance is higher with sodium phosphate (NaP) preparations, and bisacodyl is related to adverse effects. In constipated patients NaP plus bisacodyl is the most effective preparation.

Pereyra L, Cimmino D, González Malla C, Laporte M, Rotholtz N, Peczan C, Lencinas S, Pedreira S, Catalano H, Boerr L. Colonic preparation before colonoscopy in constipated and non-constipated patients: A randomized study. *World J Gastroenterol* 2013; 19(31): 5103-5110 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i31/5103.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i31.5103

INTRODUCTION

Colonoscopy has become the standard procedure for the diagnosis and treatment of colon diseases[1]. An adequate colonic cleansing is necessary for a proper evaluation of the entire colonic mucosa and therefore for achieving a high quality colonoscopy[2]. Sodium phosphate (NaP) is a small volume hyperosmotic solution that provides effective colonic cleansing in preparation for colonoscopy. In the past years the popularity of orally administered NaP has increased because of its superior tolerance by patients compared with large-volume cleansing agents such as polyethylene glycol electrolyte solutions[3-5]. Although it presents a safety profile similar to other colonic cleansing agents, serious adverse events have been reported when administered in high volume or in patients with contraindications to NaP[6]. Polyethylenglicol (PEG) solutions are the most commonly used laxatives for colonic cleansing because of their safety profile and lack of contraindication. However, unpleasant taste and large volume of PEG lead to poor compliance and result in patient dissatisfaction. The two aforementioned agents are the most frequently used for colonic cleansing in many countries and despite the significant heterogeneity between different studies comparing them for colonic preparation, a systematic review showed similar adequate preparation rates, 75% for NaP and 71% for PEG[7,8]. Numerous clinical trials have also assessed prokinetic (metoclopramide, cisapride and tegazerod)[9-13] and laxative agents (magnesium citrate and bisacodyl)[14-16] associated with standard or lower volumes of this colon cleansing agents. Sharma *et al*[14] found that pretreatment with magnesium citrate or bisacodyl in addition to half-dose of PEG was associated with better preparation quality and patient satisfaction than full-dose of PEG. To our best knowledge, there is no study directly comparing whole and half-dose of PEG and NaP alone or in combination with bisacodyl in constipated and non-constipated patients. The aim of this study was to compare the efficacy and tolerability of whole doses of NaP and PEG and half-doses of those agents in combination with bisacodyl for colonic cleansing in constipated and non-constipated patients.

MATERIALS AND METHODS

This was a randomized, double-blind, four-arm study stratified by constipation. The study was carried out in accordance with the declaration of Helsinki. All patients included in the study signed an informed consent form. The human ethics committee from our institution approved the protocol.

Study population

All patients older than 18 years old who were scheduled for an elective outpatient's colonoscopy were eligible for participating in the study and were randomized in a 6-mo period (June-December 2011). As safety issues about NaP solutions have emerged, we only included healthy patients following the United States Food and Drug Administration recommendation to avoid renal damage. Patients were excluded if they presented one or more of the following characteristics: age younger than 18 years old, were hospitalized for any reason, hypersensibility to any of the components of PEG, NaP or bisacodyl, were under more than one antihypertensive medication, presented history of diarrhea (more than 3 bowel movements a day), acute or chronic renal failure, cardiovascular disease (history of myocardial infarction, congestive heart failure, unstable angina pectoris, unstable hypertension and/or cardiac arrhythmia), ascites, electrolite inbalance (hiponatremia, hipokalemia, hipocalcemia, hipomagnesemia or hyperphosphatemia), inflammatory bowel disease, partial or subtotal colectomy, ileus or suspected intestinal obstruction and pregnancy or breastfeeding, childbearing potential without contraception.

Study design

Patients who met all the inclusion criteria and no exclusion criteria were randomly assigned to receive one of the four colonic preparations according to a computer-generated randomization list. Randomization was stratified by constipation in order to make a subgroup analysis of constipated and non-constipated patients at the end of the study. Constipation was defined according to Thompson *et al*[17] criteria. Allocation was concealed using same color, size and weight closed boxes. The nurses that provided the patients with colonic preparation, the endoscopy assistant that evaluated the preparation compliance, tolerance and adverse reactions, the data analysts; and the endoscopists who evaluated bowel cleansing quality were blinded. If the patients had doubts about the preparation they could make a telephone call to a physician that was not blinded, was not present during

Table 1 Bowel preparation quality grading score used by the endoscopists

Excellent	No fecal matter or nearly none in the colon, small-to-moderate amounts of clear liquids
Good	Small amounts of thin liquid fecal matter seen and easily suctioned, mainly distal to splenic flexure, small lesions may be missed, > 90% mucosa seen
Fair	Moderate amounts of thick liquid to semisolid fecal matter seen and suctioned, included proximal to splenic flexure, small lesions may be missed, 90% mucosa seen
Poor	Large amounts of solid fecal matter found, precluding a satisfactory study, unacceptable preparation; < 90% mucosa seen

Table 2 Characteristics of the included patients *n* (%)

Characteristics	Prep 1	Prep 2	Prep 3	Prep 4	*P* value
Patients	78	78	84	84	
Age (yr), mean ± SD	59 ± 13.2	57 ± 11.1	60 ± 13.8	59 ± 10.9	NS
Sex					
Male	37 (47)	40 (51)	41 (49)	45 (53)	NS
Female	41 (53)	38 (49)	43 (51)	39 (47)	NS
Constipation	21 (27)	16 (21)	15 (12)	24 (29)	NS
Successful cecal intubation	78 (100)	78 (100)	84 (100)	84 (100)	NS

NS: Not significant.

colonoscopy nor participated in the endoscopic quality assessment, tolerability questionnaire, or statistical analysis. To reassure that endoscopists were blinded, a blinding challenge was performed after finishing the colonoscopy by asking them which of the four different colonic cleansing agents they thought the patients had received. A kappa coefficient of agreement was used for this purpose. A kappa under 0.3 and a non-significant *P* value was considered as an adequate blinding.

Prep 1 consisted of 90 mL of NaP alone (Gadolax®, Gador Laboratory, Argentina) 45 mL with four glasses of water at 4:00 pm and the other 45 mL at 8:00 pm of the day before the study. Prep 2 consisted of 45 mL of NaP with four glasses of water and 20 mg of bisacodyl at 4:00 pm the day before the study. Prep 3 consisted of 4 L of PEG (Barex®, Dominguez Laboratory, Argentina) alone starting at 4:00 pm the day before the study at a rate of 250 mL every 15 min until finishing the solution.

Prep 4 consisted of 2 L of PEG starting at the same time and with the same rate as mentioned before for prep 3 plus 20 mg of bisacodyl. Patients in all groups were encouraged to go through the same low fiber diet during the three days before the study and to adhere to a clear liquid diet from 8:00 am to midnight on the day before colonoscopy. Before colonoscopy the patients were asked to answer a questionnaire to assess patient satisfaction, tolerability, and compliance to the preparation. The questionnaire included yes/no responses for tolerance, preparation completed, and specific symptoms (nausea, vomiting, abdominal or chest pain, dizziness, bloating, and poor sleep). Before entering the Endoscopy Unit patients were asked not to reveal their assigned preparation to the Endoscopy Unit staff. Colonoscopies were done by four colonoscopists from the Endoscopic Unit and all studies were done between 7:30 am and 1:00 pm. All studies were performed using the same Storz Videocolonoscope. The quality of colonic cleansing was graded according to a previously reported scale[13] (Table 1). All endoscopists were trained on the scale using previously selected videos of colonoscopy with different colonic cleansing quality. Endoscopists were also asked if they thought there was a need to repeat the study due to inadequate preparation.

Statistical analysis

Statistical analysis were performed using statistical software SPSS for windows 10.0. Knowing that 70% of colonic cleansings are excellent or very good[1-3], a sample size of 88 patients in each group was calculated to detect a 20% difference in primary outcome with 80% of power at a standard level of significance $a = 0.05$. Categorical variables were compared using the Fisher exact test or χ^2 test. A *P* value of less than 0.05 was considered significant. Results were analyzed according to the intention-to-treat principle. Handling of loss to follow-up: We evaluated different assumptions about the incidence of events among participants lost to follow-up and the impact of those assumptions on the estimate of effect for the primary outcome. For this purpose, we used the $RI_{LTFU/FU}$ as proposed by Akl *et al*[18]. The $RI_{LTFU/FU}$ is defined as the event incidence among those lost to follow-up relative to the event incidence among those followed up. The assumptions we evaluated by combining a range of $RI_{LTFU/FU}$ values (1, 1.5, 2, 3.5 and 5) in the intervention group and control group.

RESULTS

A total of 349 patients scheduled for outpatient colonoscopy participated in the study and were randomized to receive one of the four colonic cleansing preparations. Three patients were excluded post-randomization because they met one or more exclusion criteria, 15 patients failed to present to the procedure and 7 presented incomplete colonoscopy because of fixed angulations (4 patients) or colonic neoplasia (3 patients). Finally, of the 346 randomized patients, information about the primary outcome was obtained from 324 patients (93%) (Figure 1). There were no significant differences among the four preparation groups with respect to: age, sex, cecal intubation, and constipation (Table 2).

Blinding challenge

There was no significant concordance between the endoscopists presumption and the colonic preparation group that the patients had been assigned to ($P = 0.56$, $\kappa = 0.019$). This observation reassures that the endoscopists were unaware of the assigned groups (blinding).

Quality of colonic cleansing

We obtained information about this outcome for 93% of

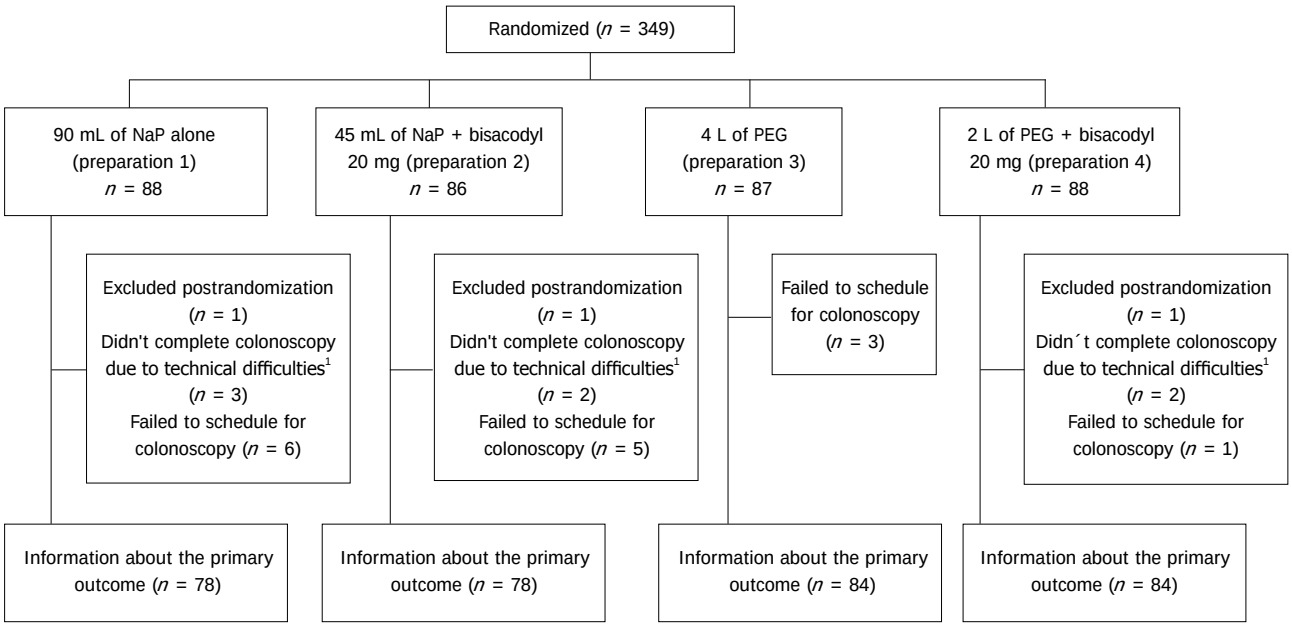

Figure 1 Flow chart of the included patients. [1]Fixed angulations or colonic neoplasia. NaP: Sodium phosphate; PEG: Polyethylenglicol.

patients. The quality of colonoscopic visualization was similar in the four different groups (Figure 2A).

Results were dichotomized into satisfactory colonic cleansing (excellent and good) and unsatisfactory (fair and poor). Satisfactory preparations were achieved in similar proportion in the different groups: prep 1, 82%, prep 2, 80%, prep 3, 79% and prep 4, 78% ($P > 0.05$) (Figure 2B). Endoscopists thought that only 6% of all the patients in this study needed to repeat the study because of inadequate colonic preparation. This was also similar between different preparations: prep 1, 3.4%, prep 2, 4.7%, prep 3, 6.8% and prep 4, 6.8% ($P > 0.05$) (Figure 2C).

We conducted a separate analysis of constipated and non-constipated patients. In the non-constipated patients, we didn´t find differences in the quality of colonic cleansing (Figure 2B) but the necessity to repeat colonoscopy was more frequent in prep 4 compared to prep 1 (11% *vs* 2%, $P < 0.05$) (Figure 2C). In constipated patients, NaP plus bisacodyl preparation (prep 2) achieved higher rate of satisfactory colonic cleansing than those receiving whole dose of PEG (prep 3): 95% *vs* 66% ($P = 0.03$) (Figure 2B).

Compliance

Both preparations containing NaP, presented better compliance than those containing PEG. Preparation was completed by 94% of the patients in prep 1, 100% of patients in prep 2, 81% of the patients in prep 3 and 87% of the patients in prep 4. Therefore, half-dose of NaP plus bysacodyl achieved the highest compliance (prep 2 *vs* 1, 3 and 4, $P < 0.01$) followed by full-dose of NaP (prep 1 *vs* 3 and 4, $P < 0.05$) (Figure 2D). In non-constipated patients, compliance was also higher in those preparations containing NaP compared to full-dose PEG: 95% (prep 1), 100 % (prep 2) *vs* 80% (prep 3) ($P < 0.05$) (Figure 2D). In constipated patients compliance was similar between different preparations.

Tolerability

The preparation was reported as tolerable in 77% of the patients in prep 1, 81% in prep 2, 82% in prep 3 and in 84% in the prep 4, there was no significant difference between the different preparations ($P > 0.05$). There was also no significant difference in tolerability between preparations in constipated and non-constipated patients (Table 3).

Symptoms profile

The most frequent adverse effects reported were: nausea (33%), bloating (30%) and abdominal pain (23%). There were no significant differences among different groups with respect to: nausea, vomiting, chest pain, bloating and dizziness (Table 3). Abdominal pain was more frequent in patients that received both preparations containing bisacodyl, prep 1, 16%, prep 2, 27%, prep 3, 19%, prep 4, 28%, but this difference didn´t reach statistical significance in the overall analysis ($P = 0.2$) (Table 3). The patients receiving NaP and bisacodyl preparations (prep 2) presented more frequently poor sleep than the other groups ($P < 0.05$) (Table 3). In non-constipated patients, abdominal pain was more frequent in those preparations containing bisacodyl: prep 2 (31%) and prep 4 (29%); compared to those without it: prep 1 (14%) and prep 3 (20%) ($P < 0.05$) (Table 3). The symptoms profile was similar between different preparations in constipated patients.

Patient preferences

Only 21% of all the patients would refuse to take the same colonic preparation in the future and almost 37% would like to try a different preparation. This finding was similar in the different groups. There was also no significant differences in patients perception in different groups

Table 3 Symptoms profile of different preparations *n* (%)

Adverse effects	Prep 1			Prep 2			Prep 3			Prep 4			*P* value		
	Overall	Non-constipated (*n* = 59)	Constipated (*n* = 22)	Overall	Non-constipated (*n* = 58)	Constipated (*n* = 20)	Overall	Non-constipated (*n* = 69)	Constipated (*n* = 15)	Overall	Non-constipated (*n* = 65)	Constipated (*n* = 20)	Overall	Non-constipated	Constipated
Tolerability	62 (77)	47 (80)	15 (68)	63 (81)	47 (81)	16 (80)	69 (82)	57 (83)	12 (80)	71 (84)	53 (82)	18 (90)	NS	NS	NS
Nausea	27 (33)	17 (29)	10 (13)	30 (38)	24 (41)	6 (30)	26 (31)	2 (32)	4 (26)	25 (29)	18 (28)	7 (35)	NS	NS	NS
Vomiting	6 (7)	2 (3)	4 (18)	3 (4)	6 (10)	3 (15)	9 (11)	2 (3)	1 (7)	6 (7)	5 (8)	3 (15)	NS	NS	NS
Abdominal pain	13 (16)	8 (14)	5 (23)	21 (27)	18 (31)	3 (15)	16 (19)	14 (20)	2 (13)	24 (28)	19 (29)	5 (33)	0.2	< 0.05[1]	NS
Bloating	25 (31)	17 (29)	8 (36)	21 (27)	15 (26)	6 (30)	27 (32)	22 (32)	5 (33)	24 (28)	16 (25)	8 (40)	NS	NS	NS
Insomnia	10 (12)	9 (15)	1 (5)	17 (21)	14 (24)	3 (15)	5 (6)	4 (6)	1 (7)	11 (13)	10 (15)	1 (5)	< 0.05[2]	NS	NS
Dizziness	12 (15)	10 (17)	2 (9)	7 (9)	6 (10)	1 (5)	7 (8.3)	5 (7)	2 (13)	8 (9)	5 (8)	3 (15)	NS	NS	NS
Chest pain	1 (1)	1 (2)	0 (0)	2 (3)	1 (2)	1 (5)	1 (1)	1 (1)	0 (0)	1 (1)	0 (0)	1 (5)	NS	NS	NS

[1]Prep 2 and 4 *vs* prep 1 and 3; [2]prep 2 *vs* prep 1, 3 and 4. NS: Not significant.

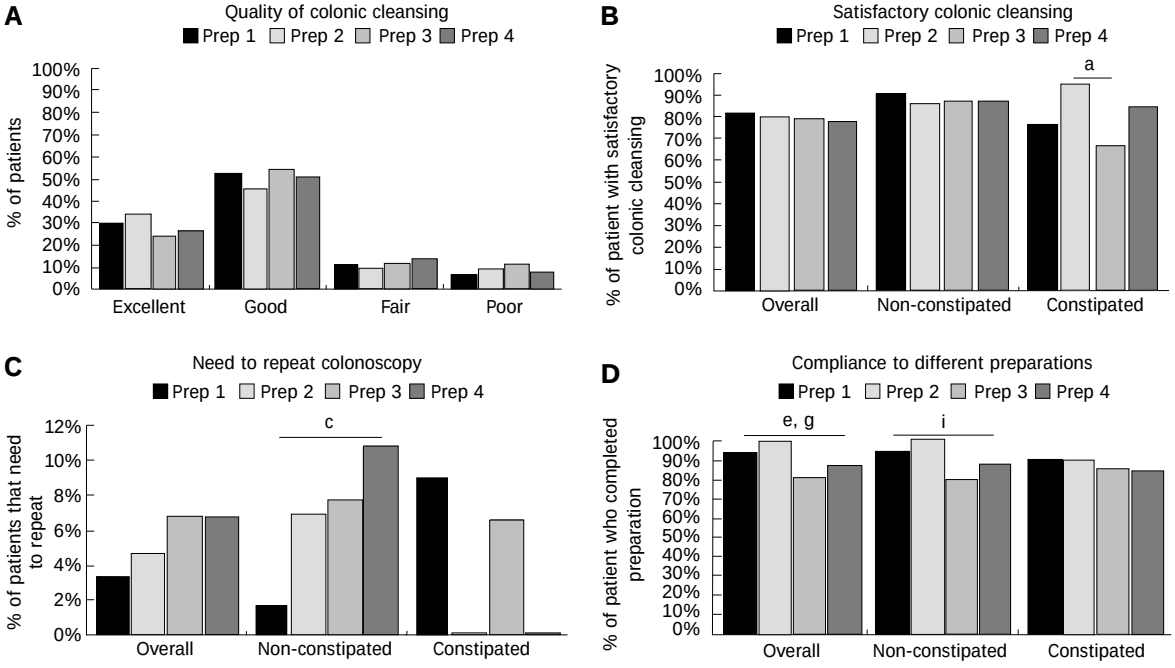

Figure 2 Efficacy and compliance of different preparations. A: Preparation quality score obtained with different preparations (no statistical difference between groups). Values are expressed as the percentage of patients. Prep 1, 90 mL of sodium phosphate (NaP); Prep 2, 45 mL of NaP followed by 20 mg of bisacodyl; Prep 3, 4 L of polyethylenglicol (PEG); Prep 4, 2 L of PEG followed by 20 mg of bisacodyl; B: Percentage of patients who had satisfactory and unsatisfactory colonic cleansing in the overall analysis and in the subgroup of constipated and non-constipated patients. Constipated patients obtained a higher rate of satisfactory colonic cleansing with prep 2, (45 mL of NaP followed by 20 mg of bisacodyl) when compared to preparation 3 (4 L of PEG) (prep 2 *vs* 3, [a]$P = 0.03$); C: Endoscopist appraisal on the necessity to repeat colonoscopy due to inadequate preparation in the overall analysis and in the subgroup of constipated and non-constipated patients. Non-constipated patients assigned to prep 4 (2 L of PEG followed by 20 mg of bisacodyl) needed to repeat colonoscopy due to inadequate preparation more often when compared to patients assigned to prep 1 (90 mL NaP) (prep 4 *vs* 1, [c]$P < 0.05$); D: Compliance to different preparations in the overall analysis and in the subgroup of constipated and non-constipated patients. Prep 2 (45 mL of NaP followed by 20 mg of bisacodyl) *vs* 1 (90 mL NaP), 3 (4 L of PEG) and 4 (2 L PEG followed by 20 mg of bisacodyl), [e]$P < 0.05$; prep 1 (90 mL NaP) *vs* 3 (4 L of PEG) and 4 (2 L of PEG followed by 20 mg of bisacodyl), [g]$P < 0.05$; prep 1 (90 mL NaP) and 2 (45 mL of NaP followed by 20 mg of bisacodyl) *vs* prep 3 (4 L of PEG), [i]$P < 0.05$.

in constipated and non-constipated patients.

Loss to follow up

None of the different assumptions of incidence of events in loss to follow up patients changed significantly the estimate of the effect in the different outcomes.

DISCUSSION

There is a growing acceptance of colorectal cancer screening with colonoscopy. Its goal is to identify and remove neoplastics polyps; therefore a high-quality preparation that lends to a clear visualization is crucial. Inadequate colonic cleansing could lead to a diminished adenoma detection rate[19-21]. This has been recently shown to be the strongest predictor of interval colorectal cancer[22,23]. However none of the different preparation agents are ideal for colonic cleansing. They present historic rates for adequate cleansing that ranges from 70% to 82%[24-26]. Tolerability and side effects are probably the main issues and represent some of the most important reasons for patient's refusal to the study[25]. In an attempt

to decrease these sides effects, many studies have evaluated different doses of conventional preparation agents and pretreatment with prokinetics or laxative agents, but there is little information about the effect of these preparations in subgroups of constipated and non-constipated patients[7]. In this study we compared two of the most used colonic cleansing agents, PEG and NaP. As in past years, there has been a strong tendency to prepare patients with half doses of this previously mentioned agents associated with bisacodyl because of commercially available preparation kits. We decided to carry out a direct comparison between whole dose of PEG and NaP alone and half doses of these two agents associated with bisacodyl in constipated and non-constipated patients. Our studies main limitations include, single centre study and the use of non-validated scale for the evaluation of primary outcome (quality of colonic preparation) and patient related outcomes (tolerability, adverse events, preferences). Nevertheless, the randomized, double-blind, four-arm study design and the constipated and non-constipated subgroup analysis could provide useful information on how to manage patients that might undergo colonoscopy. Similar to the results reported by previous studies, almost 80% of patients presented to colonoscopy with satisfactory colonic cleansing (excellent or very good). We did not find any difference with respect to quality of colonic cleansing in the different groups, even in those with half doses of NaP and PEG. Preparation quality was also similar in different groups in non-constipated patients, but endoscopists thought that there was a greater necessity to repeat the study due to an inadequate colonic cleansing in prep 4 (half dose of PEG plus bisacodyl) compared to prep 1 (whole dose of NaP) (11% *vs* 2%, $P < 0.05$). Although this is a non-validated and subjective outcome; we think it´s interesting to know endoscopist perception, because it represents what they really do in the daily practice and is a patient important outcome. In constipated patients, preparations containing bisacodyl presented higher rates of satisfactory colonic cleansing: 95% (prep 2) and 85% (prep 4) *vs* 67% (prep 3) and 77% (prep 1). Only NaP plus bisacodyl reached a statistically significant difference compared to whole dose of PEG (95% *vs* 66%, $P = 0.03$). The prokinetic effect of the bisacodyl may explain the high rates of satisfactory colonic preparations. Even though a statistical significant difference was only obtained with NaP plus bisacodyl and not with PEG plus bisacodyl, we think that this may be related to the small sample size of the constipated patients subgroup. In the overall analysis, compliance was higher in groups with preparations containing NaP, reaching 100% in the half dose NaP plus bisacodyl group and 94% in the whole dose of NaP. In non-constipated patients, compliance with NaP preparations was higher than whole doses PEG preparation. We were not able to demonstrate higher compliance rates with NaP preparations in constipated patients. However, the observed tendency to higher compliance in these groups along with evidence of previous studies lead us to believe that we were unable to find statistically significant difference due to the small sample size. Tolerability (taste, nausea, *etc.*) was similar in the different groups. Consequently, we believe that the differences in compliances were related to the volume of the preparations and probably not to tolerability. The most frequent adverse effect was nausea followed by bloating and abdominal pain. None of the different preparations were associated with an antiemetic medication, so we do not know if nausea and probably tolerance could be optimized with this association. Bisacodyl has been previously associated with abdominal cramping. In this study both groups with preparations containing bisacodyl presented higher incidence of abdominal pain: prep 1, 16%, prep 2, 27%, prep 3, 19%, prep 4, 28%, but the difference was not statistically significant. The difference was statistically significant when we analyzed the subgroup of non-constipated patients: prep 1, 14%, prep 2, 31%, prep 3, 21%, prep 4, 29% ($P < 0.05$). Curiously, constipated patients that received preparation with bisacodyl did not have higher incidence of abdominal pain. We think that constipated patients can present a motility dysfunction that could be optimized with the administration of the bisacodyl and that could explain the difference perception of abdominal pain in constipated and non-constipated patients. In the overall analysis, the combination of NaP with bisacodyl was also associated with higher rates of poor sleep than other preparations. We did not find any previous reports of this association and we do not have a specific explanation for this finding. However, it seems that the bisacodyl adverse effects profile is different in constipated and non-constipated patients, suggesting that constipated patients are less affected by these effects. Although the evaluated preparations presented a high rate of satisfactory colonic cleansing, compliance and a low profile of side effects, almost 37% of all the patients when asked, would prefer to try a different preparation in next colonoscopy. This study shows that none of the preparations agents is ideal, and highlights the need to improve bowel cleansing methods not only to get high quality colonic cleansing, but also to achieve a higher adherence to colonoscopy screening and surveillance programs. In summary, the quality of colonic cleansing and side effects profile of evaluated preparations are different in constipated and none-constipated. In non-constipated patients, preparation quality is similar with whole or half doses of NaP or PEG, alone or in combination with bisacodyl and compliance is higher with NaP preparations. Bisacodyl addition is associated with a higher incidence of adverse events. In constipated patients, the combination of NaP with bisacodyl is the most effective preparation. In this subgroup of patients, bisacodyl addition is not associated with higher incidence of adverse effects as noticed in non-constipated patients.

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COMMENTS

Background

Colonoscopy has become the standard procedure for the diagnosis and treatment of colon diseases. Adequate bowel cleansing is essential for a high-quality effective and safe colonoscopy.

Research frontiers

Numerous clinical trials have assessed the efficacy of whole or low doses of sodium phosphate (NaP) and polyethylenglicol (PEG) alone or with bisacodyl. There is no information about which is the most suitable preparation regimen for constipated and non-constipated patients.

Innovations and breakthroughs

Their randomized clinical trial compared the efficacy and tolerability of whole and half doses of NaP and PEG alone or associated with bisacodyl preparations and explored the different effect on constipated and none-constipated patients.

Applications

Compliance was higher with NaP preparations in non-constipated patients and the addition of bisacodyl was associated with higher incidence of adverse effects. Half-dose of NaP plus bisacodyl was the most effective preparation in constipated patients. Bisacodyl was not associated with adverse effects in constipated patients as noticed in non-constipated patients.

Peer review

This is a good study in which authors compare the efficacy of different doses of NaP and PEG alone or with bisacodyl for colonic cleansing in constipated and non-constipated patients. The results are interesting and suggest that in non-constipated patients, compliance is higher with NaP preparations, and bisacodyl is related to adverse effects. In constipated patients NaP plus bisacodyl is the most effective preparation.

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BRIEF ARTICLE

Diagnostic accuracy of a new point-of-care screening assay for celiac disease

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Abstract

AIM: To determine the diagnostic accuracy of a new point-of-care assay detecting anti-deamidated gliadin peptides in celiac disease (CD) patients.

METHODS: One-hundred-and-twelve patients (age range: 1.8-79.2 years old) with clinical symptoms suggestive of CD and/or first-degree relatives (FDR) of CD patients (n = 66), and confirmed CD on a gluten-free diet (GFD) (n = 46), were prospectively enrolled in the study at Gastroenterology outpatient clinics for adult patients and from the Gastroenterology Consultation Ward at the Pediatric Department of the University Hospital of Geneva. Written informed consent was obtained from all subjects enrolled. The study received approval from the local ethics committee. The original CD diagnosis had been based on serum-positive IgA anti-tissue transglutaminase enzyme-linked immunosorbent assay (ELISA) (QuantaLite™, Inova Diagnostics, San Diego, CA, United States) and on biopsy results. Serum samples from all study participants were tested by the new CD lateral flow immunochromatographic assay (CD-LFIA) device, Simtomax® Blood Drop (Augurix SA, BioArk, Monthey, Switzerland) to detect immunoglobulin (Ig)A and IgG antibodies against deamidated gliadin peptides. The diagnostic performance was evaluated using receiver operating characteristic curves with 95%CIs. A cut-off of 2 on the Rann colorimetric scale was used to calculate the device's sensitivity and specificity.

RESULTS: CD-LFIA was highly accurate in detecting untreated celiac patients. In the group of patients with CD symptoms and/or FDR, eight new cases of CD were detected by ELISA and biopsy. All of these new cases were also correctly identified by CD-LFIA. The test yielded four false positive and four false negative results. The false positive results were all within the groups with clinical symptoms suggestive of CD and/or FDR, whereas the false negative results were all within the GFD group. The test yeld a sensitivity of 78.9% (95%CI: 54.4-93.9) and specificity of 95.7% (95%CI: 89.4-98.8), and the area under the curve reached 0.893 (95%CI: 0.798-0.988). The Kappa coefficient, calculated according to the values obtained by two readers from the same device, was of 0.96 (SE: 0.06). When the GFD patients were excluded from the analysis, the area under the curve reached 0.989 (95%CI: 0.971-1.000) and the Kappa coefficient, calculated according to the values obtained by two readers from the same device, became 0.96 (SE: 0.07). Furthermore, using the Rann scale cut-off of 2 without the GFD pa-

tients, sensitivity was 100% and specificity was 93.1% (95%CI: 83.3-98.1).

CONCLUSION: The new CD-LFIA rapid screening test shows good diagnostic accuracy, sensitivity and specificity, and may rule out CD in patients with CD-related symptoms.

Key words: Celiac disease; Deamidated gliadin; Total immunoglobulin A; Screening; Point-of-care assay

Core tip: The aim of the present study was to evaluate the clinical accuracy of a new point-of-care device that is based on deamidated gliadin peptides (DGP) for diagnosis of celiac disease (CD). One-hundred-and-twelve patients with clinical symptoms suggestive of CD and/or first-degree relatives of CD patients, and patients with confirmed CD on a gluten-free diet, were prospectively enrolled in the study. The actual CD diagnosis had been based on serum-positive immunoglobulin A anti-tissue transglutaminase results by enzyme-linked immunosorbent assay and on biopsy findings. Overall evaluation shows that the new DGP-based rapid point-of-care test is an excellent screening tool for high-risk populations.

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INTRODUCTION

Celiac disease (CD) is a common T cell-mediated gluten-sensitive enteropathy. CD diagnosis remains challenging since only a minority of celiac patients presents with specific gastrointestinal symptoms and the majority of patients manifests atypical extra-intestinal symptoms that may lead to missed diagnosis or misdiagnosis[1].

The initial diagnosis of CD is made by serological testing and confirmed either by histopathologic examination of small-bowel biopsy or further blood tests, depending on the serum concentration of anti-tissue transglutaminase (tTG) autoantibodies and the patient's age. Serology markers of CD have evolved over the years, as more specific antibodies have been identified. Currently, endomysial and anti-tTG autoantibodies are considered among the most reliable CD diagnostic markers[2,3]. Although both of these markers exhibit high sensitivity and specificity, their accuracy remains controversial in patients of a very young age or with a minor degree of mucosal damage; moreover, their accuracy for monitoring CD status in patients following a gluten-free diet (GFD) remains controversial[4,5]. Very recently, a new generation of assays based on the detection of antibodies against deamidated gliadin peptides (DGP) has demonstrated very high sensitivity, as well as a diagnostic accuracy that is at least equivalent to the established serological assays[6-9].

Given the high prevalence of the disease and likelihood of missed diagnosis, several simple immunoassays have been developed as a first step toward reducing the turnaround time for result delivery and initiating patient counseling and treatment[10]. Unfortunately, these new assays feature several drawbacks, including the reliance on serum samples, requirement for some basic laboratory equipment, their lack of sensitivity to identify celiac disease and to identify patients suffering from an immunoglobulin (Ig)A deficiency[11].

To overcome these issues, a multi-analytic lateral-flow immunochromato-graphic assay (LFIA) device, the Simtomax® Blood Drop system, has been developed that is based upon the detection of both IgA and IgG anti-DGP and total IgA. In this study, this new CD-LFIA test was evaluated in a ward setting to determine its accuracy, sensitivity, and specificity as compared to the established laboratory serology assay.

MATERIALS AND METHODS

Patients

Patients visiting the gastroenterology adult outpatient clinic and the gastroenterology consultation ward in the pediatric department of the University Hospital of Geneva from April 2008 to December 2009 were prospectively enrolled in this study. Criteria for study inclusion were clinical symptoms suggestive of CD and/or first-degree relatives (FDR) of CD-confirmed patients (n = 66), and CD-confirmed patients on a gluten-free diet (n = 46). Written informed consent was obtained from all subjects prior to study participation. The study was carried out with approval from the local ethics committee board (University Hospital of Geneva application 07-217).

Diagnostic methods

The diagnosis of CD was based on results of serologic enzyme-linked immunosorbent assay (ELISA) tests (described below) and small intestine mucosal biopsy examination.

The IgA and IgG anti-tTG QuantaLite™ ELISA tests from Inova Diagnostics (San Diego, CA, United States) were used to detect serum samples from all study participants. For both tests, concentrations > 30 U/mL were considered moderate to strongly positive for CD.

Total IgA was measured by the BN II nephelometer (Dade Behring Ltd., Milton Keynes, United Kingdom) according to the manufacturer's protocol. Results were evaluated by referring to a standard curve and by using < 0.05 g/L as the cut-off point to identify IgA deficiency. For the study population, normal values ranged between

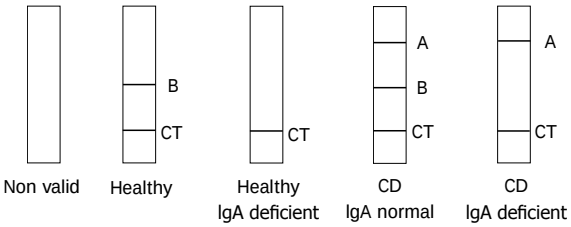

Figure 1 Celiac disease lateral-flow immunochromatographic assay visual result interpretation. CT: Control line; A: Position for detection of IgA and IgG anti-DGP; B: Position for detection of total IgA; CD: Celiac disease; IgA: Immunoglobulin A.

0.05 and 4.07 g/L, depending on the patient's age.

Small-bowel biopsies were obtained from all patients who tested positive by serology tests. The mucosal biopsy sections were analyzed by an experienced histopathologist, who assessed the following pathologic features of CD: villus atrophy, crypt hyperplasia, increased intraepithelial lymphocytes, and chronic inflammation in the lamina propria. The diagnosis of CD was subsequently confirmed according to the modified Oberhuber-Marsh classification system[12].

CD-LFIA test

Serum samples were collected from all study participants, stored at -20 ℃, and tested in duplicate on the Simtomax® Blood Drop system (Augurix SA, BioArk, Monthey, Switzerland). This CD-LFIA device was developed as an antigen direct sandwich assay capable of detecting both human IgA and IgG anti-DGP, as well as total IgA. A synthetic DGP conjugated to a carrier protein[7] was attached to the device's nitrocellulose membrane at the test line A position for detection of IgA and IgG anti-DGP. Mouse anti-human IgA was attached at the test line B position for detection of total IgA. In the test, secondary gold-conjugated antibodies bind to the patient's antibodies to form detectable complexes that are captured by the test in lines A and B. The control line, CT, is formed by the interaction of nitrocellulose-attached goat anti-mouse antibodies with the secondary gold-conjugated antibodies. All the lines are formed in 10-15 min. A CD-positive test result was indicated by detection of both the CT and A lines. IgA deficiency was indicated by absence of the B line. Figure 1 illustrates the device run with samples representative of the various diagnoses. Each sample was tested by two independent user-operators blinded to the subject's histories and laboratory findings and each of whom performed the CD-LFIA interpretations twice on two independent devices.

The CD-LFIA test lines were semi-quantitatively evaluated by using the Rann colorimetric scale (British Biocell International, Cardiff, United Kingdom). A series of five pink/red lines with a colloidal gold solution of decreasing optical density were sprayed on a card, and yielded line intensities ranging from 10 (maximum line intensity) to 2 (weakest visible line). Accordingly, the cut-off value for a positive result was set at 2. Spiked celiac serum equivalent to the ELISA QuantaLite™ cut-off value was used to set the visual limit of detection.

Statistical analysis

Statistical analyses were carried out by the STATA software (version 11; College Station, TX, United States). The StatXact-8 software (Cytel Inc., Cambridge, MA, United States) was used to calculate the 95%CIs. The diagnostic performance of the CD-LFIA test was evaluated by generating receiver operating characteristic (ROC) curves for each CD-LFIA device used and for each user-operator[13]. The areas under the ROC curves (AUCs) were provided with the corresponding 95%CIs. The "gold standard" diagnostic methods of laboratory ELISA and biopsy results were used for comparative analyses to evaluate the testing features of CD-LFIA. The cut-off of 2 Rann, which represented the delimitation between a "positive" and "negative" result (visible/invisible band) was used to calculate the CD-LFIA test' s sensitivity, specificity, and positive and negative likelihood ratios (LR+, LR-). Concordance between sample and device replicates was evaluated by calculating the Kappa coefficient and its SE.

RESULTS

Overall agreement between CD-LFIA and ELISA IgA-tTG laboratory test results

A total of 112 patients (71 females, 36 males; no sex information was available for five patients) with a mean age of 24.6 years old (median 13.9 years; range: 1.8-79.2 years) were analyzed.

Based on the laboratory values and biopsy results, a group of eight newly diagnosed celiac patients was found amongst the group of 66 patients composed of FDR and patients with clinical symptoms suggestive of CD. Thus, the CD prevalence in this study was 12.1%. All of the eight newly diagnosed CD patients were correctly identified by the CD-LFIA test (range of Rann values between 3-10). Among them, one did not undergo intestinal biopsy but had typical clinical presentation of CD and high positive titers of IgA-tTG (137 U/mL). The remaining seven had a positive intestinal biopsy (Marsh 3 and 4) and positive titers of IgA-tTG (119 -197 U/mL). Out of the 58 CD sero-negative patients, four were positive by the CD-LFIA test, however their Rann scores were just near cut-off: 2-3.

Of the 46 CD GFD patients, two patients showed selective IgA deficiency, and the CD-LFIA test detected this at 100%. Out of the 46 CD GFD patients, eleven of the CD GFD patients tested positive on the IgA-tTG ELISA, with three having high levels (116-170 U/mL) and the remaining eight having moderate levels (near the cut-off value; 30-55 U/mL). Among those 11 patients with positive IgA-tTG serology, four had negative results with the CD-LFIA test. These four patients had IgA-tTG ELISA levels near the cut-off values (36-55 U/mL for IgA-tTG for ELISA) and values of 0 Rann for CD-LFIA. The remaining 35 CD GFD patients were cor-

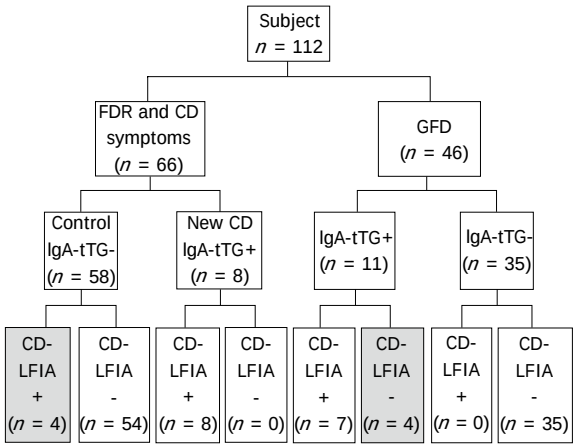

Figure 2 Histogram showing the immunoglobulin A-tissue transglutaminase enzyme-linked immunosorbent assay and celiac disease lateral-flow immunochromatographic assay test results. Text in gray indicates false-positive and false-negative results by celiac disease lateral-flow immunochromatographic assay (CD-LFIA). FDR: First-degree relatives; GFD: Gluten-free diet; Control: First-degree relatives and patients with celiac disease symptoms diagnosed as celiac disease (CD)-negative; IgA-tTG: Immunoglobulin A-tissue transglutaminase.

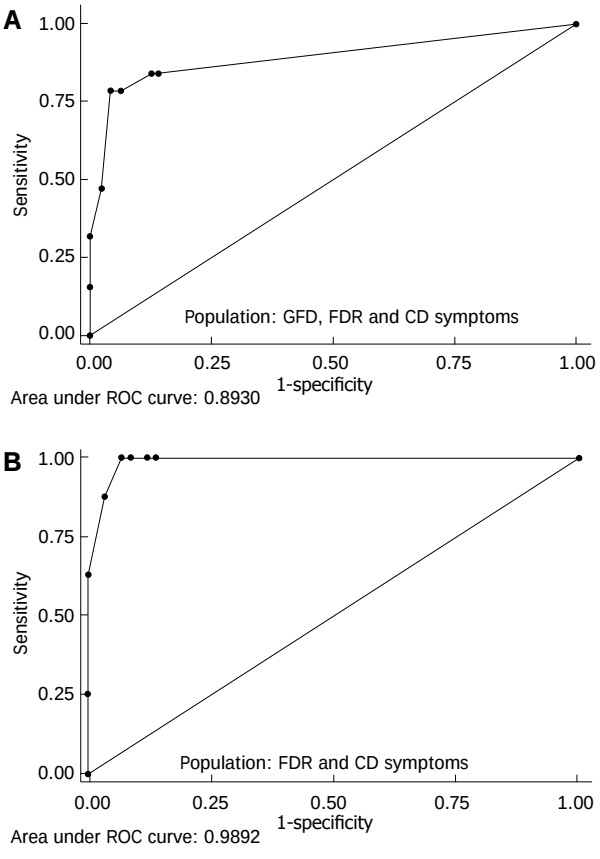

Figure 3 Diagnostic performance of the celiac disease lateral-flow immunochromatographic assay test determined by receiver operating characteristic curve analysis. A: GFD, FDR and CD symptoms; B: FDR and CD symptoms. FDR: First-degree relatives; CD: Celiac disease symptoms; GFD: Gluten-free diet; ROC: Receiver operating characteristic.

rectly identified as CD-negative by CD-LFIA. The overall agreement between the CD-LFIA test and the ELISA laboratory test results is shown in Figure 2.

Thus, CD-LFIA tests showed four false-positive results, all in the FDR and CD symptoms group. All of the ELISA laboratory test results were below the cut-off value and the Rann scores were between 2 and 3, just near the cut-off value. There were also four false-negative results obtained by the CD-LFIA device, all of which were from the CD GFD group. The serological IgA-tTG level of these patients was near the cut-off values.

Table 1 Celiac disease lateral-flow immunochromatographic assay result compared to diagnosis of celiac disease

		IgA-tTG ELISA		Total
		Positive	Negative	
GFD, FDR and CD symptoms				
CD-LFIA	Positive	15	4	19
	Negative	4	89	93
		19	93	112
FDR and CD symptoms				
CD-LFIA	Positive	8	4	12
	Negative	0	54	54
		8	58	66

Celiac disease lateral-flow immunochromatographic assay (CD-LFIA) result compared to diagnosis of celiac disease based on elevated titers of immunoglobulin A-tissue transglutaminase (IgA-tTG) in a population including gluten-free diet (GFD), first-degree relatives (FDR) and patients with celiac disease (CD)-related symptoms and FDR and patients with CD-related symptoms only. ELISA: Enzyme-linked immunosorbent assay.

Evaluation of the diagnostic performance of CD-LFIA on a population including patients monitored for compliance with GFD

The AUCs for each CD-LFIA device used and for each user-operator were 0.869 (95%CI: 0.764-0.975) and 0.893 (95%CI: 0.798-0.988), indicating excellent diagnostic performance of the test (Figure 3).

These results yield a sensitivity for the CD-LFIA device of 78.9% (95%CI: 54.4-93.9) and a specificity of 95.7% (95%CI: 89.4-98.8), as compared to the serological IgA-tTG levels detected by the ELISA laboratory tests. Considering the newly diagnosed CD patients (n = 8), the sensitivity was 100% (95%CI: 63.1-100) for both user-operators (Table 1).

Although the CD-LFIA is dependent upon the user-operator's semi-quantitative assessment of the colors of the reactive bands, the results were very reproducible between devices and user-operators. The concordance between user-operators and devices was indicated by the Kappa coefficients of 0.96 (SE = 0.06) and 0.92 (SE = 0.05), respectively.

In addition, an LR+ of 18.4 (95%CI: 7.0-51.8) and an LR- of 0.22 (95%CI: 0.08-0.46) were found for the CD-LFIA test when compared to the IgA-tTG ELISA (Table 1).

Evaluation of the diagnostic performance of CD-LFIA on a high-risk population

Exclusion of the CD GFD patients from the ROC anal-

ysis brought the AUC up to 0.989 (95%CI: 0.971-1.000), depending on the device and user-operator (Figure 3).

The kappa coefficient was 0.96 (SE = 0.07), indicating an excellent concordance between devices and user-operators.

In addition, when the CD GFD patients were excluded and the Rann cut-off of 2 was used, the sensitivity was of 100% (95%CI: 63.1-100) and the specificity remained nearly unchanged at 93.1% (95%CI: 83.3-98.1) (Table 1).

The LR+ became 14.5 (95%CI: 5.8-49.0) and the LR- became 0.00 (95%CI: 0.00-0.39), respectively (Table 1).

DISCUSSION

Diagnostic tests play a vital role in medicine, not only to confirm the presence of diseases but also to rule them out[14]. Diagnosis of CD has improved significantly in the past 20 years, as highly sensitive and specific biomarkers were identified[15]. Nevertheless, the prevalence of CD has dramatically increased over this same period (rising from a previously assumed 0.1% to up to 1.0%)[16,17]. This increase is probably largely due to identification of patients suffering from mild or atypical forms of CD. Moreover, large epidemiological screening studies have revealed that CD is a worldwide health concern[18]. Besides the improved detection methods, other etiological factors appear to have contributed to the increased prevalence[16], and, similar to other autoimmune conditions, these may include different environmental factors, such as gluten, antigens in breast milk, or from other pathogenic infections[19,20].

Unfortunately, CD remains one of the most common underdiagnosed medical conditions, with estimates of more than 90% of the patients being unrecognized[19,21]. Due to mild and atypical symptoms, the diagnosis of CD is often a challenge for many physicians, resulting in delays in diagnosis (up to 11 years[21]) and high rates of patient dissatisfaction and discomfort.

A large retrospective study of a managed-care population demonstrated that timely CD diagnosis was associated with a significant overall cost reduction that was attributable to reduced amounts of office visits, laboratory services, diagnostic and imaging support services, and endoscopy procedures[22]. Several simple, visual assays have been developed to promote the feasibility of CD screening programs[4,23-27]. However, while these assays have been demonstrated as reliable and easy-to-use, they are limited in sensitivity and lack the ability to concomitantly detect IgA deficiency[11].

Therefore, there is a clear unmet clinical need for a rapid and discriminative point-of-care test that could facilitate the management of patients consulting in primary care centers for CD-related symptoms. To this end, in this study, we compared the validity of the newly developed rapid point-of-care diagnostic device for detecting both human IgA and IgG anti-DGP to the measurements of serological IgA and IgG anti-tTG levels detected by routine laboratory ELISA. Sensitivity and specificity are two features of a diagnostic test that measure the validity of a new test as compared to a gold standard test, such as the ELISA. The ROC curves, as well as the corresponding AUCs, are effective measures of the inherent validity of a diagnostic test. Here, we found that the CD-LFIA test had a sensitivity of 100% for the detection of new CD cases, and result interpretation appeared unambiguous between multiple devices and multiple user-operators. The ROC curve indicated that, at a cut-off of 2 Rann, the device has a good discriminative ability between patients with CD and those without CD. The high values of the AUCs (up to 0.989) indicated an excellent accuracy of the CD-LFIA test. LR+ and LR- values represent measures of the performance of a diagnostic method, independent of disease prevalence[18,28]. The CD-LFIA test in this study achieved a LR+ of 14.5, indicating that patients having CD are 15 times more likely to have a positive test than those who are healthy. Moreover, the LR- of 0.0 indicated that the CD-LFIA test is very good at ruling out the disease.

The particular challenges to this test concerned interpretation of samples with weak reactivity that were exclusively representative of the CD GFD patients and would affect monitoring of CD status in this patient population. For this specific group, another approach may be required.

Here, we showed that CD-LFIA is highly accurate in detecting untreated celiac patients. It can be easily performed during the course of a consultation in primary care to test patients with symptoms suggestive of CD, and may represent a reliable alternative to the traditional laboratory assays. With specificity and sensitivity of 93.1% and 100%, respectively, and a LR-value of 0.0, CD-LFIA appears highly suitable for ruling out CD, representing an interesting tool in an exclusion diagnostic strategy. In case of positive serology, the physician can proceed to further investigations by the traditional laboratory assay. Therefore, CD-LFIA can be used as a rapid and accurate test to rule out CD in patients presenting with CD-related symptoms to primary care centers.

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COMMENTS

Background

Traditionally thought to be a rare childhood disease, celiac disease (CD) is currently recognized as a frequent condition both in adults and children and has become a widespread public health concern. CD diagnosis can be quite challenging for physicians since only a minority of celiac patients suffer from specific gastrointestinal symptoms. The majority of patients present with an atypical extra-intestinal manifestation that may not raise the physician's suspicion of CD. Laboratory-based methods, such as enzyme-linked immunosorbent assays (ELISA), remain the primary screening tool for CD. However, these tests are labor intensive and relatively high cost. Development and implementation of simple immunoassays will be a first step toward reducing the turnaround time for

result delivery and patient counseling and treatment.

Research frontiers

Serology markers of CD have evolved over the years with the identification of more disease-specific antibodies. Endomysial and anti-tissue transglutaminase (tTG) autoantibodies are currently considered among the most reliable of the CD-related markers. Although these markers exhibit a high sensitivity and specificity, their accuracy in very young children, in patients with a minor degree of mucosal damage, and for the follow-up of CD patients under a gluten-free diet remains controversial. Very recently, a new generation of assays based on the detection of antibodies against deamidated gliadin peptides (DGP) has demonstrated very high sensitivity for CD, as well as diagnostic accuracy that is at least equivalent to the traditional immunoassays.

Innovations and breakthroughs

A new rapid point-of-care serologic screening test based on detection of anti-DGP antibodies (immunoglobulin, IgA and IgG) and total IgA by a lateral flow immunochromatographic assay was evaluated in a pediatric and adult population and compared to ELISA reference laboratory serology assays. The new test was found to be rapid and highly accurate for ruling out CD in patients with CD-related symptoms.

Applications

The test can be easily performed during the course of a consultation visit and may represent a reliable alternative to the traditional laboratory assays, and appears to be highly suitable for ruling out CD in primary care centers in patients with CD-related symptoms.

Peer review

The manuscript evaluates the use of a new point-of-care assay for diagnosing CD in a clinical setting and compares its use to traditional tTG ELISA measurements. The test is based on simultaneous detection of IgA and IgG DGP antibodies and total IgA. The test shows a good accuracy in diagnosing CD. This is important as it suggests the test as a reliable alternative to laboratory assays for ruling out CD in patients with CD-related symptoms.

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BRIEF ARTICLE

Rikkunshito improves globus sensation in patients with proton-pump inhibitor-refractory laryngopharyngeal reflux

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Abstract

AIM: To investigate the effect of rikkunshito on laryngopharyngeal reflux (LPR) symptoms and gastric emptying in patients with proton-pump inhibitor (PPI)-refractory LPR.

METHODS: In total, 22 patients with LPR were enrolled. Following a 2-wk treatment with PPI monotherapy, PPI-refractory LPR patients were randomly divided into two treatment groups (rikkunshito alone or rikkunshito plus the PPI, lansoprazole). LPR symptoms were assessed using a visual analog scale (VAS) score, gastrointestinal symptoms were assessed using the gastrointestinal symptom rating scale (GSRS), and gastric emptying was assessed using the radio-opaque marker method prior to and 4 wk following treatments.

RESULTS: The 4-wk treatment with rikkunshito alone and with rikkunshito plus the PPI significantly decreased the globus sensation VAS scores. The VAS score for sore throat was significantly decreased following treatment with rikkunshito plus PPI but not by rikkunshito alone. Neither treatment significantly changed the GSRS scores. Rikkunshito improved delayed gastric emptying. We found a significant positive correlation between improvements in globus sensation and in gastric emptying (r^2 = 0.4582, P < 0.05).

CONCLUSION: Rikkunshito improved globus sensation in patients with PPI-refractory LPR, in part, because of stimulation of gastric emptying. Thus, rikkunshito is an effective treatment for PPI-refractory LPR.

Key words: Laryngopharyngeal reflux; Gastroesophageal reflux disease; Globus sensation; Gastric emptying; Rikkunshito

Core tip: Regarding the treatment of laryngopharyngeal reflux (LPR) symptoms such as globus sensation and a scratchy feeling, proton pump inhibitors (PPIs) are considered the mainstay. We investigated the effects of rikkunshito on globus sensation and gastric emptying in patients with PPI-refractory LPR.

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INTRODUCTION

Symptoms or complaints of globus sensation ("globus"), a "lump in the throat" feeling located between the upper edge of the sternum and the cricoid region, are common. Recently, gastroesophageal reflux disease (GERD) has been identified as a major cause of globus[1-3]. Stom-

ach acid reflux produces a number of extraesophageal symptoms in the laryngopharynx, commonly referred to as laryngopharyngeal reflux (LPR)[1,2], which include a hoarse voice, cough, a scratchy feeling in the throat, and globus[1-3]. However, the etiology of globus remains unclear. Recent studies have suggested the condition may be caused by hypertonicity in the upper esophageal sphincter (UES)[4,5]. We have demonstrated that elevated UES pressure resulting from gastroesophageal reflux without direct exposure of the hypopharynx to acid can cause the globus sensation[6].

Proton-pump inhibitors (PPIs) are considered the mainstay treatment for LPR[7]. However, LPR requires more aggressive and prolonged therapy than GERD, and PPIs do not improve extraesophageal symptoms in the laryngopharynx in all cases[7,8]. Furthermore, increasing evidence suggests that duodeno-gastroesophageal reflux may be related to several laryngeal disorders[9]. Thus, stimulation of gastric emptying or esophageal clearance in addition to inhibition of gastric acid secretion may be an effective treatment for LPR. Ezzat *et al*[10] reported that adding prokinetics, such as cisapride and itopride, to PPIs to treat LPR reduced the recurrence of symptoms. However, few studies have investigated the efficacy of prokinetics in the treatment of LPR.

Rikkunshito, a traditional Japanese medicine, is widely used to treat upper gastrointestinal symptoms such as gastroesophageal reflux[11,12] and dyspepsia[13,14]. Rikkunshito has been shown to accelerate gastric emptying in functional dyspeptic patients[13,14] and rats[15]. Furthermore, rikkunshito improved upper gastrointestinal symptoms in PPI-refractory GERD patients[12]. Thus, we investigated the effects of rikkunshito on globus sensation and gastric emptying in patients with PPI-refractory LPR.

MATERIALS AND METHODS

Subjects

In total, 22 patients with PPI-refractory LPR were enrolled at Tokyo Medical University Hospital, from March, 2007 to December, 2008. PPI-refractory LPR was defined as the presence of LPR symptoms (globus sensation, sore throat, excessive throat clearing) despite therapy using a standard dose of PPI for 2 or more weeks. Enrolled patients met the following inclusion criteria: (1) 20-76 years of age; (2) received standard-dose therapy with a PPI for at least 2 wk prior to commencement of the study; (3) a score of three or higher than the average gastrointestinal symptom rating scale (GSRS) score for acid reflux, abdominal pain, or indigestion; (4) had LPR symptoms (globus sensation, sore throat, or excessive throat clearing); and (5) provided written informed consent for study participation. Exclusion criteria were: (1) use of an antipsychotic drug, skeletal muscle relaxant, anti-ulcer drug (with the exception of a PPI), digestive drug, or antacid within 2 wk of the start of the present study; (2) patients who had globus sensation, laryngopharyngeal pain, or chronic cough due to an organic disease; (3) cervical spine disease; (4) sinusitis; (5) bronchial asthma; (6) patients with serious complications; (7) a history of drug hypersensitivity; (8) females who were pregnant or wished to become pregnant during the study or follow-up period, and lactating females; and (9) patients who were considered unsuitable by the chief investigator.

Study design

This prospective, randomized, comparative parallel group study examined the efficacy and safety of a therapeutic strategy using rikkunshito in patients with PPI-refractory LPR. The study was conducted according to ethical guidelines for clinical studies and with consideration of patients' human rights and privacy. The protocol was approved by the Institutional Review Board of Tokyo Medical University.

Study procedures

All patients were treated with a standard-dose PPI for at least 2 wk prior to obtaining written informed consent. After obtaining written informed consent, LPR symptoms and gastrointestinal symptoms were evaluated using a visual analog scale (VAS) score and the GSRS scores. Following treatment with the PPI, lansoprazole (30 mg/d, *qd*), for at least 2 wk, patients with PPI-refractory LPR who met the inclusion and none of the exclusion criteria were enrolled in the study. Enrolled patients were randomly divided into two groups using the envelope method: rikkunshito (7.5 g/d, *tid*) alone and rikkunshito (7.5 g/d, *tid*) plus a standard dose of lansoprazole (30 mg/d). We used a powdered extract of rikkunshito (Tsumura & Co., Tokyo, Japan) obtained by spray drying a hot water extract mixture of the following eight crude herbs: *Atractylodis lanceae Rhizoma* (4.0 g), *Ginseng radix* (4.0 g), *Pinelliae tuber* (4.0 g), *Hoelen* (4.0 g), *Zizyphi fructus* (2.0 g), *Aurantii nobilis pericarpium* (2.0 g), *Glycyrrhizae radix* (1.0 g), and *Zingiberis rhizoma* (0.5 g). LPR symptoms, gastrointestinal symptoms, and gastric emptying were evaluated before and after a 4-wk treatment regimen using rikkunshito or rikkunshito plus PPI.

Assessment of LPR symptoms and gastrointestinal symptoms

LPR symptoms of globus sensation, sore throat, and excessive throat clearing were assessed using a VAS scale. Gastrointestinal symptoms were assessed using the GSRS, a 15-item questionnaire used to assess general gastrointestinal symptoms[16]. Each GSRS item is rated on a seven-point Likert scale, from no discomfort (1) to very severe discomfort (7). According to a factor analysis, the 15 GSRS items are divided into five domains: abdominal pain (abdominal pain, hunger pain, and nausea), reflux syndrome (heartburn and acid regurgitation), diarrhea syndrome (diarrhea, loose stools, urgent need for defecation), indigestion syndrome (borborygmus, abdominal distension, eructation, increased flatus), and constipation syndrome (constipation, hard stools, feeling of incomplete evacuation).

Table 1 Subjects' characteristics

	Rikkunshito	Rikkunshito + PPI
Number of patients	11	11
Mean age (range)	55.9 (39-76)	56.6 (25-76)
Sex (male/female)	4/7	4/7
Smoking (yes/no)	5/6	3/8

There is no significant difference between the rikkunshito and rikkunshito + proton-pump inhibitor (PPI) groups (Fisher's exact test or Wilcoxon's rank sum test).

Measurement of gastric emptying using radio-opaque markers

Radio-opaque markers were used to evaluate gastric emptying according to the method proposed by Cremonini *et al*[17]. Briefly, 18 subjects swallowed a capsule containing 40 radio-opaque markers (Sitzmarks, Konsyl Pharmaceuticals, Fort Worth, TX, United States) before and after 4 wk treatment with rikkunshito or rikkunshito plus PPI. A plain abdominal radiograph was obtained 3 h after intake of the capsule, and the number of markers in the stomach was counted.

Adverse events, safety and tolerability

Safety and tolerability were assessed by recording all adverse events, and changes in hematological and clinical laboratory variables were measured at the screening visit. An adverse event was defined as any unfavorable or unintended sign, whether or not it was considered to be causally related to the drugs used in this study.

Compliance

Treatment compliance was defined as the percentage of the test drug used. A treatment compliance of at least 66.6% was considered acceptable.

Statistical analysis

Within-group treatment responses in were evaluated according to pre- and post-treatment VAS and GSRS scores using a paired *t* test or the Wilcoxon signed-rank test. Mean the pre- and post-treatment scores were compared between groups using the Wilcoxon rank-sum test. Between-group age and demographic factors were compared using the Wilcoxon rank-sum test, and the distributions of sex and smoking status were compared using Fisher's exact test. We calculated the correlation between change in globus sensation and change in gastric emptying values using the non-parametric Spearman's *r* correlation. *P* values < 0.05 were considered to indicate statistical significance. All data are expressed as mean ± SD.

RESULTS

Patient characteristics

We found no marked differences in age, sex, or smoking status between the groups (Table 1). No difference was found between pre- and post-PPI monotherapy for

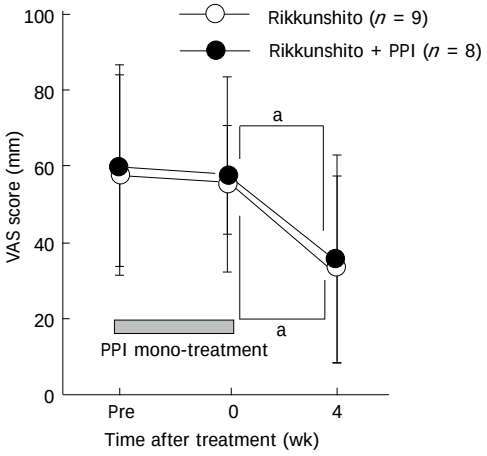

Figure 1 Effects of proton-pump inhibitor monotherapy and subsequent treatment with rikkunshito alone or rikkunshito plus proton-pump inhibitor on visual analog scale scores for globus sensation in patients with **proton-pump inhibitor-refractory laryngopharyngeal reflux.** Proton-pump inhibitor (PPI) monotherapy was delivered for at least 2 wk prior to the experiment. Each value represents the mean ± SD. [a]*P* < 0.05, significantly different from the visual analog scale (VAS) score at week 0 in each group (paired *t* test). No significant between-group differences were found at any time point.

globus sensation (VAS score, 58.7 ± 25.2 and 56.7 ± 20.1, respectively) or gastrointestinal symptoms (overall GSRS score, 2.2 ± 0.9 and 2.0 ± 0.7, respectively) in the enrolled patients.

Changes in LPR and gastrointestinal symptoms after rikkunshito or rikkunshito plus PPI treatment

The 4-wk treatment regimen significantly decreased the globus sensation VAS scores in both treatment groups (Figure 1). Furthermore, the post-treatment VAS scores were not significantly different between treatment groups.

The effects of rikkunshito alone or rikkunshito plus PPI treatments on sore throat and excessive throat clearing in patients with PPI-refractory LPR are shown in Table 2. The VAS scores for sore throat and excessive throat clearing did not decrease following the 2-wk PPI monotherapy. The VAS score for sore throat decreased after treatment with rikkunshito plus PPI but not after rikkunshito alone. The VAS score for excessive throat clearing did not change in either treatment group.

Neither the rikkunshito alone nor rikkunshito plus PPI treatment group showed a significant change in the overall GSRS or five subscale scores following the 4-wk treatment period (Table 3).

Changes in gastric emptying following rikkunshito alone or rikkunshito plus PPI treatment

Changes in gastric emptying following rikkunshito or rikkunshito plus PPI treatment are shown in Figure 2. The number of markers in the stomach tended to decrease after treatment with rikkunshito alone, but the difference was not statistically significant. However, the number of markers in the stomach was significantly decreased following treatment with rikkunshito plus PPI. We found no between-group difference in the number of markers in

Table 2 Effects of rikkunshito and rikkunshito plus proton-pump inhibitor treatments on sore throat and excessive throat clearing in patients with proton-pump inhibitor-refractory laryngopharyngeal reflux

	Week	Visual analog scale score (mean ± SD)		
		A: Rikkunshito	B: Rikkunshito + PPI	*P* (A *vs* B)
Sore throat		(*n* = 4)	(*n* = 5)	
	-2	35.4 ± 21.6	44.3 ± 30.5	0.730
	0	24.0 ± 28.1	45.2 ± 28.4	0.234
	4	24.8 ± 32.8	31.8 ± 30.2[a]	0.538
Excessive throat clearing		(*n* = 4)	(*n* = 8)	
	-2	48.0 ± 12.8	40.8 ± 32.5	1.000
	0	37.2 ± 21.5	45.7 ± 25.0	0.514
	4	39.8 ± 34.9	25.7 ± 24.2	0.569

Each value represents the mean ± SD. [a]*P* < 0.05, significantly different from the visual analog scale score at week 0 in each group (paired *t* test). No significant differences were found between the rikkunshito and rikkunshito plus proton-pump inhibitor (PPI) treatments at any time point.

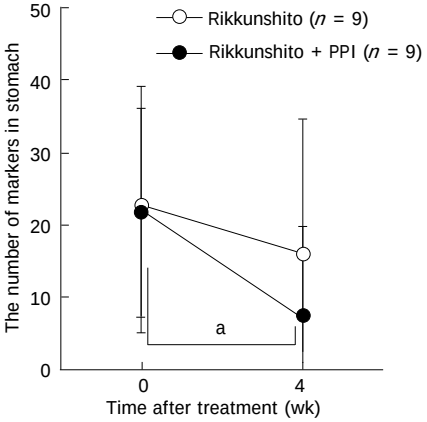

Figure 2 Effects of rikkunshito alone and rikkunshito plus proton-pump inhibitor on gastric emptying in patients with proton-pump inhibitor-refractory laryngopharyngeal reflux. Each value represents the mean ± SD. [a]*P* < 0.05, significantly different from the number of markers at week 0 in the rikkunshito + proton-pump inhibitor (PPI) group (Wilcoxon signed-rank test). We found no significant difference between treatment groups after the 4-wk treatment period (Wilcoxon rank-sum test).

the stomach following the 4-wk treatment period.

Correlation between improvement in globus sensation and improvement in gastric emptying

The correlation between improvement in globus sensation and improvement in gastric emptying is shown in Figure 3. A marked improvement in globus sensation was observed in patients with PPI-refractory LPR as gastric emptying improved. The correlation analysis revealed a significant positive correlation between the improvement in globus sensation and the improvement in gastric emptying ($r^2 = 0.4582$, $P < 0.05$).

Safety of rikkunshito

No adverse event/reaction requiring treatment occurred in any patient during the study period.

DISCUSSION

As no diagnostic gold standard is available for LPR, few studies have investigated this condition. However, previous reports indicate that 74.4% of GERD patients experience extraesophageal or atypical manifestations with prevalences of globus sensation and laryngitis/pharyngitis in GERD patients of 38.7% and 19.9%, respectively[8]. LPR requires more aggressive and prolonged therapy than GERD, and several cases in which PPIs did not improve extraesophageal symptoms in the laryngopharynx have been reported[7,8]. We examined PPI-refractory patients whose LPR symptoms of globus sensation, sore throat, or excessive throat clearing did not improve after at least 2 wk of PPI treatment. Rikkunshito has been shown to improve upper gastrointestinal symptoms in PPI-refractory GERD patients[12]; thus, we investigated the efficacy of rikkunshito in improving extraesophageal symptoms in patients with PPI-refractory LPR. Our findings indicate that a 4-wk treatment regimen of rikkunshito alone or rikkunshito plus PPI improved globus sensation in patients with PPI-refractory LPR. Two theories of LPR pathogenesis have been proposed. According to the direct impairment theory, LPR occurs when stomach acid acts directly on the hypopharynx, whereas the reflex theory holds that acid reflux in the lower esophagus causes coughing or other symptoms through a vagal reflex[1-3]. Moreover, we demonstrated previously that globus sensation can be caused by elevated upper esophageal sphincter pressure resulting from gastroesophageal reflux without direct exposure of the hypopharynx to acid[6]. Thus, acid secretion control alone is not sufficient for the treatment of LPR, which is caused by several factors. Unlike the PPIs, rikkunshito does not have an antisecretory effect[18], and, thus, may improve the globus sensation via a different mechanism. Kawahara *et al*[11] reported that rikkunshito reduced esophageal acid exposure through improved esophageal acid clearance in GERD patients. The hesperidine and atractylodin, components of rikkunshito, have been shown to improve delayed gastric emptying in L-NNA-administered rats[15,19], and rikkunshito improved upper GI symptoms *via* stimulation of gastric emptying in functional dyspeptic patients[13,14] and in patients who had undergone pylorus-preserving gastrectomy[20]. A recent study showed that rikkunshito stimulated secretion of a ghrelin, which has stimulatory effects on appetite and gastrointestinal motor activity[21,22]. Furthermore, rikkunshito and atractylodin enhance reactivity of its receptor[23]. Nahata *et al*[24] found an association between impaired ghrelin signaling and gastrointestinal motility dysfunction and demonstrated that rikkunshito restored gastrointestinal motility by improving the ghrelin response in rat GERD models. If rikkunshito reduces gastric contents, it seems reasonable that a subsequent reduction in the reflux volume may reduce acid exposure in the esophagus, pharynx, and larynx. We calculated the correlation between improved globus sensation and improved gastric emptying to investigate the association between rikkunshito-induced stimulation of gastric emptying improved globus sensation. We found

Table 3 Gastrointestinal symptom rating scale scores after 4 wk treatments of rikkunshito with or without proton-pump inhibitor

	Week	Rikkunshito (mean ± SD)	Test[1] *P* value	Rikkunshito + PPI (mean ± SD)	Test[1] *P* value	Test[2] *P* value
Overall scores	-2	2.25 ± 1.06	0.232	2.19 ± 0.73	0.375	1.000
	0	2.12 ± 0.85	-	1.96 ± 0.50	-	0.778
	4	1.83 ± 0.84	0.148	1.73 ± 0.37	0.195	0.736
Subscale scores						
Reflux syndrome	-2	2.25 ± 1.06	0.055	2.79 ± 0.91	0.170	0.369
	0	2.23 ± 1.60	-	2.45 ± 1.42	-	0.540
	4	1.94 ± 1.16	1.000	1.94 ± 0.86	0.106	0.801
Abdominal pain	-2	2.27 ± 1.29	0.168	2.33 ± 1.12	0.058	0.658
	0	1.87 ± 0.86	-	1.77 ± 0.85	-	0.914
	4	1.59 ± 0.78	0.250	1.50 ± 0.40	0.223	0.805
Indigestion syndrome	-2	2.40 ± 1.04	0.615	2.54 ± 1.29	0.551	0.844
	0	2.30 ± 1.03	-	2.20 ± 0.86	-	1.000
	4	2.17 ± 1.22	0.201	1.94 ± 0.75	0.139	0.961
Diarrhea syndrome	-2	1.77 ± 1.05	0.750	1.71 ± 0.71	1.000	0.878
	0	1.61 ± 0.68	-	1.77 ± 0.75	-	0.661
	4	1.41 ± 0.49	0.098	1.71 ± 0.68	0.866	0.345
Constipation syndrome	-2	1.77 ± 1.05	0.341	1.71 ± 0.71	0.784	0.138
	0	1.61 ± 0.68	-	1.77 ± 0.75	-	0.254
	4	1.41 ± 0.49	0.134	1.71 ± 0.68	1.000	0.883

0 week: Baseline. Test[1]: There is also no significantly different compared from gastrointestinal symptom rating scale score at week 0 (Wilcoxon's signed rank test); Test[2]: There is no significant difference between the rikkunshito with or without plus proton-pump inhibitor (PPI) treatment at each period (Wilcoxon's rank sum test).

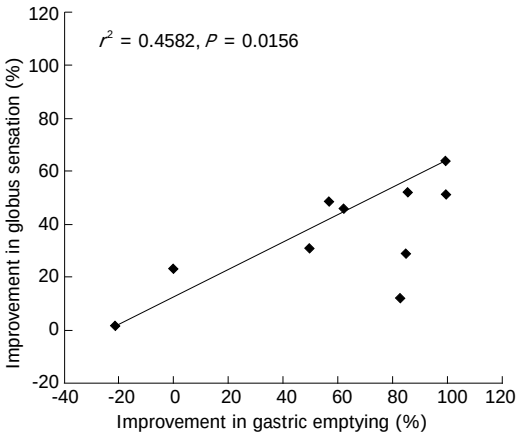

Figure 3 Correlation between improvement in globus sensation and improvement in gastric emptying. The improvement in globus sensation calculation based on pre- and post-treatment visual analog scale (VAS) scores using the following formula: Improvement (%) = [(pre-score) - (post-score)]/(post-score) × 100. Pre-score: VAS score before the start of rikkunshito or rikkunshito + proton-pump inhibitor treatment; Post-score: VAS score after the 4-wk treatment period. Improvement in gastric emptying was calculated based on the number of markers in the stomach before and after treatment.

a significant positive correlation between improved globus sensation and improved gastric emptying. Thus, the improvement in globus sensation following treatment with rikkunshito may be the result, at least in part, of improved gastric emptying. In addition to the globus sensation, patients with LPR typically experience sore throat or excessive throat clearing. Treatment with rikkunshito plus PPI, but not with rikkunshito alone, improved the tingling sensation in patients with PPI-refractory LPR in the present study, suggesting that acid may play a greater role in causing a sore throat than in globus sensation. Moreover, the LPR symptoms of globus sensation, sore throat, and excessive throat clearing may be induced by different mechanism. Johnston *et al*[25] reported absence or decreased expression of mucosal-protective proteins in laryngeal epithelial cells in 64% of patients with LPR. Thus, reducing the gastric content that passes into the laryngopharyngeal tissue *via* mucosal defenses may be an effective treatment for LPR. Rikkunshito has an effect on mucosal defenses in the gastroesophageal region, although the effect in the laryngopharynx is unclear[26,27]. In addition to the inhibitory effects of PPIs on acid, rikkunshito-induced stimulation of gastric emptying and effects on mucosal defense may contribute to the improvement in sore throat in the laryngopharynx.

The present study demonstrated that rikkunshito did not improve gastrointestinal symptoms in patients with PPI-refractory LPR assessed using the GSRS. In contrast, rikkunshito has been shown to improve upper gastrointestinal symptoms in PPI-refractory GERD patients assessed using the frequency scale for the symptoms of GERD score[12]. This discrepancy may be related to differences in the pathology and/or assessment tools used in the two studies.

In conclusion, rikkunshito treatment improved the globus sensation in patients with PPI-refractory LPR. The effect may be the result, at least in part, of the stimulation of gastric emptying. Rikkunshito plus PPI therapy may be an effective novel therapeutic strategy for PPI-refractory LPR symptoms, including globus sensation and sore throat.

COMMENTS

Background

Regarding the treatment of laryngopharyngeal reflux (LPR) symptoms such as globus sensation and a scratchy feeling, proton pump inhibitors (PPIs) are considered the mainstay. However, cases exist in which extraesophageal symp-

toms in the laryngopharynx are not improved by PPI.

Research frontiers

Recently, gastroesophageal reflux disease (GERD) has been considered a major cause of globus. However, the etiology of globus remains unclear. The authors have demonstrated that the cause of the globus sensation is elevated upper esophageal sphincter pressure, resulting from gastroesophageal reflux without direct exposure of the hypopharynx to acid.

Innovation and breakthroughs

Stimulation of gastric emptying or esophageal clearance in addition to inhibition of gastric acid secretion may also be efficacious in the treatment of LPR. It has been reported that addition of prokinetics, such as cisapride and itopride, to PPIs in the treatment of LPR reduced the recurrence of symptoms. However, there are few reports of the efficacy of prokinetics in the treatment of LPR.

Applications

Rikkunshito, a traditional Japanese medicine, has a dual action on the stomach: relaxation of the proximal stomach and contraction of the distal stomach. Recently, it was reported that rikkunshito improved upper gastrointestinal symptoms in PPI-refractory GERD patients. This was a prospective, randomized, parallel comparative study performed to examine the efficacy and safety of a therapeutic strategy using rikkunshito in patients with PPI-refractory LPR.

Peer review

The authors examined the effect of an herbal medicine "rikkunshito" on symptoms and gastric emptying in patients with LPR. The outcome of the study is interesting and important for the care of patients with PPI-refractory LPR.

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BRIEF ARTICLE

Emergency balloon-occluded retrograde transvenous obliteration of ruptured gastric varices

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Abstract

AIM: To evaluate the effectiveness and safety of emergency balloon-occluded retrograde transvenous obliteration (BRTO) for ruptured gastric varices.

METHODS: Emergency BRTO was performed in 17 patients with gastric varices and gastrorenal or gastrocaval shunts within 24 h of hematemesis and/or tarry stool. The gastric varices were confirmed by endoscopy, and the gastrorenal or gastrocaval shunts were identified by contrast-enhanced computed tomography (CE-CT). A 6-Fr balloon catheter (Cobra type) was inserted into the gastrorenal shunt *via* the right internal jugular vein, or into the gastrocaval shunt *via* the right femoral vein, depending on the varices drainage route. The sclerosant, 5% ethanolamine oleate iopamidol, was injected into the gastric varices through the catheter during balloon occlusion. In patients with incomplete thrombosis of the varices after the first BRTO, a second BRTO was performed the following day. Patients were followed up by endoscopy and CE-CT at 1 d, 1 wk, and 1, 3 and 6 mo after the procedure, and every 6 mo thereafter.

RESULTS: Complete thrombosis of the gastric varices was not achieved with the first BRTO in 7/17 patients because of large gastric varices. These patients underwent a second BRTO on the next day, and additional sclerosant was injected through the catheter. Complete thrombosis which led to disappearance of the varices was achieved in 16/17 patients, while the remaining patient had incomplete thrombosis of the varices. None of the patients experienced rebleeding or recurrence of the gastric varices after a median follow-up of 1130 d (range 8-2739 d). No major complications occurred after the procedure. However, esophageal varices worsened in 5/17 patients after a mean follow-up of 8.6 mo.

CONCLUSION: Emergency BRTO is an effective and safe treatment for ruptured gastric varices.

Key words: Emergency balloon-occluded retrograde transvenous obliteration; Gastric varices; Bleeding; Portal hypertension; Ethanolamine oleate

Core tip: As ruptured gastric varices are associated with high rates of recurrent bleeding and mortality, quick treatment is essential. Balloon-occluded retrograde transvenous obliteration (BRTO) is a minimally invasive treatment for gastric varices with a high success rate and a low recurrence rate. Emergency BRTO is an effective and safe treatment, providing temporary hemostasis of ruptured gastric varices can be achieved, allowing the sclerosant to accumulate in the varices.

Sonomura T, Ono W, Sato M, Sahara S, Nakata K, Sanda H, Kawai N, Minamiguchi H, Nakai M, Kishi K. Emergency balloon-occluded retrograde transvenous obliteration of ruptured gastric varices. *World J Gastroenterol* 2013; 19(31): 5125-5130 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i31/5125.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i31.5125

INTRODUCTION

As gastric varices have greater blood flow compared with esophageal varices, ruptured gastric varices can cause massive hemorrhage, and are associated with high rates of recurrent bleeding and mortality (45%-55%)[1,2]. Therefore, ruptured gastric varices must be treated as quickly as possible. Balloon-occluded retrograde transvenous obliteration (BRTO) is a minimally invasive treatment for gastric varices, with a high success rate and a low recurrence rate[3-7]. However, few reports have evaluated emergency BRTO for ruptured gastric varices[8,9]. In this study, we report the long-term outcomes of emergency BRTO performed within 24 h of hematemesis and/or tarry stool.

MATERIALS AND METHODS

The effectiveness and safety of emergency BRTO for ruptured gastric varices were evaluated retrospectively. Between March 1998 and December 2008, BRTO was performed for gastric varices with gastrorenal or gastrocaval shunts in 79 patients. Of these patients, emergency BRTO was performed for ruptured gastric varices within 24 h of hematemesis and/or tarry stool in 17 patients. The patients' ages ranged from 33 to 79 years, and the mean age was 58.8 years. All patients had liver cirrhosis corresponding to Child-Pugh class A in 2 patients, class B in 12 patients, and class C in 3 patients. The etiologies of liver cirrhosis were hepatitis C in seven patients, hepatitis B in three patients, alcoholic liver disease in four patients, primary biliary cirrhosis in one patient, and unknown in two patients. Mean creatinine value before BRTO was 0.82 mg/dL (normal range: 0.64-1.11 mg/dL), and we had no patients with renal dysfunction. Informed consent for BRTO was obtained from all patients.

The gastric varices were confirmed by endoscopy. According to Sarin classification[2], isolated varices in the fundus of the stomach were found in 13 of 17 patients, and gastroesophageal varices in the remaining 4 patients. Also, white plugs which indicated bleeding sites were found in 7 patients, and oozing in 3 patients. Temporary hemostasis was achieved spontaneously in 9/17 patients and by balloon compression in 8/17 patients. The presence and diameter of the gastrorenal or gastrocaval shunts were evaluated by contrast-enhanced computed tomography (CE-CT). The gastric varices drained *via* the gastrorenal shunt in 16 patients, and by the gastrocaval shunt in 1 patient. An 8-Fr sheath (Cobra type; Medikit, Tokyo, Japan) was inserted into the left renal vein through the right internal jugular vein with ultrasound-guided puncture while an 8-Fr sheath (Straight type; Medikit) was inserted into the inferior vena cava through the right femoral vein. A 6-Fr balloon catheter (Cobra type; Clinical Supply, Gifu, Japan) was inserted into the gastrorenal or gastrocaval shunt. The balloon diameter was 13 or 20 mm. In patients with a shunt diameter $\geqslant$ 13 mm, a 20 mm diameter balloon was used. A sclerosing agent, 5% ethanolamine oleate iopamidol (EOI), was infused through a balloon catheter or a microcatheter placed close to the gastric varices during balloon occlusion. In the 13 most recent cases, microcatheters were used to decrease the sclerosant dose. We prepared 5% EOI by mixing 10 mL of contrast material with 10 mL of 10% ethanolamine oleate (Oldamin; Glelan Pharmaceutical, Tokyo, Japan). The infusion of 5% EOI was continued until the entire gastric varices and feeding veins were rendered opaque. The mean dose of 5% EOI per procedure was 21.3 mL (range 2-40 mL). The balloon occlusion time ranged from 12 to 48 h. The catheters were fixed in place using sterilized tape (Hogy Medical, Tokyo, Japan). The morning after BRTO, thrombosis of the gastric varices was evaluated by CE-CT. In patients with incomplete thrombosis after the first BRTO, a second BRTO was performed the following day[7]. After complete thrombosis of gastric varices was confirmed by CE-CT, the catheters were removed. To prevent renal damage caused by EOI-related hemolysis, 4000 units of haptoglobin (Mitsubishi Pharma, Osaka, Japan) was intravenously administered to all patients[10,11]. Patients underwent endoscopy and CE-CT at 1 d, 1 wk, and 1, 3 and 6 mo after the procedure, and every 6 mo thereafter.

RESULTS

Complete thrombosis of the gastric varices was not achieved with the first BRTO in 7/17 patients because of large gastric varices. These patients underwent a second BRTO on the next day, and additional sclerosant was injected through the catheter[7]. Complete thrombosis which led to disappearance of the varices was achieved in 16/17 patients (Figures 1 and 2), while the other patient had incomplete thrombosis of the varices. None of the patients experienced rebleeding or recurrence of the gastric varices during a median follow-up of 1130 d (range 8-2739 d). However, esophageal varices worsened in 5/17 patients during a mean follow-up of 8.6 mo[12-14] (Table 1). In two of these five patients, red-colored esophageal varices were treated by endoscopic sclerotherapy. Reddening of the variceal mucosa is associated with a high risk of variceal bleeding[15].

All of the complications were transient[16], and included sclerosant-induced hematuria (7/17 patients), abdominal pain (8/17), high fever (6/17), sclerosant-induced blood pressure elevation (1/17), headache (2/17), pleural effusion (15/17), and ascites (12/17). Although extravasation of the sclerosant occurred in one patient

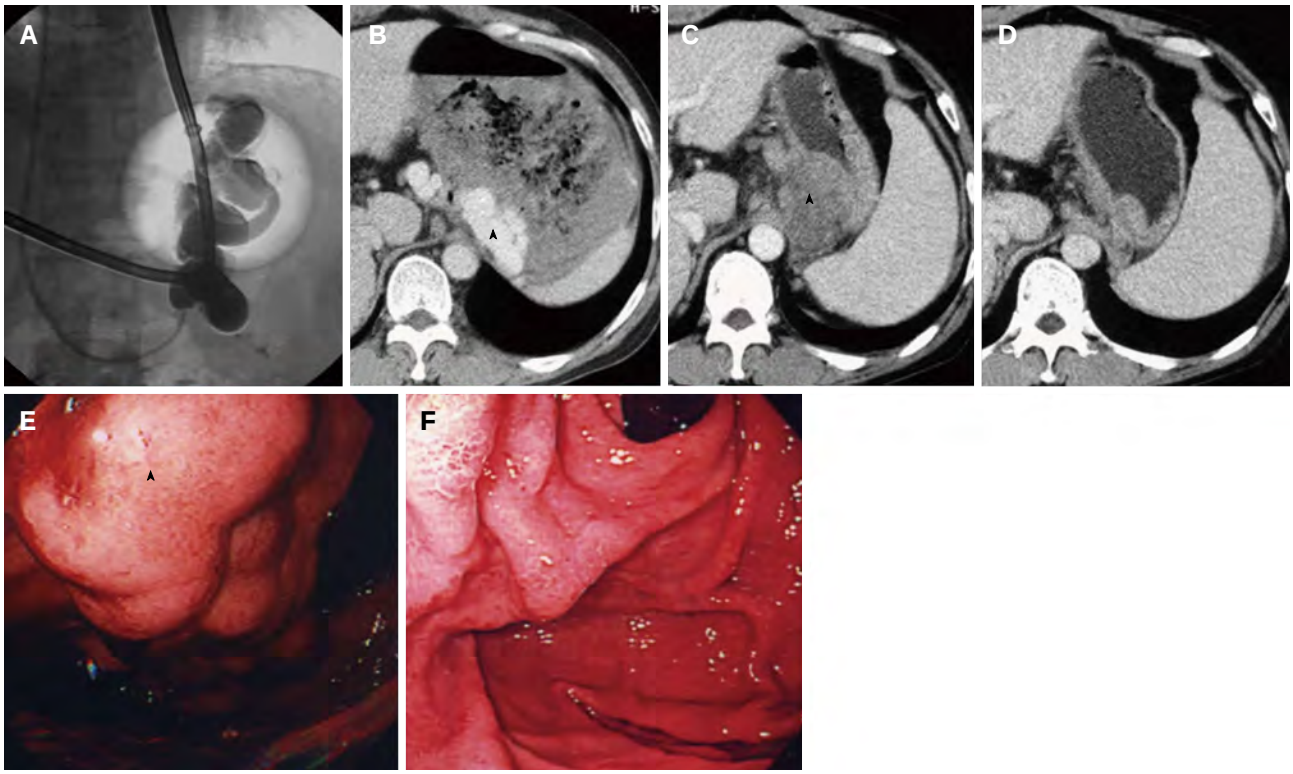

Figure 1 Gastric varices with a gastrorenal shunt (case 15). A: Balloon-occluded retrograde transvenous obliteration (BRTO) was performed 11 h after hematemesis. The gastric varices and a gastrorenal shunt were filled with 36 mL of 5% ethanolamine oleate iopamidol. A Sengstaken–Blakemore tube was inserted into the stomach for temporary hemostasis; B: Contrast-enhanced computed tomography (CE-CT) image taken before BRTO shows gastric varices (arrowhead) with a massive hematoma; C: CE-CT image taken 1 wk after BRTO shows complete thrombosis of the varices (arrowhead); D: CE-CT image taken 6 mo after BRTO shows complete disappearance of the varices; E: Endoscopy performed before BRTO shows gastric varices (arrowhead) with oozing; F: Endoscopy performed 6 mo after BRTO shows complete disappearance of the varices.

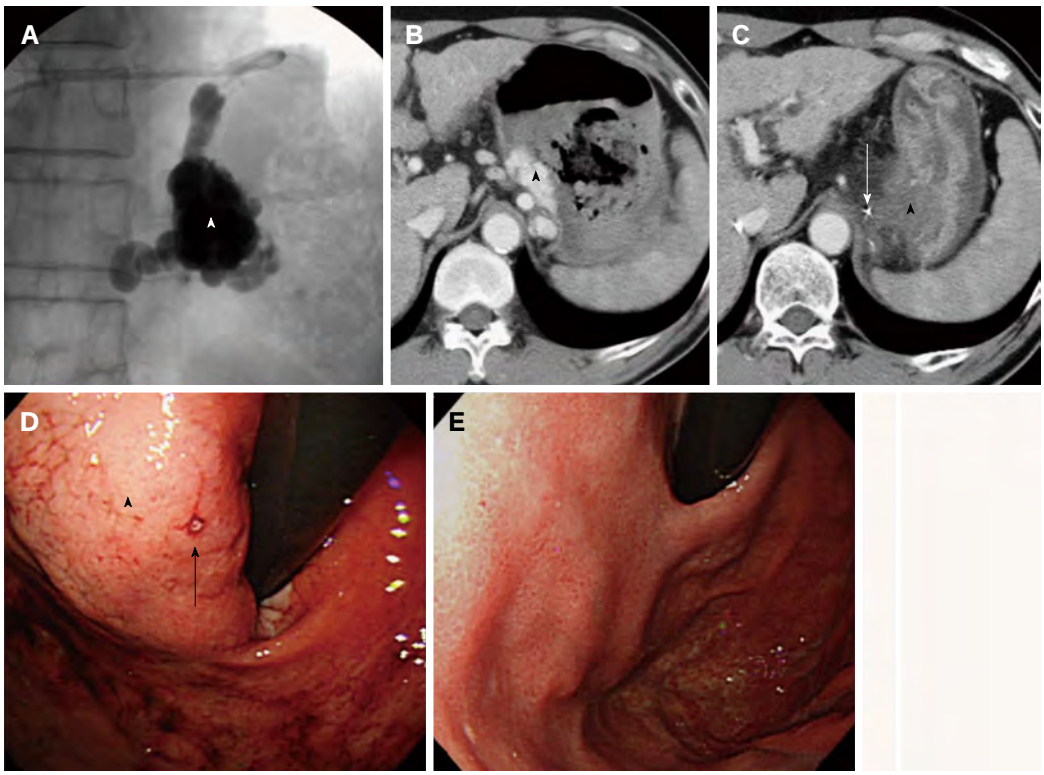

Figure 2 Gastric varices with a gastrocaval shunt (case 8). A: A balloon catheter was inserted into a gastrocaval shunt and 18 mL of 5% EOI was injected through the microcatheter that had been advanced close to the gastric varices; B: Contrast-enhanced computed tomography (CE-CT) image taken before balloon-occluded retrograde transvenous obliteration (BRTO) shows gastric varices (arrowhead) with a massive hematoma; C: CE-CT image taken the day after BRTO shows complete thrombosis of the varices (arrowhead) and that the tip of the microcatheter (arrow) is close to the varices; D: Endoscopy performed before BRTO shows bleeding site (arrow) of the gastric varices (arrowhead); E: Endoscopy performed 3 mo after BRTO shows complete disappearance of the varices.

Table 1 Patient characteristics and clinical outcomes

Case	Age (yr)	Sex	Cause of LC	Child-Pugh class	Temporary hemostasis	Drainage route	Dose of 5% EOI (mL)	Eradication of GV	Worsening of EV	Follow-up time (d)	Clinical outcome
1	63	M	HCV	B	Spontaneous	GR	30	Complete	–	205	Alive
2	59	M	HBV	B	Spontaneous	GR	40	Complete	+	2229	Alive
3	58	F	PBC	A	Spontaneous	GR	18	Complete	–	2197	HF[1]
4	67	M	HCV	A	Spontaneous	GR	12	Complete	–	2529	HCC[2]
5	79	F	Unknown	C	Spontaneous	GR	35	Complete	–	135	HF[1]
6	46	M	HCV	B	Balloon	GR	36	Complete	–	388	Alive
7	66	F	Alcohol	B	Spontaneous	GR	10 + 17	Complete	+	2739	HF[1]
8	59	M	Alcohol	B	Spontaneous	GC	18	Complete	–	1501	Alive
9	70	M	HCV	B	Spontaneous	GR	17	Complete	–	8	Alive
10	33	F	Unknown	B	Balloon	GR	30 + 10	Complete	–	1293	Alive
11	46	M	HBV	C	Balloon	GR	36 + 23	Complete	–	41	Alive
12	57	M	HBV	B	Balloon	GR	30 + 28	Complete	+	164	HF[1]
13	66	M	Alcohol	B	Balloon	GR	18 + 13	Partial	–	14	HF[1]
14	65	F	HCV	B	Balloon	GR	10 + 8	Complete	–	1130	Alive
15	51	M	HCV	B	Balloon	GR	36	Complete	+	2466	HF[1]
16	53	M	Alcohol	C	Balloon	GR	15	Complete	+	545	HF[1]
17	62	F	HCV	B	Spontaneous	GR	20 + 2	Complete	–	1835	Alive

[1]Died of hepatic failure (HF); [2]Died of hepatocellular carcinoma (HCC). LC: Liver cirrhosis; PBC: Primary biliary cirrhosis; HCV: Hepatitis C virus; HBV: Hepatitis B virus; GR: Gastrorenal shunt; GC: Gastrocaval shunt; EOI: Ethanolamine oleate iopamidol; GV: Gastric varices; EV: Esophageal varices.

during BRTO, the procedure was continued and achieved complete thrombosis of the varices. No major complications, such as renal failure, pulmonary embolism, or liver failure, occurred after the procedure.

DISCUSSION

The cumulative risk for hemorrhage from gastric fundal varices has been reported to be 16%, 36% and 44% at 1, 3 and 5 years, respectively[17]. Ruptured gastric varices are also associated with high rates of rebleeding and mortality (45%-55%)[1,2]. Therefore, ruptured gastric varices must be treated as quickly as possible.

As most patients with ruptured gastric varices are in a critical state because of hypovolemic shock, surgical treatment and transjugular intrahepatic portosystemic shunts (TIPS) are too invasive and risky. The mortality of patients with esophageal varices undergoing emergency surgery was reported to be 38.4%[18]. Although TIPS is a treatment for portal hypertension to decrease the portal pressure, Miller-Catchpole[19] reported some of the problems of TIPS, which included technical failure, restenosis or occlusion of the shunt, dislocation of the stent, and hepatic encephalopathy. Overall, 21% of patients (86/416) died because of bleeding, liver failure, or multiple organ failure. Only 50% of patients had improvements in gastric fundal varices after TIPS[20]. Furthermore, the cumulative gastric variceal bleeding rate at 1 year was 20% in patients who underwent TIPS compared with 2% in patients who underwent transcatheter sclerotherapy ($P < 0.01$) (Kaplan-Meier method and Log-rank test)[21]. Although percutaneous transhepatic obliteration (PTO) may achieve temporary embolization of gastric varices, the varices recur very quickly[22]. Because gastric varices usually have many feeding veins, it is difficult to embolize all of them by PTO. Arai *et al*[23] reported that PTO achieved a success rate of 44% (8/18) but the recurrence rate of gastric varices was 38% (3/8). By contrast, BRTO was found to have a success rate of 81% (75/93) and the recurrence rate of gastric varices was just 4% (3/75). Although the Baveno Consensus[24] suggests endoscopic cyanoacrylate injection for bleeding from isolated gastric varices, it is also difficult to apply endoscopic methods, such as endoscopic injection sclerotherapy (EIS), endoscopic variceal ligation (EVL), and sclerotherapy using cyanoacrylate, to ruptured gastric varices because of their extensive blood supply. Additionally, the mortality rate of EIS in patients with bleeding gastric varices was 55%[1]. EVL using a rubber band[25] has also been associated with a high risk of adverse outcomes for treating ruptured gastric varices, as it often causes re-rupture during the procedure, and has a high incidence of rebleeding. The rebleeding rate of EVL was significantly higher than that of endoscopic obturation using cyanoacrylate (54% *vs* 31%; $P = 0.0005$)[26]. Endoscopic-guided injection of cyanoacrylate into the varices may induce multiple organ embolisms, such as cerebral infarction[27] and pulmonary embolisms[28,29], which are caused by leakage of the sclerosant into the systemic circulation. Furthermore, cyanoacrylate treatment of gastric variceal bleeding has a high rate of early bleeding (15.5%-20.5%)[30,31]. By contrast, BRTO is a minimally invasive treatment of gastric varices that is associated with a high success rate and a low recurrence rate[3-7]. Therefore, it may be much more effective than surgery, TIPS, PTO or endoscopic treatment for critical patients.

Ethanolamine oleate is a sclerosant that damages endothelial cells and induces thrombus formation in the vessel. EOI was prepared by mixing ethanolamine oleate with contrast medium to monitor the movement of EOI under fluoroscopy. To prevent EOI-related complications caused by excess sclerosant, we believe that < 40 mL of

5% EOI should be used during individual BRTO procedures. If complete thrombosis of large gastric varices is not achieved, a second BRTO can be performed the following day, and additional sclerosant can be injected through the catheter that was left in place overnight[7]. To decrease the sclerosant dose, 50% glucose solution[32] or polidocanol foam[33] may be used during BRTO procedures. Haptoglobin has also been intravenously administered to prevent renal failure[10,11].

In our study, the BRTO procedure achieved temporary hemostasis in all of the patients. If active bleeding from the gastric varices continues during the procedure, then 5% EOI may be unable to control the bleeding, because it will leak into the gastric lumen. This displaces the sclerosant and prevents it from accumulating in the gastric varices. In such situations, transportal or transesophageal sclerotherapy with cyanoacrylate and coils may be necessary. The coils serve as a scaffold to trap the cyanoacrylate preventing pulmonary embolism[34-36]. If temporary hemostasis by balloon compression is achieved, we perform BRTO. On the other hand, if temporary hemostasis by balloon compression is not achieved and the gastric varices continue to spurt blood, we perform transportal or transesophageal sclerotherapy with cyanoacrylate and coils.

Esophageal varices worsened in 5/17 patients in this study. The occlusion of a gastrorenal shunt probably induced the esophageal varices through another collateral route. BRTO was reported to significantly increase the portal systemic pressure gradient[13] and increase the bleeding rates of coexisting esophageal varices[12]. Other major risk factors identified for worsening of esophageal varices after BRTO were the presence of esophageal varices, higher Child-Pugh class, and higher resistance index assessed by endoscopic color Doppler ultrasonography before BRTO[14]. Therefore, esophageal varices should be endoscopically checked every 6 mo after BRTO.

As ruptured gastric varices are associated with high rates of recurrent bleeding and mortality, quick treatment is essential. BRTO is a minimally invasive treatment for gastric varices with a high success rate and a low recurrence rate. Emergency BRTO is an effective and safe treatment, providing temporary hemostasis of ruptured gastric varices can be achieved, allowing the sclerosant to accumulate in the varices.

COMMENTS

Background

As gastric varices have greater blood flow compared with esophageal varices, ruptured gastric varices can cause massive hemorrhage, and are associated with high rates of recurrent bleeding and mortality. Therefore, ruptured gastric varices must be treated as quickly as possible.

Research frontiers

In this study, the authors demonstrate the long-term results of emergency balloon-occluded retrograde transvenous obliteration (BRTO) for ruptured gastric varices.

Innovations and breakthroughs

Sixteen of 17 patients had complete thrombosis leading to disappearance of gastric varices. One patient had incomplete thrombosis leading to reduction of varices.

Applications

Patients who have ruptured gastric varices and gastrorenal or gastrocaval shunts can be treated with emergency BRTO within 24 h of hematemesis and/or tarry stool.

Terminology

Emergency BRTO is a procedure where a balloon catheter is inserted into a draining vein of gastric varices, and the sclerosant can be injected into the varices through the catheter during balloon occlusion. The sclerosant damages endothelial cells of the varices resulting in thrombosis and disappearance of the varices.

Peer review

The authors reported the results of a retrospective study on patients who underwent emergency BRTO for ruptured gastric varices. Emergency BRTO is an effective and safe treatment, providing temporary hemostasis of ruptured gastric varices can be achieved, allowing the sclerosant to accumulate in the varices.

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BRIEF ARTICLE

Connective tissue diseases in primary biliary cirrhosis: A population-based cohort study

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Abstract

AIM: To establish the frequency and clinical features of connective tissue diseases (CTDs) in a cohort of Chinese patients with primary biliary cirrhosis (PBC).

METHODS: Three-hundred and twenty-two Chinese PBC patients were screened for the presence of CTD, and the systemic involvement was assessed. The differences in clinical features and laboratory findings between PBC patients with and without CTD were documented. The diversity of incidence of CTDs in PBC of different countries and areas was discussed. For the comparison of normally distributed data, Student's t test was used, while non-parametric test (Wilcoxon test) for the non-normally distributed data and 2×2 χ^2 or Fisher's exact tests for the ratio.

RESULTS: One-hundred and fifty (46.6%) PBC patients had one or more CTDs. The most common CTD was Sjögren's syndrome (SS, 121 cases, 36.2%). There were nine cases of systemic sclerosis (SSc, 2.8%), 12 of systemic lupus erythematosus (SLE, 3.7%), nine of rheumatoid arthritis (RA, 2.8%), and 10 of polymyositis (PM, 3.1%) in this cohort. Compared to patients with PBC only, the PBC + SS patients were more likely to have fever and elevated erythrocyte sedimentation rate (ESR), higher serum immunoglobulin G (IgG) levels and more frequent rheumatoid factor (RF) and interstitial lung disease (ILD) incidences; PBC + SSc patients had higher frequency of ILD; PBC + SLE patients had lower white blood cell (WBC) count, hemoglobin (Hb), platelet count, γ-glutamyl transpeptidase and immunoglobulin M levels, but higher frequency of renal involvement; PBC + RA patients had lower Hb, higher serum IgG, alkaline phosphatase, faster ESR and a higher ratio of RF positivity; PBC + PM patients had higher WBC count and a tendency towards myocardial involvement.

CONCLUSION: Besides the common liver manifestation of PBC, systemic involvement and overlaps with other CTDs are not infrequent in Chinese patients. When overlapping with other CTDs, PBC patients manifested some special clinical and laboratory features which may have effect on the prognosis.

Key words: Cirrhosis; Biliary; Connective tissue disease; Sjögren's syndrome; Systemic sclerosis; Raynaud phenomenon

Core tip: This study demonstrated that primary biliary cirrhosis (PBC) is a complicated disease that not only involves the liver but also often coexists with other connective tissue diseases (CTDs). Evaluation of our cohort of 322 Chinese PBC patients showed that Sjögren's syndrome was the CTD that most frequently coexisted with PBC. In addition, it was also shown that when CTDs coexist with PBC, the clinical features and the disease course are different from those in patients with PBC alone. Our collective results suggest that Chinese patients with PBC may benefit from assessment of systemic involvement and screening for CTDs through detection of autoantibodies.

Wang L, Zhang FC, Chen H, Zhang X, Xu D, Li YZ, Wang Q, Gao LX, Yang YJ, Kong F, Wang K. Connective tissue diseases in primary biliary cirrhosis: A population-based cohort study. *World J Gastroenterol* 2013; 19(31): 5131-5137 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i31/5131.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i31.5131

INTRODUCTION

Primary biliary cirrhosis (PBC), which predominantly affects middle-aged women, is histologically characterized by chronic non-suppurative destructive cholangitis. Although the liver is the chief target, PBC may involve multiple systems, such as interstitial lung disease (ILD)[1,2], pulmonary artery hypertension (PAH)[3], and nephritis[4]. PBC is also associated with other connective tissue diseases (CTDs) and autoimmune disorders, such as Sjögren's syndrome (SS), systemic sclerosis (SSc), and systemic lupus erythematosus (SLE).

These co-existing conditions frequently increase the difficulty in making a diagnosis and treating the disease. They may also change the natural course and prognosis of PBC. We have established a database of PBC patients admitted to our hospital during the past ten years, to serve as a resource of data for studies of PBC features and outcomes. In this report, we describe our analysis of these patients' data to determine the frequencies of extrahepatic lesions and association of CTDs.

MATERIALS AND METHODS

Patients

Chinese patients with PBC (294 women, 28 men; age mean: 53 years, range: 20-81 years old), who attended our hospital during 2002-2012, were prospectively entered into our collective database and retrospectively analyzed in this study. PBC diagnosis was made according to the criteria published in the guidelines of the American Association for the Study of Liver Diseases[5,6]. The majority (91.9%) of the patients resided in northern China, with 23.6% of those individuals being from Beijing.

Diagnostic criteria for CTDs and definition of organ involvement

Diagnosis of SS was made if the patient fulfilled the 2002 European diagnostic criteria[7]. Diagnosis of SSc (including scleroderma) was made according to the 1980 American College of Rheumatology (ACR) criteria[8]. Diagnosis of SLE was made according to the 1997 revised ACR criteria[9] and the 2009 Systemic Lupus International Collaborating Clinic revision of the ACR classification criteria for SLE[10]. Diagnosis and classification of rheumatoid arthritis (RA) were made according to the 1987 revised ACR[11] and 2010 ACR/European League Against Rheumatism criteria[12]. Diagnoses of polymyositis (PM) or dermatomyositis (DM) were made according to the criteria reported by Bohan and Peter[13], and diagnosis of mixed connective tissue disease (MCTD) was made according to the 1987 Alarcon-Segovia criteria[14].

Renal involvement was defined by persistent protein urea of > 0.5 g/d, and/or glomerular haematuria, and/or cellular casts[15]. Cardiac involvement was defined by the presence of cardiomyopathy, pericarditis, or arrhythmia.

Statistical analysis

SPSS version 11.5 (SPSS Inc., Chicago, IL, United States) was used for statistical analysis of the data. Main results were presented as mean ± SD. According to the type and distribution of the data, the statistical significance was estimated by Student's *t* test, Wilcoxon test, or $2 \times 2\ \chi^2$ or Fisher's exact tests. *P* values < 0.05 were considered to be statistically significant.

RESULTS

General characteristics of PBC patients

Of the 322 PBC patients enrolled in the study, the mean time from onset of symptoms to diagnosis was 5.8 years. Anti-nuclear antibody (ANA) was present in 87.0% of the patients, while anti-mitochondrial antibody (AMA) was present in 90.9% of the patients, among which 90.3% were also positive for the M_2 subtype of AMA (AMA-M_2). Seventy-two (22.4%) of the total patients underwent liver biopsy (Table 1).

CTDs in PBC patients and inter-study comparison with other countries

One-hundred and fifty (46.6%) of the patients had CTDs, 11 (3.4%) of which had two or more CTDs (Figure 1). SS (121 cases, 36.2%) was the most frequent CTD represented. There were nine cases of SSc (2.8%), 12 of SLE (3.7%), 9 of RA (2.8%), and 10 of PM (3.1%) in this cohort. no DM or MCTD coexisted with PBC.

The incidence of PBC + SS in the current study was significantly higher than that reported in either the United

Table 1 Baseline characteristics of the study population

Characteristic	Cohort representation
Sex, female/male	294/28
Age (yr)	53 ± 12
Duration of disease (yr)	5.8 ± 3.5
Mayo risk score	4.5 ± 1.1
Positive ANA	275/316 (87.0)
Positive AMA	290/319 (90.9)
Positive AMA-M2	262/290 (90.3)
Titers of AMA-M2 (IU/mL)	139.6 ± 107.9
Liver biopsy	72 (22.4)

Data are presented as mean ± SD, ratio or *n* (%). ANA: Anti-nuclear antibody; AMA: Anti-mitochondrial antibody; AMA-M2: M2 subtype of anti-mitochondrial antibody.

Table 2 Inter-study comparison of patterns of connective tissue diseases in primary biliary cirrhosis patients *n* (%)

	Wang *et al* China	Watt *et al*[16] United Kingdom	Marasini *et al*[17] Italy
PBC	322	160	170
CTDs in PBC	150 (46.6)	84 (53.0)	62 (36.5)
PBC + SS	121 (37.6)	40 (25.0)[a]	6 (3.5)[a]
PBC + SSc	9 (2.8)	12 (8.0)[a]	21 (12.3)[a]
PBC + SLE	12 (3.7)	2 (1.3)	3 (1.8)
PBC + RA	9 (2.8)	27 (17.0)[a]	3 (1.8)
PBC + PM	10 (3.1)	0 (0.0)	1 (0.6)
PBC + MCTD	0 (0.0)	0 (0.0)	1 (0.6)

[a]$P < 0.05$ *vs* Wang *et al* (China, the current study). PBC: Primary biliary cirrhosis; CTDs: Connective tissue diseases; SS: Sjögren's syndrome; SSc: Systemic sclerosis; SLE: Systemic lupus erythematosus; RA: Rheumatoid arthritis; PM: Polymyositis; MCTD: Mixed connective tissue disease.

Kingdom study[16] (37.6% *vs* 25.0%, $P = 0.006$) or the Italy study[17] (37.6% *vs* 3.5%, $P = 0.000$), while the frequency of PBC + SSc was much lower (2.8% *vs* 8.0%, $P = 0.017$; 2.8% *vs* 12.3%, $P = 0.000$). The frequency of RA in the current study was less than that in the UK study (2.8% *vs* 17.0%, $P = 0.000$) but about the same as in the Italy study. The frequencies of PBC + SLE, + PM and + MCTD were not different between the three studies (Table 2).

Primary biliary cirrhosis patients with and without connective tissue diseases

There were no significant differences between PBC patients with and without CTDs in terms of sex, age, incidences of Raynaud's phenomenon (RP) or PAH, or levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), ANA, AMA, or AMA-M2 (Table 3).

Compared with PBC patients, the PBC + SS patients had significantly higher incidence of fever (6.4% *vs* 15.7%, $P = 0.010$) and ILD (7.6% *vs* 22.3%, $P = 0.000$), while the PBC + RA patients had significantly higher incidence of arthralgia (21.5% *vs* 100%, $P = 0.000$) and the PBC + SSc patients also had significantly higher incidence of ILD (7.6% *vs* 33.3%, $P = 0.035$). Patients with PBC + PM were likely to have cardiac involvement, most frequently cardiomyopathy (40% *vs* PBC patients: 2.9%, $P = 0.001$), and renal involvement was more common in patients with

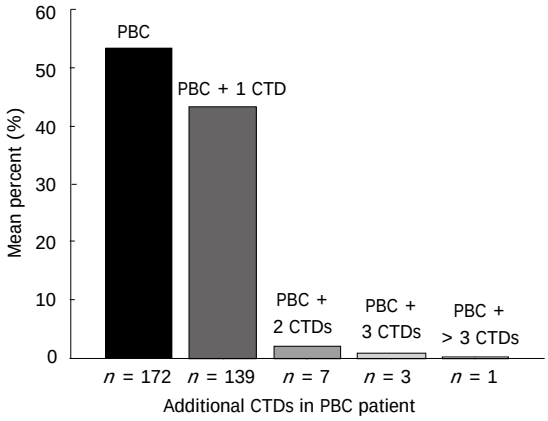

Figure 1 Percentage of primary biliary cirrhosis patients with varying numbers of connective tissue diseases. CTDs: Connective tissue diseases; PBC: Primary biliary cirrhosis.

PBC + SLE (33.3% *vs* PBC patients: 5.2%, $P = 0.006$).

The most common disease coexisting with PBC was SS. Compared to PBC patients without SS, the PBC + SS patients had higher serum level of immunoglobulin G (IgG; 17.1 ± 6.2 g/L *vs* 21.0 ± 12.4 g/L, $P = 0.004$), faster erythrocyte sedimentation rate (ESR; 41 ± 28 mm/h *vs* 57 ± 38 mm/h, $P = 0.032$), and higher rates of positivity for rheumatoid factor (RF; 19.9% *vs* 76.7%, $P = 0.000$). There were no significant differences in the clinical characteristics of patients who had PBC + SSc and those with PBC alone.

Compared to PBC patients, the PBC + SLE patients had lower white blood cell count (WBC; 5.7 ± 2.6 × 10^9/L *vs* 4.0 ± 1.1 × 10^9/L, $P = 0.005$), level of hemoglobin (Hb; 126 ± 20 g/L *vs* 102 ± 20 g/L, $P = 0.003$), platelet count (PLT; 189 ± 82 × 10^9/L *vs* 96 ± 75 × 10^9/L, $P = 0.001$), serum levels of γ-glutamyl transpeptidase (γ-GT; 320 ± 340 U/L *vs* 207 ± 153 U/L, $P = 0.048$), and IgM (4.5 ± 4.7 g/L *vs* 1.9 ± 1.2 g/L, $P = 0.001$). Compared to PBC patients without RA, patients with PBC + RA had lower Hb (126 ± 20 g/L *vs* 110 ± 11 g/L, $P = 0.001$), but higher levels of serum alkaline phosphatase (ALP, 250 ± 221 U/L *vs* 487 ± 411 U/L, $P = 0.047$) and IgG (17.1 ± 6.2 g/L *vs* 22.2 ± 5.1 g/L, $P = 0.004$), ESR (41 ± 28 mm/h *vs* 76 ± 30 mm/h, $P = 0.004$), and ratio of positive RF (19.9% *vs* 100.0%, $P = 0.009$). Compared to PBC patients, patients with PBC + PM had higher WBC count (5.7 ± 2.6 × 10^9/L *vs* 7.9 ± 3.4 × 10^9/L, $P = 0.048$).

DISCUSSION

Autoimmune diseases exhibit an increased immune response to self-antigens, occur predominantly in females, and share some similar pathogenic pathways or genetic etiologies[18,19]. Consequently, it is common for more than one autoimmune condition to occur in a single patient. For instance, the classic model of SS shows its secondary nature to SLE[20] and SSc overlapping with PM[21]. Similarly PBC often overlaps with other autoimmune diseases and conditions, thereby causing not only liver damage but

Table 3 Clinical features and laboratory results of patients with primary biliary cirrhosis alone and patients with primary biliary cirrhosis plus one other connective tissue diseases

	PBC (n = 172)	PBC + SS (n = 121)	PBC + SSc (n = 9)	PBC + SLE (n = 12)	PBC + RA (n = 9)	PBC + PM (n = 10)
Female/male	153/19	112/9	9/0	12/0	7/2	7/3
Age (yr)	53 ± 11	53 ± 12	51 ± 6[1]	50 ± 9[1]	59 ± 121	53 ± 8[1]
Fever	11 (6.4)	19 (15.7)[a]	0 (0)	3 (25.0)	1 (11.1)	1 (10.0)
RP	32 (18.6)	23 (19.0)	4 (44.4)	5 (41.7)	0 (0)	0 (0)
Arthralgia	37 (21.5)	31 (25.6)	2 (22.2)	4 (33.3)	9 (100)[a]	1 (10)
ILD	13 (7.6)	27 (22.3)[a]	3 (33.3)[a]	0 (0)	1 (11.1)	2 (20)
PAH	11 (6.4)	13 (10.7)	2 (22.2)	1 (8.3)	0 (0)	0 (0)
Cardiac	5 (2.9)	3 (2.5)	0 (0)	0 (0)	0 (0)	4 (40.0)[a]
Renal	9 (5.2)	8 (6.6)	1 (11.1)	4 (33.3)[a]	0 (0)	0 (0)
WBC (10^9/L)	5.7 ± 2.6	5.0 ± 2.9	5.7 ± 2.8[1]	4.0 ± 1.1[1,a]	5.7 ± 2.4[1]	7.9 ± 3.4[1,a]
Hb (g/L)	126 ± 20	118 ± 18	120 ± 23[1]	102 ± 20[1,a]	110 ± 11[1,a]	127 ± 16[1]
PLT (10^9/L)	189 ± 82	135 ± 69	190 ± 103	96 ± 75[a]	247 ± 142	206 ± 9[1]
ALT (U/L)	79 ± 76	91± 75	76 ± 62[1]	69 ± 72[1]	56 ± 46[1]	73 ± 56[1]
AST (U/L)	76± 62	90 ± 64	96 ± 41[1]	69 ± 55[1]	79 ± 52[1]	73 ± 33[1]
ALP (U/L)	250 ± 221	287 ± 224	291 ± 166[1]	173 ± 98[1]	487 ± 411[1,a]	153 ± 98[1]
γ-GT (U/L)	320 ± 340	309 ± 290	344 ± 346[1]	207 ± 153[1,a]	239 ± 166[1]	264 ± 275[1]
IgG (g/L)	17.1 ± 6.2	21.0 ± 12.4[a]	17.3 ± 5.8[1]	15.8 ± 5.2[1]	22.2 ± 5.1[1,a]	16.4 ± 4.4[1]
IgM (g/L)	4.5 ± 4.7	4.3 ± 3.9	3.4 ± 1.8[1]	1.9 ± 1.2[1,a]	4.2 ± 3.6[1]	5.4 ± 3.1[1]
ESR (mm/1h)	41 ± 28	57 ± 38[a]	47 ± 34[1]	47 ± 25[1]	76 ± 30[1,a]	48 ± 20[1]
RF+	31/156 (19.9)	92/120 (76.7)[a]	4/8 (50.0)	5/11 (45.5)	9/9 (100)[a]	4/10 (40.0)
ANA	142/169 (84.0)	111/119 (93.3)	9/9 (100)	12/12 (100)	8/9 (88.9)	10/10 (100)
AMA	153/171 (89.5)	109/120 (90.8)	8/9 (88.9)	11/12 (91.7)	8/9 (88.9)	10/10 (100)
AMA-M2 (IU/mL)	147± 125	130 ± 105	119 ± 115[1]	160 ± 116[1]	139 ± 118[1]	172 ± 138[1]

Data are presented as mean ± SD, ratio or n (%).[1]Non-normally distributed data compared with PBC patients by the Wilcoxon test. [a]$P < 0.05$ *vs* PBC patients. PBC: Primary biliary cirrhosis; SS: Sjögren's syndrome; SSc: Systemic sclerosis; SLE: Systemic lupus erythematosus; RA: Rheumatoid arthritis; PM: Polymyositis; WBC: White blood cell count; Hb: Hemoglobin; PLT: Platelet count; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; γ-GT: γ-glutamyl transpeptidase; IgG: Immunoglobulin G; IgM: Immunoglobulin M; ESR: Erythrocyte sedimentation rate; RF: Rheumatoid factor; ANA: Anti-nuclear antibody; AMA: Anti-mitochondrial antibody; AMA-M2: M2 subtype of anti-mitochondrial antibody.

also extrahepatic injury.

An epidemiological study from United States showed that one-third of 1032 patients with PBC were affected by another autoimmune disease, most commonly SS, RP, autoimmune thyroid disease, scleroderma, or SLE[22]. Yet another United States-based study reported that about 72% of the PBC patients also had SS, and 20% of PBC patients had joint disease[23]. Similarly, a previous study of the United Kingdom showed that 53% of the PBC patients had at least one additional autoimmune condition, with the most common being SS, autoimmune thyroid disease, RA, and SSc[16]. None of these patients had concomitant PM or DM. An Italian-based study of 170 PBC cases showed that the highest-frequency CTD was SSc (21 cases, 12.3%)[17].

The data from the current study showed that SS was the most common CTD that coexisted with PBC. The frequency was higher compared to rates reported in Europe[16,17]. SS and PBC are both characterized by immune-mediated progressive destruction of the epithelial tissues, with SS mainly affecting the salivary and lacrimal glands and PBC mainly affecting the small bile ducts[24]. Clinically, many PBC patients also present with dry eyes or mouth (47%-73%), and focal lymphocytic infiltration of labial glands (26%-93%)[25-27]. Nevertheless, in these PBC patients, anti-SSA or anti-SSB antibodies were rarely detected, and sequelae were milder than in the primary SS patients. They were also found to express lower levels of human leukocyte antigen-B8, DR3, and DRW52 compared to the primary SS patients[24]. Perhaps, only PBC patients who met the criteria of SS and also had exact anti-SSA or anti-SSB antibodies had really overlapping SS. In the current study, all of the PBC patients who met the criteria of SS were included[7], regardless of whether specific antibodies were present or not, which likely explains the particularly high number of PBC + SS patients in the current study's cohort. Compared to patients with PBC only, the PBC + SS patients were more likely to have fever and elevated ESR, suggesting that the inflammatory reaction may have been more severe in the concomitant cases. The PBC + SS cases also showed higher serum IgG levels and more frequent RF and ILD incidences; thus, treatment with glucocorticoids or immunosuppressive agents, in addition to ursodesoxycholic acid, might be beneficial for these cases.

SSc was the first reported CTD to coexist with PBC[28]. Although the known molecular targets of SSc and PBC are distinct, the two diseases share similar outcomes: sclerosis in the case of SSc and cirrhosis in the case of PBC. As both conditions result in fibrogenesis, there may be some similar epitopes or sequences in the target antigens of the two diseases that are involved in the effects on the fibrogenic pathway. According to the data from the Italian-based study[17], SSc was the most frequent comorbidity in PBC; moreover, a future study suggested that this rate might be underestimated[29]. In the Chinese-based study, the frequency of PBC + SSc was much lower than that of PBC + SS. In China, SSc cases

with only skin involvement usually consult a dermatologist for diagnosis and treatment, instead of a rheumatologist. It is likely that many cases of PBC with SSc remain undiagnosed. On the other hand, prevalence of SSc has been reported to be much higher in North America and Australia than in Japan, another Asian country[30]. The exact epidemiologic data for SSc in China is not available, but considering the similarity in genetic backgrounds of Asian ethnicities it is possible that the incidence and prevalence of SSc in China may be close to that in Japan, and lower than that in Europe. Such a situation may partially explain the observed low frequency of PBC + SSc in our Chinese cohort. Recent study from United Kingdom have demonstrated that patients affected by both PBC and SSc manifested a less aggressive form of liver disease, suggesting an active interaction between the two conditions[31]. Such characteristics were not observed in the current study's Chinese cohort. Specifically, there were no significant differences in the results of laboratory tests from the PBC patients and the PBC + SSc patients; however, the latter had higher frequency of ILD due to the existence of SSc.

PBC mostly affects middle-aged women, while the majority of SLE cases occur in women of childbearing age[32]. Therefore, the likelihood of co-existence of PBC and SLE is theoretically low. In fact, the reported frequencies of PBC + SLE in PBC are 1.25%-1.80%[16,17] and in SLE are 1.4%-7.5%[33-35]. In the current Chinese cohort, the frequency of PBC + SLE was 3.7%, which was higher than that of PBC + SSc (2.8%). Compared to patients with PBC alone, the PBC + SLE patients had lower WBC count, Hb, and PLT, and higher frequency of renal involvement, all of which are distinctive features of SLE. The coexistence of SLE in PBC patients appeared to be associated with much less extensive liver damage, as reflected by lower γ-GT and IgM levels. These finding suggest that SLE may protect against progression of PBC by inducing a slower progression to cirrhosis and delaying the need for liver transplantation[36,37].

Arthralgia is a non-specific symptom, which is very common in CTD, and inflammation of multiple joints with arthralgia is characteristic of RA. A study from the United States indicated that the rate of prevalence of RA in PBC patients did not differ from that in healthy controls[22]. However, the incidence of RA in PBC patients in the current study (2.8%) was higher than the incidence of 0.5%-1.0%[38] reported worldwide, but less than that reported in the United Kingdom study[16]. In the current cohort, the PBC + RA patients had lower Hb levels but higher serum levels of IgG, faster ESR and a higher ratio of RF positivity. They also had elevated serum ALP level, from which we conclude that coexistence of RA maybe a negative-prognosis factor for PBC[36,37].

Regarding the overlap of PBC and PM/DM, the current data did not confirm that it was as rare as reported in the previous studies in the literature. Interestingly, no cases of PBC + DM were detected. In contrast, there have been several case reports of PBC complicated by PM[39-41], and many of these cases have been asymptomatic or showing mild (early) histological changes. Higher WBC count meant more severe inflammation in PBC + PM. The PBC + PM patients in our Chinese cohort showed a tendency towards myocardial involvement, and that rate was much higher than that in the PM/DM patients[42]. It is unclear why the heart is particularly involved in PBC complicated with PM. Treatment with high-dose steroids or even pulse therapy is a particularly effective strategy[43] and has been shown to decrease mortality[44]. It is intriguing to consider that the pathogenesis of this syndrome might be related to the presence of various subtypes of AMA[45]; however, further studies are necessary to investigate whether the preferential myocardial involvement is a diagnostic finding in patients with PBC and PM.

There are several limitations inherent to the current study's design, which may have affected the results. Less than one-fourth of the patients underwent liver biopsy, which precluded our ability to perform statistical analyses of the differential pathologic features in patients with CTDs and those without CTDs. In addition, the retrospective and descriptive nature of the study restricted our investigations to only the fundamental relationship between PBC and CTDs. Future studies should be designed to investigate the relation with genetics and immune regulator factors to help identify common and distinct pathways involved in pathogeneses of the various CTDs. Finally, the follow-up was relatively short, and longer-term follow-up will help to determine the differential prognosis and mortality profiles of the various CTDs.

In conclusion, many CTDs coexist with PBC, which suggests that PBC and CTDs may share similar pathogenic mechanisms. When various CTDs coexist with PBC, different manifestations and some specific organ involvement may appear. PBC is a systemic autoimmune disease and not organ-specific. Clinicians should screen for CTDs in PBC patients, especially those who have RP, renal manifestation, or signs of involvement of other organ systems. Detailed medical history should be obtained, and laboratory examination of autoantibodies, such as ANA, should be performed to screen for co-existing CTDs and PBC.

COMMENTS

Background

Primary biliary cirrhosis (PBC) is often thought of as an organ-specific autoimmune disease which mainly targets the liver. However, accumulating evidence has indicated that PBC may involve multiple systems and may be associated with other connective tissue diseases (CTDs). It remains unknown whether these complicated PBC cases have distinctive clinical features and/or prognoses, especially in ethnic Chinese.

Research frontiers

The current study assessed the frequency of extrahepatic lesions and the association of CTDs in a cohort of 322 Chinese patients with PBC. In addition, the clinical and laboratory features were compared between the subsets of PBC patients with and without various CTDs.

Innovations and breakthroughs

According to some studies from Europe, systemic sclerosis is the CTD that

most frequently coexists with PBC. However, in the current study of a Chinese cohort, Sjögren's syndrome was the CTD that most frequently coexisted with PBC. This report is the first retrospective cohort study to investigate the differences in the clinical features and extrahepatic involvement between PBC patients with and without CTDs.

Applications

PBC is a systemic autoimmune disease and without organ-specificity. It is necessary to evaluate CTDs in PBC patients, especially those with Raynaud phenomenon, renal manifestation, and signs of involvement of multiple organ systems. Detailed collection of medical history and laboratory examination of related autoantibodies should be performed to help diagnose cases of coexisting CTDs and PBC.

Terminology

PBC is characterized by chronic non-suppurative destructive cholangitis and presence of anti-mitochondrial antibody. It ultimately progresses to cirrhosis and hepatic failure. PBC is an autoimmune liver disease that may involve multiple systems and may be associated with other CTDs.

Peer review

This is a descriptive study in which the authors analyzed the frequency and clinical features of CTDs in a cohort of 322 Chinese patients with PBC. The results showed that there were some interesting manifestations in the PBC patients with other CTDs and suggest that assessment of systemic involvements and examination of associated autoantibodies may be beneficial for patients with PBC.

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BRIEF ARTICLE

Preclinical evaluation of herpes simplex virus armed with granulocyte-macrophage colony-stimulating factor in pancreatic carcinoma

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Abstract

AIM: To investigate the therapeutic efficacy and mechanisms of action of oncolytic-herpes-simplex-virus encoding granulocyte-macrophage colony-stimulating factor ($HSV^{GM\text{-}CSF}$) in pancreatic carcinoma.

METHODS: Tumor blocks were homogenized in a sterile grinder in saline. The homogenate was injected into the right armpit of each mouse. After vaccination, the mice were randomly assigned into four groups: a control group, a high dose $HSV^{GM\text{-}CSF}$ group [1×10^7 plaque forming units (pfu)/tumor], a medium dose $HSV^{GM\text{-}CSF}$ group (5×10^6 pfu/tumor) and a low dose $HSV^{GM\text{-}CSF}$ group (5×10^5 pfu/tumor). After initiation of drug administration, body weights and tumor diameters were measured every 3 d. Fifteen days later, after decapitation of the animal by cervical dislocation, each tumor was isolated, weighed and stored in 10% formaldehyde solution. The drug effectiveness was evaluated according to the weight, volume and relative volume change of each tumor. Furthermore, GM-CSF protein levels in serum were assayed by enzyme-linked immunosorbent assays at 1, 2, 3 and 4 d after injection of $HSV^{GM\text{-}CSF}$.

RESULTS: Injection of the recombinant mouse HSV encoding GM-CSF resulted in a significant reduction in tumor growth compared to the control group, and dose-dependent effects were observed: the relative tumor proliferation rates of the low dose, medium dose and high dose groups on 15 d after injection were 45.5%, 55.2% and 65.5%, respectively. The inhibition rates of the tumor weights of the low, middle, and high dose groups were 41.4%, 46.7% and 50.5%, respectively. Furthermore, the production of GM-CSF was significantly increased in the mice infected with $HSV^{GM\text{-}CSF}$. The increase in the GM-CSF level was more pronounced in the high dose group compared to the other two dose groups.

CONCLUSION: Our study provides the first evidence that $HSV^{GM\text{-}CSF}$ could inhibit the growth of pancreatic cancer. The enhanced GM-CSF expression might be responsible for the phenomenon.

Key words: Pancreatic carcinoma; Gene therapy; Animal test; Herpes-simplex-virus encoding granulocyte-macrophage colony-stimulating factor

Core tip: Herpes-simplex-virus encoding granulocyte-macrophage colony-stimulating factor ($HSV^{GM\text{-}CSF}$) is an engineered oncolytic virus. The key features of $HSV^{GM\text{-}CSF}$ include the deletion of both copies of $\gamma_1$34.5 and the

ICP47 gene as well as interruption of the *ICP6* gene and insertion of the therapeutic gene GM-CSF. Our study provides the first evidence that HSV^{GM-CSF} could inhibit the growth of pancreatic cancer in a dose-dependent manner. Enhanced GM-CSF expression might be responsible for the phenomenon.

Liu H, Yuan SJ, Chen YT, Xie YB, Cui L, Yang WZ, Yang DX, Tian YT. Preclinical evaluation of herpes simplex virus armed with granulocyte-macrophage colony-stimulating factor in pancreatic carcinoma. *World J Gastroenterol* 2013; 19(31): 5138-5143 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i31/5138.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i31.5138

INTRODUCTION

Pancreatic cancer is a rapidly fatal malignancy with one-year relative survival rates less than 30% and nearly all patients die from their disease within 7 years of surgery[1,2]. More than 80% patients are unsuitable for radical resection. Further more, it is insensitive to current chemotherapy, radiotherapy and immunotherapy.

Gene therapy of pancreatic carcinoma is considered a novel model, and has become an emerging research area in recent years. Successful drugs for gene therapy may result in prolonged survival. Oncolytic herpes simplex virus encoding granulocyte-macrophage colony-stimulating factor (HSV^{GM-CSF}) is an attenuated, replication-competent oncolytic virus. It can activate the host's own immune system against infected tumor cells. Some clinical trials of HSV for the treatment of various cancers have been completed, providing preliminary data about its safety and effectiveness[3-7]. However, there is little data for pancreatic cancer.

Therefore, we conducted a preclinical evaluation of effects of HSV^{GM-CSF} on pancreatic cancer and explored the mechanisms that may be involved in any antitumor response.

MATERIALS AND METHODS

Experimental chemical

The OrienGene Biotechnology Ltd. (Beijing, China) provided the mouse recombinant GM-CSF herpes simplex virus (HSV^{GM-CSF}) (OrienX010).

Experimental cell and animals

Panc-2 cells: All cells used in this study represent mouse pancreatic carcinoma cell lines. Panc-2 cells were grown in Dulbecco's modification of Eagle's medium.

Animals: Female C-57B mice (4-6 wk, 16-18 g) were provided by the Experimental Animal Center, Peking Union Medical College. The Committee of Animal Care and Use of the university approved the experimental protocol, which met the regulatory requirements of Tumor Hospital, Chinese Academy of Medical Science for the use of experimental animals. All mice were bred in a standard environment and were provided with free access to food and water.

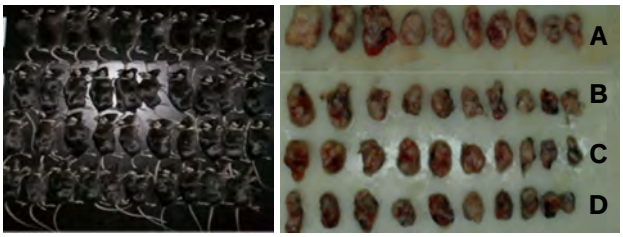

Figure 1 Inhibition of herpes simplex virus encoding granulocyte-macrophage colony-stimulating factor on the proliferation of PANC-2 pancreatic carcinoma xenografts in mice. A: Control group; B: High dosage; C: Middle dosage; D: Low dosage. Fifteen days later, after decapitation of the animals by cervical dislocation, each tumor was isolated.

Experimental procedure

Injection of transplanted tumors and drug administration in mice followed standard methods used internationally. The Discussion Draft of Guidance Principles of Pharmacodynamics of Antitumor Drugs[8] and Anticancer Drug Development Guide: Preclinical Screening, Clinical Trials and Approval[9] were used for guidance. Panc-2 cells were first recovered and amplified for collection of tumor cells, of which a total of 1×10^7-1×10^8 plaque forming units (pfu) virus were subcutaneously injected into each mouse. When the resulting tumor had grown to 2-3 cm in diameter, the tumor tissue was dissected under sterile conditions and cut into blocks of 2 mm^3 with sterile scissors. The tumor blocks were homogenized in a sterile grinder with normal saline. The homogenate was injected into the right armpit of each mouse. After vaccination, the mice were randomly grouped for intratumoral administration of drugs. After initiation of drug administration, body weights and tumor diameters were measured every 3 d. Fifteen days later, after decapitation of the animals by cervical dislocation, each tumor was isolated, weighed and stored in 10% formaldehyde solution (Figure 1). The drug effectiveness was evaluated according to the weight, volume and relative volume change of each tumor.

GM-CSF quantification by enzyme-linked immunosorbent assay

In vivo blood collected by tail vein bleed was centrifuged, and serum was collected and stored at -20 ℃. Mouse GM-CSF concentration was determined by an enzyme-linked immunosorbent assay (ELISA) (Abcam Inc, MA, United States), according to manufacturer's protocol, for cells infected with 1×10^7, 5×10^6 and 5×10^5 pfu/mL.

Animal grouping and drug administration

Three days after vaccination of tumors, the mice were randomly divided into groups with the weights of the

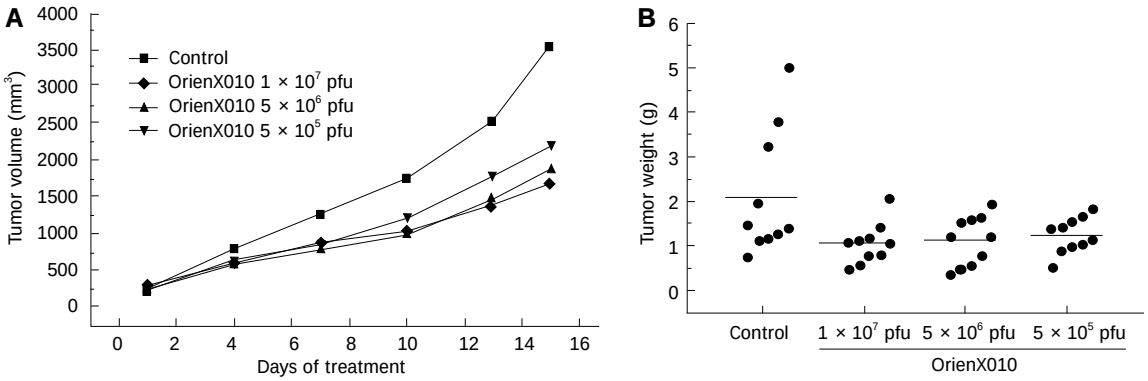

Figure 2 Effect of OrienX010 on the tumor volume (A) and tumor weight (B) of PANC-2 pancreatic carcinoma xenografts in mice. pfu: Plaque forming units.

Table 1 Inhibition of OrienX010 on the growth of PANC-2 pancreatic carcinoma xenografts in mice

Administration	Host (mice) reaction		Tumor reaction					
	Animal number beginning/end	Body weight, g (mean ± SD) beginning/end	Tumor weight, g (mean ± SD)	*Z*	Tumor volume (mm^3)	*J*	RTV	T/C (%)
	10/10	16.5 ± 1.2/19.8 ± 1.9	2.10 ± 1.41		3555.8 ± 1849.8		15.4 ± 5.7	
Tumor injection × 4	10/10	16.4 ± 0.9/18.2 ± 2.4	1.04 ± 0.45	50.50%	1668.3 ± 661.9	53.10%	7.0 ± 2.5[b]	45.5
Tumor injection × 4	10/10	16.0 ± 0.6/18.6 ± 1.3	1.12 ± 0.55	46.70%	1869.8 ± 846.6	47.40%	8.5 ± 4.4[b]	55.2
Tumor injection × 4	10/10	16.2 ± 0.8/18.5 ± 0.8	1.23 ± 0.39	41.40%	2200.3 ± 826.5	38.10%	10.1 ± 4.2[a]	65.6

[a]$P < 0.05$, [b]$P < 0.01$ *vs* control group; *Z*: Inhibition rate of tumor weight; *J*: Inhibition rate of tumor volume; RTV: Relative tumor volume.

animals being similar in each group: control group, high dose group (1×10^7 pfu/tumor), middle dose group (5×10^6 pfu/tumor) and low dose group (5×10^5 pfu/tumor). The drug was administered via intratumoral injections of 0.1 mL/tumor on the first day.

Statistical analysis

Data were expressed as mean ± SD. The inhibition rate of tumor proliferation = (tumor weight of control group - tumor weight of drug group)/tumor weight of control group × 100%. Tumor volume (V) = $1/2ab^2$ (a = tumor major diameter; b = tumor minor diameter). The inhibition rate of tumor volume proliferation = (tumor volume of control group - tumor volume of drug group)/tumor volume of control group × 100%. Relative tumor volume (RTV) = Vt/Vo (Vo = tumor volume pre-drug, Vt = tumor volume measured each time after drug administration). The relative tumor proliferation rates (T/C) = RTV of drug group/RTV of control group × 100%. SPSS13 was used for statistical analysis of inter-group difference using t tests and for plotting of the tumor volume, relative growth curve of volume-time, tumor weight and related tables and figures.

RESULTS

The tumor volume on day 15 post-treatment in the control group was 3555.8 ± 1849.8 mm^3. The tumor volume of the group treated with a single intratumoral injection of low dose virus was 2200.3 ± 826.5 mm^3 ($P < 0.05$ *vs* control). For the middle dose virus group, the tumor volume on day 15 post-treatment was 1869.8 ± 846.6 mm^3 ($P < 0.05$ *vs* control). The tumor volume on day 15 post-treatment of the high dose virus group was 1668.3 ± 661.9 mm^3 ($P < 0.01$ *vs* control) (Figure 2A). The inhibition rates of the tumor volumes of dose of the low, middle, and high dose groups were 38.1%, 47.4% and 54.3%, respectively. Thus, $HSV^{GM\text{-}CSF}$ could inhibit pancreatic cancer in a dose-dependent manner. The relative tumor proliferation rates of the low, middle, and high dose groups were 45.5%, 55.2% and 65.5%, respectively (Table 1).

The present study showed that the tumor weight of the control group was 2.10 ± 1.41 g, 1.23 ± 0.39 g in the low dose group ($P > 0.05$ *vs* control), 1.12 ± 0.55 g in the middle dose group ($P > 0.05$ *vs* control), and 1.04 ± 0.45 g in the high dose group ($P < 0.05$ *vs* control). The inhibition rates of the tumor weights of the low, middle, and high dose groups were 41.4%, 46.7% and 50.5%, respectively (Figure 2B). Only the high dose group showed a significant difference compared with the control group ($P < 0.05$). There was no significant difference in mouse body weight among these four groups ($P > 0.05$) (Figure 3). Also, none of the mice died or showed skin ulceration/necrosis at the tumor location during the experiment.

The results of serum GM-CSF protein level showed that $HSV^{GM\text{-}CSF}$ significantly increased GM-CSF production, peaking at day 3 after treatment (Figure 4). There may be a correlation between the dose of $HSV^{GM\text{-}CSF}$ and the GM-CSF protein level.

Dissection of the mice at 15th day after administration of the drug showed no adhesions around the tumors, and there were no ascites or metastasis of the tumors in the peritoneal cavity; the tumors appeared as gray in color, had uniform textures and showed no necrosis.

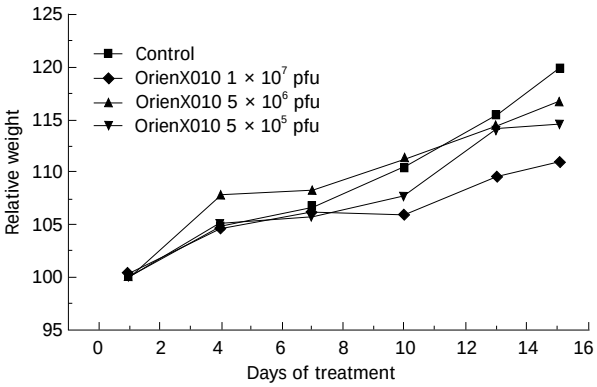

Figure 3 Changes in relative mouse body weights. pfu: Plaque forming units.

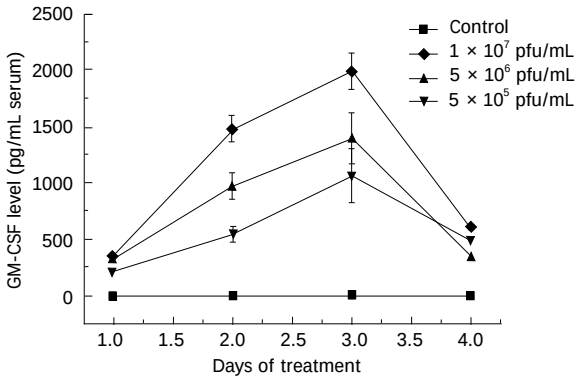

Figure 4 Quantification of expressed granulocyte-macrophage colony-stimulating factor. pfu: Plaque forming units; GM-CSF: Granulocyte-macrophage colony-stimulating factor.

DISCUSSION

Compared to the traditional therapeutic methods, gene therapy is a recent and active research field. Since 1999, Germany, the United Kingdom and the United States have approved gene therapy projects for pancreatic carcinoma to enter clinical stage Ⅰ/Ⅱ trials, some of which are complete, providing preliminary data about its safety and effectiveness. The data suggested that the gene drugs were well tolerated in cancer patients and could suppress tumor growth[3-7]. The aim of the present study was to evaluate the efficacy of HSV$^{GM\text{-}CSF}$, an attenuated, replication-competent oncolytic virus, for treating mouse pancreatic carcinoma.

HSV$^{GM\text{-}CSF}$ is an engineered oncolytic virus. It belongs to a conditional replication HSV-1 mutant that uses the differences in cellular structure and metabolic pathways between tumors and normal tissues and retains the genes related to virus replication. The key features of HSV$^{GM\text{-}CSF}$ include the deletion of both copies of $\gamma_1$34.5 and *ICP47* genes, as well as interruption of the *ICP6* gene and insertion of the therapeutic gene *GM-CSF*. GM-CSF is a pleiotropic cytokine secreted by many kinds of cells, including activated lymphocytes, macrophages and endothelial cells. Several previous studies demonstrated that GM-CSF was one of the most potent cytokines[5] that could influence the immune response in several ways, including recruitment and stimulation of antigen-presenting cells, such as dendritic cells, and induction of myeloid precursor cells to proliferate and differentiate into monocytes, macrophages, neutrophils and eosinophils[10]. Viral lysis and the mechanism mediated by the transgene protein, represent two parallel mechanisms of tumor destruction that can be achieved using HSV$^{GM\text{-}CSF}$.

The encouraging results of the present study suggested that the proliferation speed of tumors in the mouse experimental groups was reduced after 15 d of administration of HSV$^{GM\text{-}CSF}$ compared with the control group ($P < 0.05$). The reduction of tumor growth was dose-dependent. However, there was no obvious difference in the host response between the different dosages, which may be related to the small differences in drug dosages and could be resolved with the promotion of pharmacological techniques for high-concentration drugs.

The sera of cells infected with the three doses of the virus showed high expression of GM-CSF. There was no GM-CSF secretion in the control group. The results suggested that the HSV$^{GM\text{-}CSF}$ enhanced *GM-CSF* gene expression. Additionally, the increase was more pronounced in the group injected by the high dose virus than in the middle and low dose groups. There may be a correlation between the dose of HSV$^{GM\text{-}CSF}$ and the GM-CSF protein level. These results indicated that HSV$^{GM\text{-}CSF}$ could regulate immunity in cancer-bearing mice. The increased GM-CSF levels might be responsible for the dose-dependent relationship between the drug and the tumor response.

Currently, HSV vectors alone, and HSV vectors armed with GM-CSF or other recombinant genes have been successfully tested for safety in humans and have exhibited efficacy in preclinical animal models against various human cancers. And HSV mutant has been shown to be an effective strategy for lysing tumor cells *in vitro* and in multiple experimental animal models[10-16]. Geevarghese *et al*[17] evaluated the anti-tumor effects of NV1020 (another HSV-1 mutant), which showed that the NV1020 stabilized liver metastases in patients, and extended survival by resensitizing the cancer cells to chemotherapy. Both Yang *et al*[18] and Malhotra *et al*[19] suggested that the HSV vectors armed with GM-CSF had a significantly better antitumor effect compared to treatment with HSV vectors alone in mouse colon cancer. Furthermore, Derubertis *et al*[20] declared that mouse colorectal cancer hepatic metastases could be suppressed by HSV vectors armed with GM-CSF. HSV$^{GM\text{-}CSF}$ combined with cisplatin-based chemoradiotherapy was well tolerated in patients with stage Ⅲ/Ⅳ head and neck cancer. The present study showed that HSV$^{GM\text{-}CSF}$ enhanced the inhibition rate of mouse pancreatic cancer by regulating the expression of GM-CSF. The results were similar to those provided in previous studies[19,20].

Although the agent was highly attenuated and replication restricted, the use of a virus still raises concerns about viral proliferation and dissemination. During the experimental period, the body weights of the mice in the

experimental groups were similar to the control group at the beginning, and gradually and stably increased. At the later stages, the body weights slowly increased, particularly in the high-dosage group, compared to the control group, but there was no statistical difference. In addition, there was no occurrence of treatment-related death or ulceration/necrosis of the skin, suggesting that the drug is safe and effective, with low toxicity and side effects, and is tolerated by mice. The existence of antiviral drugs, such as ganciclovir, provides us with a further margin of safety.

In the present study, the transplanted tumor cell was injected into the armpits of mice and the drug was administrated by intratumoral injection. If applied in a clinic, the drug could be administered through a fine needle puncture technique with the guidance of CT/endoscopic ultrasonography or through vascular intervention, which several research centers have proved to be effective. Mulvihill *et al*[21] performed a clinical trial of intratumoral injection of the *ONYX-015* gene with the guidance of CT, while Löhr *et al*[22] reported their experimental results of clinical stage I and II trials of drug administration via vascular intervention.

Previous studies indicated that the HSV vector had a significant effect on multiple solid tumors[23-26], and could enhance the effect of other combined common therapies, such as radiotherapy and chemotherapy. Most studies that combined viral gene therapy with other therapies observed a synergistic effect in preclinical models[27-29]. Recently a stage I / II clinical trial of combined HSV with radiotherapy in head and neck tumors ended and showed no obvious side effects[7]. We are performing experimental research using an injection solution of recombined mouse $HSV^{GM\text{-}CSF}$ combined with radiotherapy to find a new approach in treating pancreatic carcinoma.

During the last two decades, gene therapy has made great progress. Simultaneous use of basic research and clinical experiments may become one of the fastest-developing areas in the field of medicine in the next 10 years. The development of gene therapy has proved difficult, and application in the clinic is still a long way off. The immunity, safety, transduction rate and tissue specificity of current vectors require further study and improvement, which is a common problem in gene therapy. The vectors used in the clinic in the future should have the advantage of combining non-viral vectors and alternative viral vectors that can be customized according to different requirements to express the target gene in specific tissues, and effectively modulate their expression level and duration.

COMMENTS

Background

Pancreatic cancer is a rapidly fatal malignancy with one-year relative survival rates less than 30%; nearly all patients die from their disease within 7 years of surgery. Gene therapy of pancreatic carcinoma is considered a novel model, and has become an emerging research area in recent years.

Research frontiers

The gene therapy model for pancreatic carcinoma has emerged recently. Successful drugs for gene therapy may result in prolonged survival. Oncolytic herpes simplex virus encoding granulocyte-macrophage colony-stimulating factor ($HSV^{GM\text{-}CSF}$) is an attenuated, replication-competent oncolytic virus. It can activate the host's own immune system against infected tumor cells. Some clinical trials of HSV for the treatment of various cancers had been completed, providing preliminary data about its safety and effectiveness. However, there is little data for pancreatic cancer.

Innovations and breakthroughs

$HSV^{GM\text{-}CSF}$ is an engineered oncolytic virus. It is a conditional replication HSV-1 mutant that utilizes differences in cellular structure and metabolic pathways between tumor and normal tissues, and retains the genes related with virus replication. The key features of $HSV^{GM\text{-}CSF}$ include the deletion of both copies of $\gamma_1$34.5 and *ICP47* gene as well as interruption of the *ICP6* gene and insertion of the therapeutic gene GM-CSF.

Peer review

The study is very interesting. Liu *et al* investigated the therapeutic efficacy of oncolytic $HSV^{GM\text{-}CSF}$ in a mouse model of pancreatic carcinoma and explored mechanisms that may be involved in the antitumor response. The authors provide evidence that $HSV^{GM\text{-}CSF}$ could inhibit the growth of pancreatic cancer.

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BRIEF ARTICLE

HMGB1 gene polymorphisms in patients with chronic hepatitis B virus infection

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Abstract

AIM: To characterize high mobility group box chromosomal protein 1 (*HMGB1*) polymorphisms in patients infected with hepatitis B virus (HBV) and determine the different patterns in patient subgroups.

METHODS: A total of 1495 unrelated Han Chinese HBV carriers were recruited in this hospital-based case-control study. The *HMGB1 1176 G/C* polymorphism was genotyped by polymerase chain reaction-restriction fragment length polymorphism assay.

RESULTS: A significant association was observed between *HMGB1 1176 G/C* polymorphism and outcome of HBV infection. The subjects bearing 1176G/G genotype had an increased risk of susceptibility to chronic hepatitis B, liver cirrhosis and severe hepatitis B when compared with those bearing at least one 1176C allele.

CONCLUSION: Patients with 1176G/G genotype of *HMGB1* gene are more likely to have a progressive status in HBV infection.

Key words: High mobility group box chromosomal protein 1; Hepatitis B virus; Polymorphism; Intron

Core tip: We analyzed the relationship between the high mobility group box chromosomal protein 1 (*HMGB1*) *1176 G/C* polymorphism and the susceptibility and outcome to hepatitis B virus (HBV) infection in a large hospital-based case-control study. Our results indicated that patients with 1176G/G genotype of *HMGB1* gene are more likely to have a progressive status in HBV infection. Our study emphasizes the importance of HMGB1 in the pathophysiology of HBV-related diseases on the population level and will provide researchers new clue for the further basic research in pathogenesis of chronic HBV infection.

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INTRODUCTION

Hepatitis B virus (HBV) infection is associated with a variety of diseases, including asymptomatic carrier (AsC), fulminant hepatitis, chronic hepatitis (CHB), liver cirrhosis (LC), and hepatocellular carcinoma (HCC). Persistent

HBV infection has been considered as a multifactorial and polygenic disorder with viral, environmental and genetic components. HBV genomic variability and a number of conventional risk factors, including age, gender, concurrent infection with hepatitis C virus, hepatitis D virus and human immune deficiency virus, are clearly the important factors contributing to the incidence of persistent HBV infection[1-4]. However, segregation analysis and twin studies strongly support the role of host genetic components in determining the chronicity of HBV infection[5,6]. A known and unknown number of identified or unidentified genes are likely to modify the susceptibility to persistent HBV infection[7-10]. Single nucleotide polymorphism (SNP) is currently believed to be a powerful tool for identifying genetic susceptibilities to common complex diseases[11,12].

The intranuclear architectural protein termed high mobility group box chromosomal protein 1 (HMGB1) has recently been identified as a potent proinflammatory mediator when passively released to extracellular by necrotic cells, as opposed to apoptotic cells that will induce inflammation[13,14]. Furthermore, HMGB1 can also be actively secreted by stimulated macrophages or monocytes[15-17]. Active secretion from living inflammatory cells and passive release from necrotic cells implicate that HMGB1 may play a central role in proinflammatory reactions. It is well known that HBV infection is closely related with cytokines. Polymorphisms of cytokine gene, such as human leukocyte antigen, estrogen receptor alpha (*ESR1*), have been reported to be associated with HBV infection[18-21]. However, so far there has been no report on the association between *HMGB1* gene and HBV infection. We conducted a hospital-based case-control study including more than one thousand subjects with HBV infection to characterize the relationship between *HMGB1* gene polymorphism and HBV infection.

MATERIALS AND METHODS

Patients

Patients with HBV infection were randomly selected from the outpatient and inpatient referral center affiliated to the Institute for Infectious Diseases of Southwest Hospital treated between February 2002 and February 2012. Informed consent was obtained from all the patients to participate in the study. Participants finally included in the current study were from a subset of unrelated individuals from the referral center. The diagnostic criteria for chronic HBV infection were as follows: persistent presence of hepatitis B surface antigen (HBsAg), absence of anti-hepatitis B surface antibodies (anti-HBs), presence of anti-core IgG antibodies (anti-HBc), and presence of hepatitis B early antigen (HBeAg) or anti-hepatitis B e antibodies (anti-HBe) for 6 mo or longer despite of virus replication. Asymptomatic carriers had no fluctuation of serum alanine aminotransferase (ALT) levels and no obvious clinical symptoms. Chronic hepatitis B had a serum ALT fluctuation, 1 × the upper limit of normal (ULN) < ALT < 5 × ULN, with or without other abnormal hepatic functions. Severe hepatitis B (SHB), which is currently equal to acute-on-chronic liver failure, presents the following symptoms: (1) fatigue with striking gastrointestinal tract symptoms; (2) rapidly worsening jaundice, with serum total bilirubin (TBIL) 10 times higher than ULN, or with a daily increase ≥ 17.1 μmol/L; (3) hemorrhagic tendency with international normalised ratio ≥ 1.5 or prothrombin activity ≤ 40% where other causes have been excluded; (4) progressive reduction in liver size; and (5) occurrence of hepatic encephalopathy. Liver cirrhosis and HCC were confirmed by liver biopsy, ultrasound, and/or computerized tomography scan. Healthy control individuals were recruited from Red Cross blood donor centers with or without anti-HBs, but HBsAg, anti-HBc, HBeAg, and anti-HBe were negative.

DNA extraction

The leukocytes genomic DNA from 5 mL whole blood was isolated using Miller's method[22]. DNA samples were diluted to 8 ng/μL and distributed into 96-well plates (DNA panels), with 94 samples and 2 controls (DNA-free water) in each plate.

Gene polymorphism

We used the current recommendations of human genome SNP described at http://www.ncbi.nlm.gov/SNP under accession number NT024524. The higher allele variation frequency selected in position 1176 G/C, the intron 4 of *HMGB1* gene, was studied to determine whether any association identified was specific to HBV infection. The SNP was named in a same way to *HMGB1* (1176G/C).

Genotype

The genotyping was analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. Appropriate primer pairs (sense 5'-3' GTCTCCTTTGCCCAGTGTATCTC and anti-sense 5'-3'GTACACAGCCTTTGTCTGAGTCTG) were designed by Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, United States). PCR condition was as follows: one cycle of predenature 3 min at 95 ℃, 30 cycles of denature 30 s at 94 ℃, hybridization for 30 s at 54 ℃, an extension cycle of 50 s at 72 ℃, and a last cycle of delay 5 min at 72 ℃. Restriction enzyme BcLI (recognition site T/GATCA) was obtained from NEB; the fragments were separated by electrophoresis on 3% agarose gel and stained with ethidium bromide for visualization under ultraviolet light. The observed genotypes were also identified by direct sequencing before large-scale test was started.

Statistical analysis

An allele frequency was directly calculated by its genotype. The observed genotype frequencies and allele frequency were compared using χ^2 test between the variables to determine if they were in Hardy-Weinberg equilibrium.

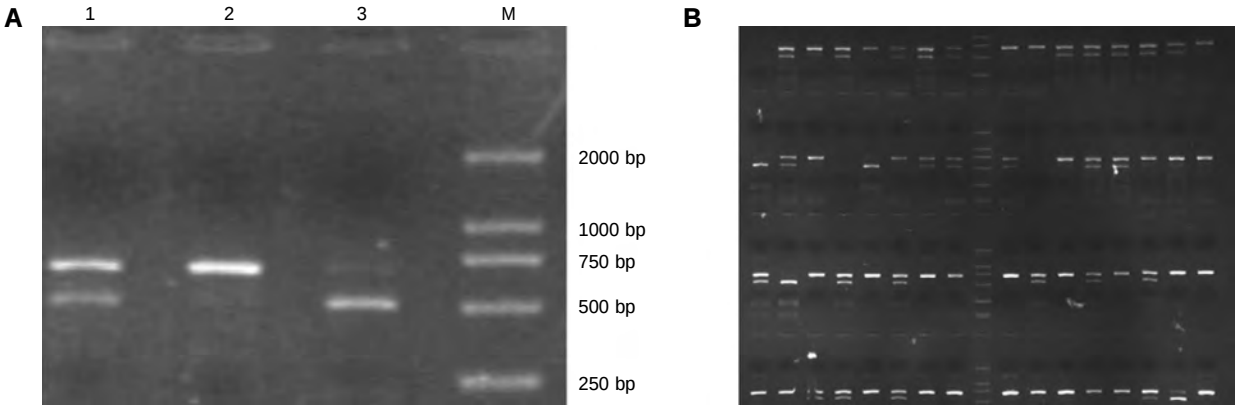

Figure 1 High mobility group box chromosomal protein 1 (1176G/C) restriction fragment length polymorphism genotyping. A: The typical pattern of three genotypes; B: Panel genotyping. 1: GC genotype; 2: GG genotype; 3: CC genotype; M: Marker DL2000.

Table 1 High mobility group box chromosomal protein 1 polymorphism (1176G/C) between various clinical subgroups infected with hepatitis B virus

	Sex	Age (yr)	Genotype			Allele frequency	
	(M/F)	(mean ± SD)	GG	CC	GC	G	C
AsC (n = 199)	116/83	34.762 ± 11.282	107	9	83	0.7462	0.2538
AHB (n = 15)	11/4	30.201 ± 10.221	9	1	5		
CHB (n = 929)	730/199	34.312 ± 11.549	572	33	324	0.6530	0.3470
SHB (n = 157)	129/28	39.989 ± 11.792	91	6	60	0.7707	0.2293
LC (n = 175)	142/33	41.950 ± 11.437	104	13	58	0.7600	0.2300
HCC (n = 20)	14/6	49.256 ± 12.232	10	1	9		
LC + CHB (n = 1104)	872/232	35.461 ± 11.642	676	46	382	0.7853	0.2147
LC + CHB + SHB (n = 1261)	1001/260	36.001 ± 11.852	767	52	442	0.7835	0.2165

There was age difference in any two subgroups except between AsC and CHB, $P < 0.05$; There was sex difference between AsC and any other subgroups, $P < 0.05$; AsC: Asymptomatic carrier; AHB: Acute hepatitis B; CHB: Chronic hepatitis B; SHB: Severe hepatitis B; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma.

An observed $P > 0.01$ was considered in Hardy-Weinberg equilibrium, and $P < 0.05$ was considered significantly different between the variables. All the analyses were performed with SPSS11.0 statistical software (SPSS Inc., Chicago, IL, United States).

RESULTS

HMGB1 1176G/C polymorphism genotyping

HMGB1 1176G/C polymorphism was genotyped by PCR-RFLP assay (Figure 1). A total of 1495 clearly diagnosed and genotyped patients were enrolled. The clinical characteristics, such as age and sex, are listed in Table 1. Apparently, age or sex difference existed in the studied subgroups. Hardy-Weinberg equilibrium by χ^2 test showed $P = 0.494 > 0.01$ (Table 1), which confirmed that the studied group was in Hardy-Weinberg equilibrium.

Case-control association study

Because age or sex difference existed in the studied subgroups, it is essential to detect the association between observed SNP and HBV infected subgroups, and age and sex factors were considered by logistic regression (Table 2). A statistically significant difference in the distribution of *HMGB1* polymorphism (1176G/C) was observed between subgroups of AsC and LC (OR = 1.571, 95%CI: 1.108-2.227, $P = 0.011$, codominant model); AsC and CHB (OR = 1.354, 95%CI: 1.085-1.689, $P = 0.007$, codominant model); AsC and CHB + SHB + LC (OR = 1.401, 95%CI: 1.010-1.944, $P = 0.044$, recessive model); OR = 1.329, 95%CI: 1.070-1.651, $P = 0.010$, codominant model, AsC and CHB + LC (OR = 1.406, 95%CI: 1.011-1.956, $P = 0.043$, recessive model; OR = 1.355, 95%CI: 1.088-1.687, $P = 0.007$, codominant model).

DISCUSSION

HMGB1 is a nuclear DNA-binding protein, which also functions as a pleiotropic cytokine, implicated in the pathology of several different immune-mediated diseases. The human *HMGB1* gene is located on chromosome 13. Kornblit *et al*[23] firstly elaborated six polymorphisms and four mutations identified in the *HMGB1* gene, located in -1615A/G, 982C/T, 3814C/G, 1779T/G, -196C/A, 1808C/G, 4519_4521delGAT, -1377delA,1747delT, 1888insT, respectively. In other studies, several associations have been observed, revealing the importance of the genetic variation in the *HMGB1* gene. In their report, the -1377delA$^{A/-}$ genotype or the -1377delA$^{-/-}$ genotype

Table 2 Association between high mobility group box chromosomal protein 1 (1176G/C) single nucleotide polymorphism and hepatitis B virus infected subgroups

Subgroup	Dominant model		Recessive model		Codominant model	
	P	OR (95%CI)	*P*	OR (95%CI)	*P*	OR (95%CI)
LC/CHB	0.075	0.533 (0.267-1.065)	0.945	0.988 (0.701-1.392)	0.120	0.844 (0.682-1.045)
LC/SHB	0.183	0.508 (0.188-1.376)	0.747	1.075 (0.693-1.669)	0.326	0.872 (0.664-1.146)
LC/AsC	0.168	2.320 (0.702-7.672)	0.108	1.572 (0.906-2.728)	0.011	1.571 (1.108-2.227)
AsC/SHB	0.473	1.616 (0.436-5.999)	0.489	1.204 (0.711-2.040)	0.206	1.254 (0.883-1.782)
AsC/CHB	0.238	1.660 (0.716-3.851)	0.052	1.389 (0.997-1.935)	0.007	1.354 (1.085-1.689)
SHB/CHB	0.916	1.050 (0.425-2.592)	0.474	1.137 (0.800-1.614)	0.462	1.090 (0.866-1.371)
SHB + LC/CHB + AsC	0.362	0.760 (0.421-1.371)	0.824	0.971 (0.747-1.262)	0.316	0.918 (0.777-1.085)
AsC/CHB + SHB + LC	0.330	1.505 (0.662-3.421)	0.044	1.401 (1.010-1.944)	0.010	1.329 (1.070-1.651)
AsC/CHB + LC	0.256	1.619 (0.704-3.721)	0.043	1.406 (1.011-1.956)	0.007	1.355 (1.088-1.687)

The association was analyzed by logistic regression analysis with adjustment for covariates, including age, sex, and alcohol consumption. Dominant model: GG + GC/CC, recessive model: GG/GC + CC, codominant model: GG/GC/CC; *P* and OR values were calculated by logistic regression. AsC: Asymptomatic carrier; AHB: Acute hepatitis B; CHB: Chronic hepatitis B; SHB: Severe hepatitis B; LC: Liver cirrhosis; OR: Odds ratio.

showed a significant association with delayed mortality, independent of age and number of the systemic inflammatory response syndrome (SIRS) criteria[24]. Subsequent estimation revealed that several polymorphisms have a potential regulatory impact on HMGB1 transcription. Genetically determined risk factors associated with early and late mortality and death due to infection have been identified, explaining some of the inherited risks in this heterogeneous patient population. Associations between genetic variation and disease severity parameters are also established. Studies of association between *HMGB1* polymorphisms and disease have been also reported with allogeneic hematopoietic cell transplantation (HCT)[25]. Patient homozygosity or heterozygosity for the-1377delA minor allele is associated with increased risk of relapse and increased relapse-related mortality. Furthermore, patient homozygosity for the 3814C/G minor allele is associated with increased overall survival and progression-free survival. Patient carriage of the 2351insT minor allele can reduce the risk from grade II to IV acute graft-versus-host disease whereas donor homozygosity is associated with chronic acute graft-versus-host disease. These findings suggest that the inherited variation in HMGB1 is associated with outcome after allogeneic HCT following myeloablative conditioning regimens. Zeng *et al*[26] found that three SNPs act as tag SNPs for the entire *HMGB1* gene in multiple organ dysfunction syndromes. The rs2249825 and the haplotype TCG can be used as relevant risk estimate for the development of sepsis in patients with major trauma.

As is well known, the susceptibility of HBV infection is closely related to the variation of some important genes. Deng *et al*[27] have demonstrated that polymorphisms at the *ESR1* gene locus are associated with persistent HBV infection. Subsequently, Yan *et al*[18] have also demonstrated an association between cis-acting regulatory variation of the *ESR1* gene and hepatitis B virus-related liver cirrhosis. Some important variations of cytokine gene also influenced the susceptibility to HBV infection. Deng *et al*[28] have found that the novel regulatory polymorphism G-201A in the promoter of interferon gamma-inducible protein of 10 kilodaltons (*IP-10*, *CXCL10*) gene might be a part of the genetic variation underlying the susceptibility of individuals to disease progression of chronic HBV infection. In another study, Yan *et al*[29] demonstrated that the -592C allele and the -1082A-819C-592C haplotype in the *IL-10* gene promoter were associated with an increased susceptibility to acute liver failure in HBV carriers.

Nevertheless, there are few reports about the association between *HMGB1* gene and HBV infection, especially reports about the association between *HMGB1* polymorphisms and HBV infection. In this study, we used the current recommendations of human genome SNPs described at http://www.ncbi.nlm.gov/SNP under accession number NT024524. The higher allele variation frequency was selected in position 1176 G/C, the intron 4 of *HMGB1* gene. There has been no report about this SNP up to date. We genotyped the polymorphisms of 1495 cases, including AsC, CHB, SHB, LC and HCC. The distribution of *HMGB1* 1176G/C genotypes in studied sample of unrelated men and women from the referral center were in Hardy-Weinberg equilibrium ($P > 0.01$), so it is important to consider whether our studied sample could be representative. Yan *et al*[18] and Deng *et al*[28] had scanned the polymorphisms on the same cohort. Because differences in age or sex existed in the studied subgroups, we detected the association by logistic regression between observed SNP and HBV infected subgroups, and the age and sex factors were considered. As a result, there was statistically significant evidence of association. The fraction calculated by relative risk indicated that *HMGB1* 1176G/G genotype was more susceptible to CHB, LC and SHB than 1176C/C and 1176G/C genotype. In other words, the patients with 1176G/G genotype of *HMGB1* gene are more likely to have a progressive status in HBV infection. The results suggest that allele 1176G is closely related to the ponderance of disease. These findings underscore a potentially important role of *HMGB1* in influencing the development of HBV infection.

In another study, we had successfully cloned and analyzed 154 bp nucleotides in intron 4 near the fourth

exon-intron boundary, and found that the region contained sequences *1176 G/C* polymorphism characteristic of an enhancer using PGL3 reporter gene systems. We demonstrated that the SNP 1176 G/C could affect the function. Furthermore, this activity was enhanced by the SNP: G→C change in position 1176, providing the basis for molecular investigations of the *HMGB1* gene in HBV infection. Subsequent reports would focus on this investigation.

In conclusion, our results showed that the *HMGB1* 1176G/G genotype was related to the outcomes of hepatitis B infection, and patients with 1176G/G genotype of *HMGB1* gene are more likely to have a progressive status in HBV infection. The subjects bearing 1176G/G genotype have an increased risk of susceptibility to CHB, LC and SHB compared with those bearing at least one 1176C allele. However, further work is needed to validate our results, and clarify more potential functions of human *HMGB1* gene.

COMMENTS

Background

Chronic hepatitis B virus (HBV) infection is a serious public health problem worldwide. Host genetic factors play a role in determining to the outcome and progression of the infection. A large number of studies on the association between cytokine gene polymorphisms and the risk of chronic hepatitis B (CHB) have been conducted. High mobility group box 1 (HMGB1) functioned as a pleiotropic cytokine and implicated in the pathology of several different immune-mediated diseases. However, there has been no report about the association between *HMGB1* gene and HBV infection.

Research frontiers

HMGB1 has recently been identified as a potent proinflammatory mediator when passively released extracellularly by necrotic cells, as opposed to apoptotic cells that will induce inflammation. Furthermore, HMGB1 can also be actively secreted by stimulated macrophages or monocytes. Active secretion from living inflammatory cells and passive release from necrotic cells implicate that HMGB1 may play a central role in proinflammatory reactions.

Innovations and breakthroughs

This study characterize the relationship between *HMGB1* gene polymorphism and HBV infection, and concluded that the *HMGB1 1176G/G* genotype was related to the outcomes of hepatitis B infection, and patients with 1176G/G genotype of *HMGB1* gene are more likely to have a progressive status in HBV infection.

Applications

The study results suggest that the subjects bearing *HMGB1 1176G/G* genotype have an increased risk of susceptibility to CHB, liver cirrhosis and severe hepatitis B compared with those bearing at least one 1176C allele, which will provide new clue for the further basic research in pathogenesis of chronic HBV infection.

Peer review

The authors have done a good job and found an association between the 1176G/C polymorphism of *HMGB1*, a proinflammatory mediator, and hepatitis B virus infection. The results are interesting and suggest that HMGB1 is a mediator of the immune response to HBV infection.

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BRIEF ARTICLE

Radical lymph node dissection and assessment: Impact on gallbladder cancer prognosis

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Abstract

AIM: To investigate the lymph node metastasis patterns of gallbladder cancer (GBC) and evaluate the optimal categorization of nodal status as a critical prognostic factor.

METHODS: From May 1995 to December 2010, a total of 78 consecutive patients with GBC underwent a radical resection at Liaocheng People's Hospital. A radical resection was defined as removing both the primary tumor and the regional lymph nodes of the gallbladder. Demographic, operative and pathologic data were recorded. The lymph nodes retrieved were examined histologically for metastases routinely from each node. The positive lymph node count (PLNC) as well as the total lymph node count (TLNC) was recorded for each patient. Then the metastatic to examined lymph nodes ratio (LNR) was calculated. Disease-specific survival (DSS) and predictors of outcome were analyzed.

RESULTS: With a median follow-up time of 26.50 mo (range, 2-132 mo), median DSS was 29.00 ± 3.92 mo (5-year survival rate, 20.51%). Nodal disease was found in 37 patients (47.44%). DSS of node-negative patients was significantly better than that of node-positive patients (median DSS, 40 mo *vs* 17 mo, χ^2 = 14.814, P < 0.001), while there was no significant difference between N1 patients and N2 patients (median DSS, 18 mo *vs* 13 mo, χ^2 = 0.741, P = 0.389). Optimal TLNC was determined to be four. When node-negative patients were divided according to TLNC, there was no difference in DSS between TLNC < 4 subgroup and TLNC ⩾ 4 subgroup (median DSS, 37 mo *vs* 54 mo, χ^2 = 0.715, P = 0.398). For node-positive patients, DSS of TLNC < 4 subgroup was worse than that of TLNC ⩾ 4 subgroup (median DSS, 13 mo *vs* 21 mo, χ^2 = 11.035, P < 0.001). Moreover, for node-positive patients, a new cut-off value of six nodes was identified for the number of TLNC that clearly stratified them into 2 separate survival groups (< 6 or ⩾ 6, respectively; median DSS, 15 mo *vs* 33 mo, χ^2 = 11.820, P < 0.001). DSS progressively worsened with increasing PLNC and LNR, but no definite cut-off value could be identified. Multivariate analysis revealed histological grade, tumor node metastasis staging, TNLC and LNR to be independent predictors of DSS. Neither location of positive lymph nodes nor PNLC were identified as an independent variable by multivariate analysis.

CONCLUSION: Both TLNC and LNR are strong predictors of outcome after curative resection for GBC. The retrieval and examination of at least 6 nodes can influence staging quality and DSS, especially in node-positive patients.

Key words: Gallbladder neoplasms; Lymphatic metastasis; Lymph node excision; Lymph node ratio; Prognosis

Core tip: The presence or absence of lymph node metastasis is an important prognostic factor in patients with curatively resected gallbladder cancer (GBC). The present study evaluates the prognostic impact of number, location and ratio of involved lymph nodes, in addition to well described prognostic parameters, in patients with curatively resected GBC. The results demonstrate that total lymph node count and lymph node ratio are more appropriate to stratify GBC patients with regards to prognosis; removal and pathological examination of at least six lymph nodes can influence staging quality and disease-specific survival especially in node-positive patients.

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INTRODUCTION

Gallbladder cancer (GBC) is one of the most common malignancies of the biliary tract with poor prognosis, because it is usually detected at an advanced stage due to no specific symptoms. Treatment options for GBC have evolved over the last decade, as it has become well accepted that patients benefit from radical resection[1-4]. The spread modes of GBC are direct, lymphatic, vascular, neural, intraperitoneal and intraductal. Lymph node is one of the most common sites of metastasis of GBC. The presence or absence of lymph node metastasis is an important prognostic factor in patients with curatively resected GBC[5-8]. However, the method of optimally categorizing lymph nodal involvement in GBC remains controversial[9,10]. It is increasingly being recognized that an inadequate number of lymph nodes examined may adversely influence survival and lead to understaging of GBC[11]. Some investigators have highlighted the importance of metastatic lymph node count as a means of stratification, while others rely on the location of involved nodes[12]. Some investigators have emphasized the total number of lymph nodes resected during operation[13,14]. Recent studies have also demonstrated the influence of involved lymph node count and metastatic to examined lymph nodes ratio (LNR) on survival of patients with GBC[15,16]. The present study evaluates the prognostic impact of number, location and ratio of involved lymph nodes, in addition to well described prognostic parameters, in patients with curatively resected gallbladder cancer.

MATERIALS AND METHODS

Patient population

From May 1995 to December 2010, a total of 78 consecutive patients with GBC underwent a radical resection at Liaocheng People's Hospital. A radical resection was defined as removing both the primary tumor and the regional lymph nodes of the gallbladder. Cancer arising in the cystic duct was also included as gallbladder cancer. Eight patients with early pT stages (Tis or T1) were excluded due to their resection of only simple cholecystectomy without lymphadenectomy. Eleven patients were excluded due to incomplete clinicopathologic data or follow-up loss. As a result, 78 patients were retrospectively reviewed; these included 46 women and 32 men ranging in age from 33 years to 82 years (median, 59 years).

Table 1 Number of radical resection procedures and their relationship with tumor node metastasis stages

TNM stage procedure	0	I	II	IIIA	IIIB	IVA	IVB	Sum
C + N	1	2	1[1]		3			7
C + WR + N		2	4	6	5		2	19
C + S4aS5 + N				8	9		1	18
C + ELH + N							1	1
C + ERH + N						2		2
C + BD + N		1[2]						1
C + WR + BD + N		1	3	1	3	1	1	10
C + S4As5 + BD + N				1	3	1	1	6
C + CH + BD + N						1		1
C + S4aS5 + other + N				3	1	1		5
C + S4As5 + BD + other + N						1		1
C + ERH + BD + other + N						1		1
HPD + N						2	4	6
Sum	1	6	8	19	24	10	10	78

[1]Tumor of the patient infiltrated the serosa at the visceral surface of the gallbladder bottom; [2]This patient was an incidental gallbladder cancer with a diagnosis confirmed during the initial operation by frozen section with a preoperative diagnosis of choledochal cyst. C: Cholecystectomy; N: Lymphadenectomy; WR: Wedge resection of the gallbladder fossae; S4aS5: Liver resection of segments Ⅳa and Ⅴ; ELH: Extended left hepatectomy; ERH: Extended right hepatectomy; CH: Central hepatectomy; BD: Resection of the bile duct; HPD: Hepatopancreaticoduodenectomy; Other: Other organ tissue resection; TNM: Tumor node metastasis.

Radical resection procedures

Radical resection procedures consisted of cholecystectomy, *en bloc* hepatic resection, and lymphadenectomy with or without bile duct excision. Lymphadenectomy included *en bloc* clearance of cystic duct, pericholedochal, hepatic artery, portal vein, periduodenal and peripancreatic lymph nodes. Celiac artery, perigastric, superior mesenteric artery and para-aortic nodal clearances were not performed routinely in every patient, but if there was any evidence of tumor infiltration or metastasis to the near organ or tissues, these nodes would be cleared by an extended radical operation such as pancreaticoduodenectomy. The extent of liver resection was guided by the extent of the tumor's liver infiltration, and the guiding principle is acquiring a negative surgical margin while at the same time preserving the maximal amount of liver parenchyma. A 2-cm non-anatomical wedge of gallbladder fossa was performed if the tumor was confined to gallbladder, and formal resection of segments Ⅴ and Ⅳ a was performed if there was gross liver involvement.

The operative procedures are shown in Table 1. All patients underwent lymphadenectomy. The operative pro-

cedures included cholecystectomy (n = 8), wedge resection (n = 29), resection of segments Ⅳa and Ⅴ (n = 30), resection of the bile duct (n = 20), extended hepatectomy (n = 5), hepatopancreaticoduodenectomy (n = 6), with other organ tissue resection (n = 7), portal vein resection and reconstruction (n = 2), proper or right hepatic artery resection (n = 3).

Pathological examination

Immediately after resection, the operating surgeon separated the lymph nodes from the node-bearing adipose tissues of the fresh surgical specimen, which were then divided by the surgeon into individual node groups according to their locations. The specimen was then fixed in 10% buffered formaldehyde solution. Primary tumor was examined to determine the histologic type, tumor grade, depth of infiltration, tumor involvement of excised contiguous viscera and resection margins. Histologic grade was determined based on the areas of tumor with highest grade. Lymph node metastasis was defined as tumor cells detected on histopathologic examination using hematoxylin and eosin stain.

The lymph nodes retrieved were examined histologically for metastases routinely from each node. The positive lymph node count (PLNC) as well as the total lymph node count (TLNC) was recorded for each patient. Here, PLNC and TLNC represented the sum of regional, celiac artery, perigastric, superior mesenteric artery and paraaortic nodes evaluated in the patient. Then the metastatic to examined LNR was calculated.

Patient follow-up after resection

Of 78 patients, one died during the hospital stay because of liver failure after the definitive resection, giving an in-hospital mortality rate of 1.28%. Patients discharged to home were followed up regularly every 1-6 mo, with a median follow-up time of 26.50 mo (range, 2-132 mo). Adjuvant chemoradiation therapy was administered to 23 patients at the discretion of the individual surgeons. Only deaths from tumor recurrence were treated as failure cases in the analysis of disease-specific survival (DSS), whereas those from other causes were recorded as censored cases. The survival time in each patient was defined as the interval between the date of definitive resection and the date of last follow up or death.

Statistical analysis

Categorical variables were compared using the Pearson χ^2 test. Numerical variables were compared using paired samples t test. Survival curves were constructed using the Kaplan-Meier method, and differences in survival were evaluated with the log rank test. Cox regression analysis was used to identify independent predictors of disease-specific survival using factors found to be significant by univariate analysis. The IBM SPSS 16.0 software (SPSS Inc., Chicago, IL, United States) was used for all statistical evaluations. All tests were two-tailed and P values less than 0.05 were considered statistically significant.

RESULTS

Pathologic features

Pathological findings were documented using the American Joint Committee on Cancer (AJCC) cancer staging manual (7th edition)[17]. Resection margin status was judged as no residual tumor (R0) in all 78 patients. The primary tumor was pTis in 1 patient, pT_1 in 7 patients, pT_2 in 12 patients, pT_3 in 44 patients, and pT_4 in 14 patients. The lymph node stage was N0 in 41 patients, N1 in 31 patients and N2 in 6 patients. The M stage was M0 in 74 patients and M1 in 4 patients. Of the metastasis patients, 1 was a single metastasis lesion on the visceral peritoneum and the other 3 were liver metastases. Then the patients were classified according to tumor node metastasis (TNM) staging: stage 0 (n = 1), stage I (n = 6), stage Ⅱ (n = 8), stage ⅢA (n = 19), stage ⅢB (n = 24), stage ⅣA (n = 10) and stage ⅣB (n = 10).

Distribution of lymph nodes metastasis

A total of 465 lymph nodes taken from the 78 studied patients were evaluated. TLNC ranged from 1 to 24 (median, 4) per patient. According to the AJCC cancer staging manual (7th edition)[17], the topographical distribution of the analyzed lymph nodes included 361 first-station nodes and 104 second-station nodes (Table 2). There were significantly more first-station nodes per patient (median = 4; range: 1-12) than second-station nodes (median = 0; range: 0-12) (t = 10.46, P < 0.001).

Of the 78 studied patients, 37 (47.44%) had a total of 98 positive lymph nodes. The number of positive nodes per patient ranged from 1 to 9 (median = 2). There were 5 (25.00%) of 20 patients with pTis to pT_2 stage who had positive nodal disease, whereas 32 (55.17%) of 58 patients with pT_3 to pT_4 stage had positive nodal disease. The occurrence of lymph node metastasis was increased obviously with the advance of pT stage (χ^2 = 5.430, P = 0.020).

The topographical distribution of all positive lymph nodes is shown in Table 2. Among the 37 node-positive patients, the prevalence of nodal disease was highest in the pericholedochal (n = 20, 54.05%) or the cystic duct (n = 18, 48.65%) node group, followed by the periportal (n = 12, 32.43%), hepatic artery (n = 10, 27.03%), postero-superior pancreaticoduodenal (n = 6, 16.22%), hilar (n = 4, 10.81%), and right celiac (n = 1, 2.70%) node groups. The paraaortic, superior mesenteric artery and perigastric nodes were not involved in any of our patients.

Of 13 patients with a single positive node, 11 (84.62%) had nodal disease in either the pericholedochal (n = 6) or cystic duct (n = 5) node group, suggesting that initial nodal involvement occurred primarily in these node groups.

Analysis of the topographical distribution of positive lymph nodes may be helpful to derive the route of lymphatic spread from GBC (Table 2). In this study, GBC primarily spread to the first-station nodes, then to the second-station nodes.

Table 2 Topographical distribution of 465 lymph nodes evaluated in 78 patients with gallbladder cancer *n* (%)

Node group	Patients with node group evaluated	Lymph nodes evaluated	Patients with positive nodes	Positive nodes
Cystic duct[1]	41 (53.95)	46 (9.89)	18 (23.08)	19 (19.39)
Pericholedochal[1]	68 (81.18)	146 (31.40)	20 (25.64)	29 (29.59)
Periportal[1]	47 (60.26)	74 (15.91)	12 (15.38)	18 (18.37)
Hepatic artery[1]	48 (61.54)	69 (14.84)	10 (12.82)	12 (12.24)
Posterosuperior pancreaticoduodenal[2]	22 (28.21)	56 (12.04)	6 (7.69)	12 (12.24)
Hilar[1]	18 (23.08)	26 (5.59)	4 (5.13)	6 (6.12)
Right celiac[2]	8 (10.26)	21 (4.52)	1 (1.28)	2 (2.04)
Perigastric[2]	4 (5.13)	6 (1.29)	0 (0.00)	0 (0.00)
Superior mesenteric artery[2]	6 (7.69)	11 (2.37)	0 (0.00)	0 (0.00)
Paraaortic[2]	6 (7.69)	10 (2.15)	0 (0.00)	0 (0.00)
Sum	78 (100)	465 (100)	37 (47.44)	98 (100)

[1]First-station nodes; [2]Second-station nodes; according to the American Joint Committee on Cancer cancer staging manual (7th edition). Here, hilar lymph nodes classified as first-station nodes and perigastric lymph nodes classified as second-station nodes.

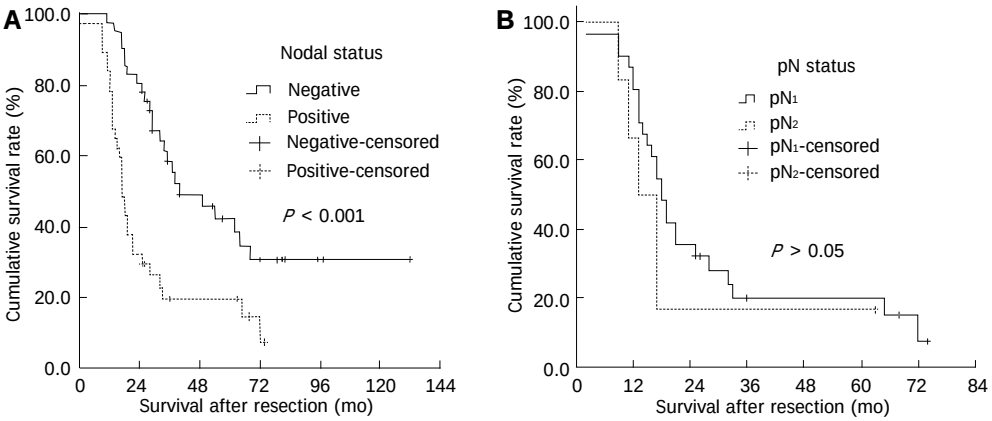

Figure 1 Kaplan-Meier survival estimates stratified. A: Lymph node status (negative *vs* positive; median disease-specific survival, 40 mo *vs* 17 mo); B: pN status in nodal positive patients (pN1 *vs* pN2; median disease-specific survival, 18 mo *vs* 13 mo).

Survival after regional lymphadenectomy

Of the overall patients, there were 22 patients who survived more than 3 years and 16 patients survived more than 5 years by the end of the follow-up; the median DSS was 29.00 ± 3.92 mo (5-year survival rate, 20.51%). The postoperative DSS of node-negative patients was significantly better than that of node-positive patients (median DSS, 40 mo *vs* 17 mo, $\chi^2 = 14.814$, $P < 0.001$, Figure 1A). Most node-negative patients achieved long-term survival after R0 resection (5-year survival rate, 26.83%). Of the 37 node-positive patients after an R0 resection, only 5 patients survived more than 5 years (5-year survival rate, 13.51%).

We then focused on a subgroup of 37 node-positive patients who had undergone an R0 resection for survival analysis; they comprised 31 N1 stage patients and 6 N2 stage patients. The postoperative DSS was not significantly different between N1 node-positive patients (median survival time, 18 mo; 5-year survival rate, 12.90%) and N2 node-positive patients (median survival time, 13 mo; 5-year survival rate, 16.67%) ($\chi^2 = 0.741$, $P = 0.389$, Figure 1B). Of the 5 patients with node-positive disease who survived for more than 5 years, there were two patients who underwent a pancreaticoduodenal lymph node dissection with hepatopancreaticoduodenectomy for suspected N2 nodal disease. These findings suggested that regional lymphadenectomy could achieve an acceptable rate of long-term survival even in patients with advanced stage of nodal metastasis, provided that an R0 resection is feasible.

Cut-off values for the TNLC, PNLC and LNR

Based on the magnitude of the Log-rank test χ^2 statistic, the optimal cut-off value was four nodes for the number of TLNC. Based on these results, the number of TLNC was placed into two categories in subsequent analyses (< 4 or ≥ 4, respectively). DSS of TLNC < 4 group was worse than that of TLNC ≥ 4 group (median DSS, 18 mo *vs* 33 mo, $\chi^2 = 5.606$, $P = 0.018$, Figure 2A). When node-negative patients were divided according to TLNC, there was no difference in DSS between TLNC < 4 subgroup ($n = 60$) and TLNC ≥ 4 subgroup ($n = 21$) (median DSS, 37 mo *vs* 54 mo, $\chi^2 = 0.715$, $P = 0.398$, Figure 2B). For node-positive patients, DSS of TLNC < 4 subgroup was worse than that of TLNC ≥ 4 subgroup (median DSS, 13 mo *vs* 21 mo, $\chi^2 = 11.035$, $P < 0.001$, Figure 3A). Moreover, for node-positive patients, a new cut-off value of six nodes for the number of TLNC clearly stratified

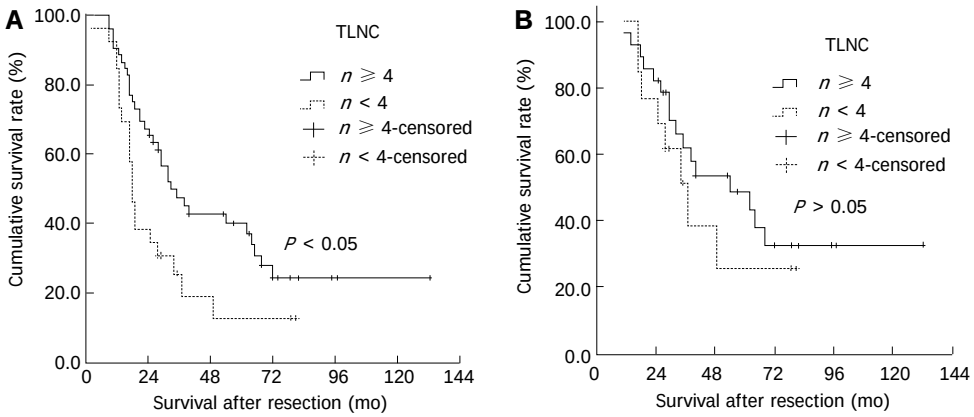

Figure 2 Kaplan-Meier survival estimates stratified for total lymph node count status (< 4 or ⩾ 4, respectively). A: In 78 patients who underwent an R0 resection (median disease-specific survival, 18 mo *vs* 33 mo); B: In 41 node-negative patients (median disease-specific survival, 37 mo *vs* 54 mo). TLNC: Total lymph node count.

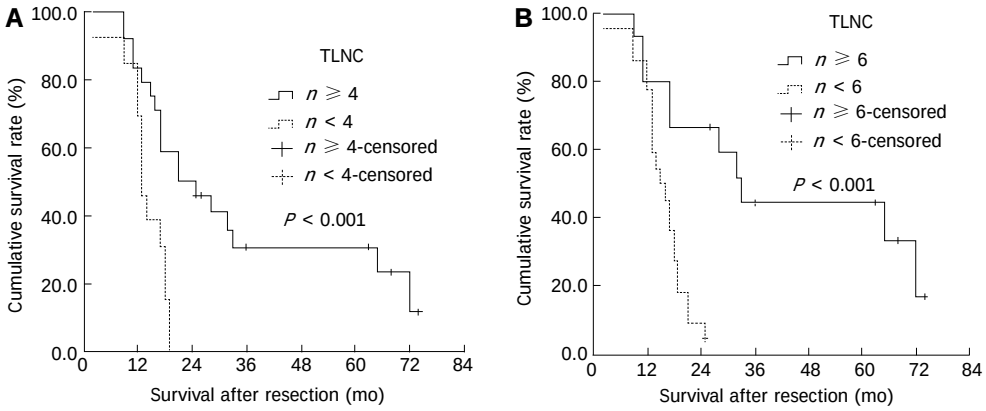

Figure 3 Kaplan-Meier survival estimates stratified for total lymph node count status in 37 node-positive patients. A: $n < 4$ or $\geqslant 4$, respectively; median disease-specific survival, 13 mo *vs* 21 mo; B: $n < 6$ or $\geqslant 6$, respectively; median disease-specific survival, 15 mo *vs* 33 mo. TLNC: Total lymph node count.

them into 2 separate survival groups (< 6 or ⩾ 6, respectively; median DSS, 15 mo *vs* 33 mo, $\chi^2 = 11.820$, $P < 0.001$, Figure 3B).

DSS progressively worsened with increasing PLNC and LNR, but no definite cut-off value could be identified. Based on the previous literature, we left the cut-off value as 3 nodes for PLNC and 50% for LNR separately[13,16].

Factors influencing disease-specific survival after resection

Univariate analyses identified liver invasion, venous invasion, pT classification, pN classification, pM classification, TNM staging, lymph node invasion, TLNC, PLNC, LNR and histological grade as significant prognostic factors (Table 3).

The univariately significant variables were then entered into multivariate analysis. Histological grade, TNM staging, TLNC and LNR remained as independently significant variables (Table 4). Neither location of positive lymph nodes nor PLNC were identified as an independent variable by multivariate analysis.

DISCUSSION

Studies have demonstrated that the presence or absence of lymph node metastasis is an important prognostic factor in patients with curatively resected GBC[5,13,18-20]. Patients with lymph node metastasis have significantly worse survival than those with negative nodes[1,21]. With the increasing safety of hepatic and pancreatic surgery, various radical procedures have been advocated to improve the curative outcome for advanced GBC[22-24]. Recent data also suggest that aggressive resection may improve long-term survival, even in patients with advanced stage disease[3,12,25].

It had been confirmed that the main lymphatic pathway of the gallbladder descends along the common bile duct and into the retroportal nodes, then to the posterosuperior of the head of the pancreas or around the hepatic artery, and finally to the paraaortic nodes near the left renal vein[26-28]. Based on these detailed anatomical studies, it has been suggested that lymphatic metastasis from GBC spreads widely through the hepatoduodenal ligament towards the peripancreatic region and beyond. In

Table 3 Univariate analysis of clinical and histopathologic variables

Variable	Number of patients	Survival rate		*P* value
		3-yr	5-yr	
Age (yr)				0.222
< 60	41	34.15%	17.07%	
≥ 60	37	24.32%	18.92%	
Sex				0.523
Female	46	28.26%	17.39%	
Male	32	31.25%	18.75%	
Cholelithiasis				0.374
Present	25	24.00%	16.00%	
Absent	53	32.08%	18.87%	
Type of radical resection				0.179
Extended cholecystectomy	7	42.86%	42.86%	
Partial hepatectomy[1]	37	32.43%	13.51%	
Partial hepatectomy and EBD resection	23	30.43%	26.09%	
Extended hepatectomy[2]	5	0.00%	0.00%	
Hepatopancreaticoduodenectomy	6	50.00%	33.33%	
Hepatic infiltration				0.005
Present	41	14.63%	4.88%	
Absent	37	51.35%	37.84%	
Bile duct infiltration				0.238
Present	17	29.41%	23.53%	
Absent	61	32.79%	19.67%	
Venous invasion				0.001
Present	10	0.00%	0.00%	
Absent	68	36.76%	23.53%	
Perineural invasion				0.539
Present	9	22.22%	22.22%	
Absent	69	33.33%	20.29%	
Lymph node involvement				< 0.001
Present	37	16.22%	13.51%	
Absent	41	46.34%	26.83%	
pT classification[3]				0.001
pT_0-pT_2	20	60.00%	45.00%	
pT_3-pT_4	58	22.41%	12.07%	
pN classification[3]				< 0.001
pN_0	41	46.34%	26.83%	
pN_1	31	16.13%	12.90%	
pN_2	6	16.67%	16.67%	
pM classification[3]				0.002
pM_0	74	33.78%	21.62%	
pM_1	4	0.00%	0.00%	
TNM staging[3]				< 0.001
0-Ⅱ	15	80.00%	60.00%	
Ⅲ	43	23.26%	11.63%	
Ⅳ	20	15.00%	10.00%	
TLNC				0.018
< 4	26	15.38%	7.69%	
≥ 4	52	40.38%	26.92%	
Number of positive lymph nodes				< 0.001
0	41	46.34%	26.83%	
< 3	24	16.67%	16.67%	
≥ 3	13	15.38%	7.69%	
LNR				< 0.001
0	41	46.34%	26.83%	
< 50	15	33.33%	33.33%	
≥ 50	22	4.55%	0.00%	
Histological type				0.706
Adenocarcinoma	69	33.33%	20.29%	
Others	9	22.22%	22.22%	
Histological grade				0.042
G1-G2	58	36.21%	24.14%	
G3-G4	19	15.79%	5.26%	

[1]Includes wedge resection and resection of segments Ⅳa and Ⅴ; [2]Includes extended right hepatectomy, extended left hepatectomy and central hepatectomy; [3]According to the American Joint Committee on Cancer cancer staging manual (7th edition). G1: Well differentiated; G2: Moderately differentiated; G3: Poorly differentiated; G4: Undifferentiated; EBD: Endoscopic balloon dilatation; TNM: Tumor node metastasis; TLNC: Total lymph node count; LNR: Lymph node ratio.

Table 4 Results of Cox multivariate regression analysis

Variable	Parameter estimate	Wald χ^2	*P*	Hazard ratio	95%CI
Tumor node metastasis staging		20.559	< 0.001		
0-Ⅱ				1.000	
Ⅲ	-3.112	19.846	< 0.001	0.045	0.011-0.175
Ⅳ	-1.044	9.341	0.002	0.352	0.180-0.688
Lymph node ratio	2.424	20.247	< 0.001	11.293	3.929-32.465
Total lymph node count	-0.147	14.273	< 0.001	0.864	0.800-0.932
Histological grade	-0.755	5.512	0.019	0.470	0.250-0.883

this study, initial nodal involvement occurred primarily in the cystic duct or pericholedochal nodes, followed by periportal and hepatic artery nodes. Posterosuperior pancreaticoduodenal and right celiac lymph nodes were involved in 16.22% of node-positive patients and were classified as N2 disease, according to the 7th edition of AJCC classification. However, we observed that the categorization of patients as having N2 disease did not adversely influence DSS as compared to those with N1 disease. Hence, we believe that even patients with N2 lymph node metastasis can achieve an ideal survival if radical lymphadenectomy is performed. An addition of pancreaticoduodenectomy could result in an R0 resection by removing extensive peripancreatic nodal disease in a select group of patients[22,23,29]. Furthermore, Murakami *et al*[30] suggested that it is lymph node metastasis but not para-aortic lymph node metastasis that is associated independently with longer survival by multivariate analysis. In this study, six patients were treated with pancreaticoduodenectomy and two patients survived more than five years.

The high propensity for lymphatic spread in GBC renders adequate lymphadenectomy indispensable for improving patient outcomes after resection[8,19]. However, because of the rarity of disease and low resectability rates, which limit the ability to perform large cohort studies or prospective randomized trials, the optimal extent of lymphadenectomy remains unresolved and there are no uniform evidence-based guidelines on the issue[9,10]. Accuracy of nodal staging depends on a critical number of lymph nodes analyzed; insufficient number of nodes retrieved during surgery or examined pathologically leads to underestimation of disease stage[14]. Although the 6th edition of AJCC suggests a minimum of three lymph nodes to be assessed for appropriate pathologic nodal staging of gallbladder cancer, the basis of recommendation is not clear, and there are no established standards. A large population-based study on the SEER database demonstrated that of the 2835 resected patients with T1-T3 M0 GBC, only 5.3% had a lymphadenectomy of three or more lymph nodes[31]. Also, Ito *et al*[14] independently suggested that retrieval and evaluation of at least six lymph nodes improves risk stratification after resection in node-negative patients. These observations indicate that retrieval of a larger number of lymph nodes than previously practiced is warranted not only for accurately staging the nodal status, but also for improving survival due to better clearance of nodal disease[13].

Although a greater number of examined nodes might improve the survival of the disease, the results of our study suggest that retrieval and evaluation of at least four nodes is perhaps optimal. Furthermore, TLNC significantly correlated with DSS in node-positive patients and allowed better prognostic substratification of these patients. For node-positive patients in this study, we can get a new cut-off value of six nodes for the number of TLNC that clearly stratifies them into 2 separate survival groups, which is more optimal than four nodes. But no definite cut-off value of TLNC could be identified for node-negative patients. Since the TLNC-survival relationship was observed only in node-positive patients and not in those node-negative patients, we believe that a higher count not only helps in stage purification but also helps improve therapeutic benefit, which is more serious in node-positive patients. These findings should heighten awareness about the importance of TLNC amongst surgeons performing lymphadenectomy for suspected node-positive patients. We believe that adequate lymphadenectomy is indispensable for improving the prognosis after radical resection in patients with GBC.

Endo *et al*[32] first suggested that the PLNC is more useful in assessing nodal status than the location of positive nodes in GBC. Sakata *et al*[12] additionally showed that the number, but not location, of positive nodes independently determined prognosis after resection. The burden of nodal disease (PLNC) also had an impact on prognosis; there was significantly reduced DSS observed in this study with involved nodes. The DSS progressively worsened with increasing PLNC; however, we were not able to identify any specific cut-off value. The use of PLNC as a prognostic factor might be limited by inherent bias of inadequate number of lymph nodes retrieved or histologically examined which leads to the phenomenon of "stage migration". However, many recent studies (including this study) have reported a number of long-term survivors after resection for GBC with multiple positive lymph nodes[11,29,30,33]. These observations indicate that regional lymph node dissection for GBC provides long term survival for selected patients with multiple positive lymph nodes, provided that R0 resection is feasible.

LNR has been shown to be an important predictor of survival for many gastrointestinal tract cancers after surgery because it is a better and reproducible method of stratifying nodal status which incorporates not only the burden and biology of disease (PLNC) but also the quality of lymphadenectomy and pathologic examination (TLNC)[34-36]. Negi *et al*[16] first found that LNR, and

not PLNC, was an independent prognostic factor in their study cohort comprising 57 patients with a relatively small TLNC. Our study suggests that, along with tumor TNM staging, LNR is an independent prognostic factor and another important lymph nodal variable in patients undergoing curative resection for GBC. The prognostic impact of LNR was observed in the entire group, including the subgroup of patients with positive nodes, even though we could not find an optimal cut-off value in this study. LNR is of particular value in patients who cannot adequately be staged because of the limited number of lymph nodes evaluated. In the case of insufficient lymph node evaluation, LNR will more accurately reflect the nodal status than the number of positive nodes in GBC. Patients with high LNR after radical resection might need adjuvant chemoradiation therapy to improve their prognosis.

The strengths of our study include the reasonably sized cohort of patients managed in a single institution using a standardized treatment approach. The current study has several limitations: the retrospective nature of the analysis, the relatively small number of patients spanning a long period of time, some variability in the degree of nodal dissection, and the short follow-up time for some patients. The observations need to be confirmed in larger, especially population-based, cohort. We believe, however, that these limitations did not greatly affect the results of the study as the differences between groups were too marked to have resulted from bias. In addition, the role of TLNC and LNR in assessing the nodal status for GBC is now more clearly defined than previously, based on the current study. Our results thus provide useful information for accurately staging nodal disease, predicting prognosis after resection, and selecting candidates for adjuvant chemoradiation therapy after resection.

The results of the present study demonstrate that, rather than categorizing GBC patients based on PLNC or location of involved nodes, TLNC and LNR are more appropriate tools to stratify patients with regards to prognosis. Our data also suggest that removal and pathological examination of at least six lymph nodes can influence staging quality and disease-specific survival especially in node-positive patients. This knowledge should heighten awareness amongst surgeons about the importance of performing lymphadenectomy for suspected node-positive patients, aiming to retrieve and examine an adequate number of lymph nodes.

COMMENTS

Background

Lymph node is one of the most common sites of metastasis of gallbladder cancer (GBC). The presence or absence of lymph node metastasis is an important prognostic factor in patients with curatively resected GBC. However, the method of optimally categorizing lymph nodal involvement in GBC remains controversial.

Research frontiers

It is increasingly being recognized that an inadequate number of lymph nodes examined may adversely influence survival and lead to understaging of GBC. Some investigators have highlighted the importance of metastatic lymph node count as a means of stratification while others rely on the location of involved nodes. Some investigators emphasized the total number of lymph nodes resected during operation. Recent studies have also demonstrated the influence of involved lymph node count and metastatic to examined lymph nodes ratio (LNR) on survival of patients with GBC.

Innovations and breakthroughs

The presence or absence of lymph node metastasis is an important prognostic factor in patients with curatively resected GBC. The present study evaluates the prognostic impact of number, location and ratio of involved lymph nodes, in addition to well described prognostic parameters, in patients with curatively resected GBC. The results demonstrate that total lymph node count (TLNC) and LNR are more appropriate to stratify GBC patients with regards to prognosis, and removal and pathological examination of at least six lymph nodes can influence staging quality and disease-specific survival especially in node-positive patients.

Applications

The study results suggest that TLNC and LNR are more appropriate to predict the prognosis of GBC patients, while surgeons need to achieve clearance and pathologically examine at least six lymph nodes to improve staging quality and disease-specific survival especially in node-positive patients.

Peer review

The lymph node is one of the most common sites of metastasis of GBC. The presence or absence of lymph node metastasis is an important prognostic factor in patients with curatively resected GBC.

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BRIEF ARTICLE

Effects of SAHA on proliferation and apoptosis of hepatocellular carcinoma cells and hepatitis B virus replication

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Abstract

AIM: To investigate the effects of suberoylanilide hydroxamic acid (SAHA) on proliferation and apoptosis of a human hepatocellular carcinoma cell line (HepG2.2.15) and hepatitis B virus (HBV) replication.

METHODS: HepG2.2.15 cells were treated with different concentrations of SAHA. Cell morphology was examined by confocal laser scanning microscopy, and cell proliferation was determined using a MTT colorimetric assay. Flow cytometry was used to detect apoptosis and determine cell cycle phase, while hepatitis B surface antigen and hepatitis B e antigen content were measured using chemiluminescence. Reverse transcription polymerase chain reaction was performed to measure HBV DNA in cell lysate.

RESULTS: Cell proliferation rates were significantly reduced by the addition of SAHA. The inhibitory effect of SAHA on cell proliferation was both time- and dose-dependent. After 24 h of treatment with SAHA, the early cell apoptotic rate increased from 3.25% to 21.02% (P = 0.041). The proportion of G_0/G_1 phase cells increased from 50.3% to 65.3% (P = 0.039), while that of S phase cells decreased from 34.9% to 20.6% (P = 0.049). After 48 h of treatment, hepatitis B surface antigen and hepatitis B e antigen content increased from 12.33 ± 0.62 to 25.42 ± 2.67 (P = 0.020) and 28.92 ± 1.24 to 50.48 ± 1.85 (P = 0.026), respectively. Furthermore, HBV DNA content increased from 4.54 ± 0.46 to 8.34 ± 0.59 (P = 0.029).

CONCLUSION: SAHA inhibits HepG2.2.15 cell proliferation, promotes apoptosis, and stimulates HBV replication. In combination with anti-HBV drugs, SAHA may potentially be used cautiously for treatment of hepatocellular carcinoma.

Key words: Human hepatocellular carcinoma; HepG2.2.15 cells; Suberoylanilide hydroxamic acid; Hepatitis B virus

Core tip: HepG2.2.15 cells were treated with different concentrations of suberoylanilide hydroxamic acid (SAHA). Hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) content were measured using chemiluminescence. Reverse transcription polymerase chain reaction was performed to measure hepatitis B virus (HBV) DNA in cell lysate. Results found that, the inhibitory effect of SAHA on cell proliferation was both time- and dose-dependent. After 24 h of treatment, the early cell apoptotic rate increased. After 48 h of treatment, HBsAg and HBeAg content both increased. Furthermore, HBV DNA content increased. In combination with anti-HBV drugs, SAHA may potentially be used cautiously for treatment of hepatocellular carcinoma.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors. The worldwide incidence of HCC ranks fifth out of all malignant tumors, and the number of patients with HCC in China accounts for more than half of total cases in the world[1]. Etiological factors of HCC vary for different countries and areas. Histone deacetylase inhibitors (HDACIs) are a series of new anticancer drugs with a wide scope of application. In recent years, HDACIs have generated considerable interest due to their high efficiency to inhibit a variety of solid tumors with low toxicity[2-6]. In the current study, the effects of suberoylanilide hydroxamic acid (SAHA), a potent HDACI, on proliferation and apoptosis of human HCC cells HepG2.2.15 and hepatitis B virus (HBV) replication were investigated. The study objective was to characterize a potentially new treatment option for HCC.

MATERIALS AND METHODS

Cell culture and treatment

HepG2.2.15 cells (obtained from the Cell Center of the Chinese Academy of Medical Sciences; prepared by transfection of HepG2 cells with HBV genome) were maintained in DMEM (HyClone Laboratories, Inc., New England, United States) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 U/mL streptomycin and 380 mg/L G418 in a thermostatic and sealed incubator (37 ℃, 5% CO_2). About 100 mmol/L SAHA (Sigma-Aldrich Corp, Missouri, United States) in dimethylsulfoxide (DMSO) was prepared and stored at -20 ℃ until further use. HepG2.2.15 cells were divided into a control group and several treatment groups to receive different concentrations of SAHA. The adherent cells were washed down with 0.25% trypsin, followed by passage.

Inhibition of cell proliferation

An MTT colorimetric assay was used to monitor inhibition of cell proliferation by the addition of different concentrations of SAHA to cell culture medium. For three 96-well plates, 100 μL HepG2.2.15 cells (1×10^5 cells/mL) was added to each well and incubated for 12 h at 37 ℃ in 5% CO_2. Once cells were adhered to the wells, SAHA was added to a final concentration of 2.5, 5, 7.5 or 10 μmol/L. Wells without SAHA were used as negative controls. After the addition of SAHA, a culture plate was incubated for 24, 48 or 72 h. Cell morphology was examined by confocal laser scanning microscopy (CLSM). Then, 20 μL of MTT (5 mg/mL) was added to each well. After incubation for 4 h, 150 μL of DMSO was added, followed by mixing for 10 min. Lastly, absorbance (A) at 490 nm was measured using a microplate reader. The inhibition rate of cell proliferation was calculated as follows: Cell proliferation inhibition rate (%) = $(1 - A_{\text{SAHA group}}/A_{\text{Negative control group}}) \times 100\%$.

Detection of apoptosis and determination of cell cycle phase

Control group and SAHA groups (2.5 and 5 μmol/L) were cultured for 24 and 48 h respectively. The single cell suspension was then prepared. After centrifugation at 2000 *g* for 5 min, the cell pellet was resuspended in 0.5 mL PBS (final concentration, $1\text{-}5 \times 10^5$ cells/mL). For detection of apoptosis, binding buffer (500 μL) and 5 μL annexin V-fluorescein isothiocyanate (Annexin V-FITC) were added to the cell resuspension, followed by 5 μL propidium iodide (PI) (Nanjing KGI Biological Technology Co., Ltd., Nanjing, China). After incubation for 5-15 min (room temperature, avoiding light), samples were subjected to flow cytometry (FCM). For determination of cell cycle phase, 5 mL of obtained cell resuspension was infused into 70% cold ethanol, followed by fixation at 4 ℃ overnight. During the next day, the cell solution was centrifuged at 800 r/min for 15 min, followed by two phosphate buffer saline (PBS) washes and resuspension in 0.4 mL PBS. RNaseA was added to a final concentration of 50 μg/mL, followed by digestion for 30 min in a 37 ℃ water bath. Lastly, PI was added to a final concentration of 65 μg/mL, followed by incubation for 30 min. After filtration through a nylon mesh, FCM was conducted.

Determination of hepatitis B surface antigen and hepatitis B e antigen content

HepG2.2.15 cells (2.5×10^5 cells/mL) were plated onto a 6-well plate. In triplicate, 1 μL SAHA (7.5 μmol/L) or an equivalent volume of DMSO was added to an individual well. After incubation for 72 h, cells were centrifuged at 800 r/min for 5 min. The supernatant was collected, and the hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) content were quantitated using quantitative chemiluminescence detection kits in i4000sR automatic chemiluminescence immunoassay analyser (R.D. Abbott Company, Inc., California, United States).

Determination of HBV DNA content

A 6-well cell culture plate was prepared as previously described above. HBV negative and positive controls were prepared as follows: 100 μL of cell supernatant was mixed with an equal amount of DNA extraction liquid (shaking for 15 s), followed by centrifugation at 12000 *g* for 10 min to remove supernatant. Then, 20 μL of DNA extraction liquid was added to the sediment, followed by incubation for 10 min in a 100 ℃ water bath.

HBV-polymerase chain reaction (PCR) reaction liquid (35.6 μL) and Taq enzyme (0.4 μL) were mixed in a 0.2 mL Eppendorf tube. Two μL of treated sample supernatant was then added to each tube and centrifuged at 8000 *g* for several seconds. Quantitative fluorescent PCR was performed under the following conditions: 95 ℃ for 3 min; 94 ℃ for 15 s (40 cycles); 60 ℃ for 30 s (40 cycles).

Statistical analysis

Data were expressed as mean ± SD. Statistical analysis

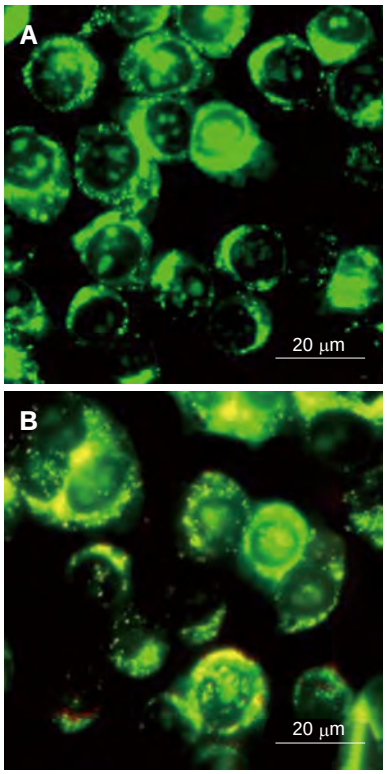

Figure 1 Cell morphology changes in confocal laser scanning microscopy. A: Control group; B: Suberoylanilide hydroxamic acid group.

was performed using SPSS 16.0 statistical software. Single factor analysis of variance and *t* tests were conducted for comparison among multiple groups. $P < 0.05$ was considered as statistically significant.

RESULTS

Effect of SAHA on cell morphology

CLSM showed that proliferation of untreated HepG2.2.15 cells was rapid, and the cells were compactly arranged. The adjacent cells fused into pieces, with clear edge. The cytoplasm was small, with a large nucleus. In SAHA-treated groups, cell proliferation rate was significantly slower. There were sparse adherent cells, with blurred configuration. The cytoplasm increased, presenting morphological changes similar to normal cells (Figure 1).

Effect of SAHA on cell proliferation

Multiple concentrations of SAHA could inhibit proliferation of HepG2.2.15 cells, and the inhibitory rate increased with increasing concentrations of SAHA ($P < 0.05$). With each SAHA concentration, the inhibition rate gradually increased with prolonged treatment time ($P < 0.05$). Taken together, the inhibitory effect of SAHA on cell proliferation was time- and dose-dependent (Table 1).

Effect of SAHA on cell apoptosis and cell cycle

After 24 h of treatment with 2.5 μmol/L SAHA, early apoptosis rate of HepG2.2.15 cells increased from 3.25% to 16.28% ($P = 0.032$), and the middle-late apoptosis rate increased from 1.08% to 5.16% ($P = 0.035$). In the 5 μmol/L SAHA group, early and middle-late apoptosis rate increased from 3.25% to 21.02% ($P = 0.041$) and 1.08% to 10.70% ($P = 0.045$), respectively (Table 2 and Figure 2). After 24 h of treatment with 2.5 and 5 μmoL/L SAHA, the proportion of G_0/G_1 phase cells increased from 50.3% to 69.9% and 65.3%, respectively, and the proportion of S phase cells decreased from 34.9% to 22.3% and 20.6%, respectively. Most cells were arrested in the G_0/G_1 phase (Table 3).

Table 1 Inhibition rate of suberoylanilide hydroxamic acid on HepG2.2.15 cells

SAHA (μmol/L)	24 h	48 h	72 h
2.5	8% ± 0.54%	15% ± 1.52%	23% ± 1.39%
5.0	13% ± 0.63%	22% ± 1.68%	34% ± 1.61%
7.5	28% ± 1.56%	39% ± 1.67%	50% ± 1.70%
10.0	42% ± 1.72%	51% ± 1.82%	66% ± 1.76%

$P < 0.05$ for comparison among different concentration and different treatment time, respectively. SAHA: Suberoylanilide hydroxamic acid.

Table 2 Effect of suberoylanilide hydroxamic acid on apoptosis rate of HepG2.2.15 cells

Group	24 h		48 h	
	Early apoptosis rate	Middle-late apoptosis rate	Early apoptosis rate	Middle-late apoptosis rate
Control	3.25%	1.08%	3.58%	1.26%
2.5 μmol/L SAHA	16.28%	5.16%	23.06%	8.42%
5.0 μmol/L SAHA	21.02%	10.70%	26.44%	17.55%

SAHA: Suberoylanilide hydroxamic acid.

Table 3 Effect of suberoylanilide hydroxamic acid on proportion of HepG2.2.15 cells with different phases

Group	24 h			48 h		
	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
Control	50.3%	34.9%	14.8%	46.3%	38.2%	15.5%
2.5 μmol/L SAHA	69.9%	22.3%	7.8%	70.9%	26.1%	3.0%
5.0 μmol/L SAHA	65.3%	20.6%	14.1%	68.9%	25.8%	5.3%

SAHA: Suberoylanilide hydroxamic acid.

HBsAg and HBeAg content and HBV DNA content

Positive expression of HBsAg and HBeAg in the SAHA group and control group, respectively, was observed. After 48 h of treatment with SAHA, HBsAg and HBeAg content increased from 12.33 ± 0.62 to 25.42 ± 2.67 ($P = 0.020$) and 28.92 ± 1.24 to 50.48 ± 1.85 ($P = 0.026$), respectively, and HBV DNA content increased from 4.54 ± 0.46 to 8.34 ± 0.59 ($P = 0.029$).

DISCUSSION

Abnormality of any step of epigenetics can affect gene

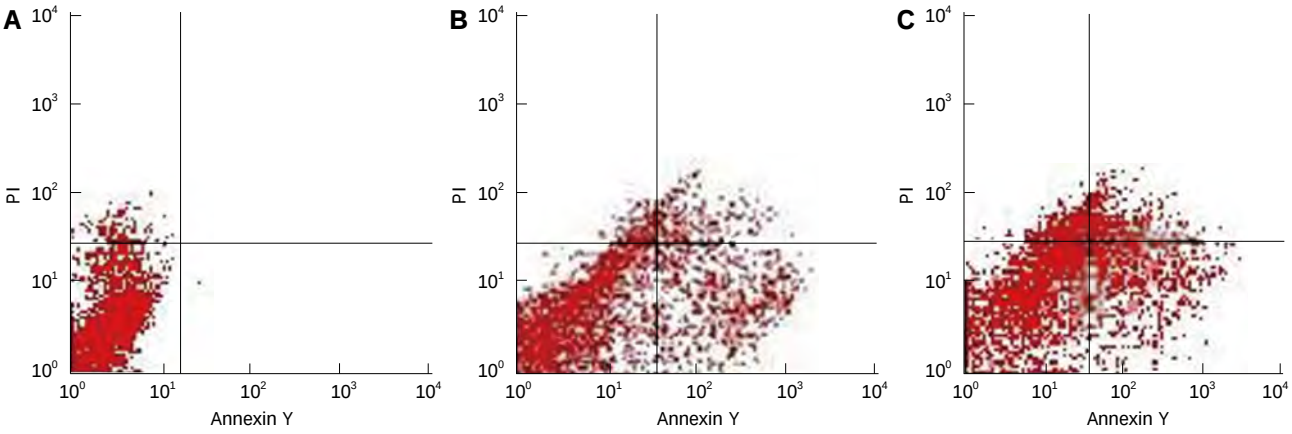

Figure 2 Effect of suberoylanilide hydroxamic acid on apoptosis of HepG2.2.15 cells. A: Control group; B: 2.5 μmol/L suberoylanilide hydroxamic acid (SAHA) group; C: 5.0 μmol/L SAHA group. PI: Propidium iodide.

expression or function, leading to the occurrence of disease, such as cancer. As a main epigenetic pattern, histone acetylation is closely related with tumor occurrence. HDACIs are often used to alter histone acetylation for treatment of cancer[7-9]. SAHA is a broad-spectrum HDACI, and was approved for treating T-cell lymphoma in 2006 (in phase I and II clinical trial). It has obvious inhibitory effects on histone deacetylase, and can inhibit the growth of HCC cells by arresting cell cycle progression and inducing cell differentiation and apoptosis. HDACIs have been shown to exhibit a broad-spectrum inhibitory activity on blood and solid tumors[10,11].

Unrestricted division and proliferation is an important feature of tumor cells. Detection of an inhibitory effect on tumor cell proliferation is a basic index for screening of anti-tumor drugs. In this study, CLSM and a MTT colorimetric assay were used to show that cell proliferation rates was significantly decreased by treatment with SAHA. Specifically, the number of cells was reduced, and adherent cells became sparse. Time- and dose-dependencies of SAHA inhibition on cell proliferation were evident. The cytoplasm increases, presenting morphological changes similar to normal cells, which is consistent with results from a previous study[12].

Apoptosis is programmed cell death. The process of apoptosis and the clearance of apoptotic cells is one of the most important factors for maintaining liver health. In this study, after 24 h of treatment with 5 μmol/L SAHA, early apoptosis rate and middle-late apoptosis rate of HepG2.2.15 cells increased from 3.25% to 21.02% (P = 0.029) and 1.08% to 10.70% (P = 0.045), respectively, indicating that SAHA may interfere with the balance between apoptosis and anti-apoptosis, induce the expressions of pro-apoptotic genes (*Bmf*, *Bim*, *TRAIL* and *DR5*)[13], and activate the expression of transcription factor E2F1. Furthermore, SAHA can induce the expression of apoptosis signal-regulating kinase 1 (ASK1), which promotes apoptosis of tumor cells through the death receptor and intracellular apoptotic pathways[14,15]. In addition, SAHA can activate the expression of pro-apoptotic proteins, including Bax and Bak, and inhibit expression of anti-apoptosis proteins, including Bcl-2 and Bcl-xL, thus inducing apoptosis of tumor cells[16]. In the extracellular apoptotic pathway, activated caspase-8 can cleave Bid to truncated Bid (tBid), as well as cause Cyt C release and Bax expression, leading to activation of caspase-9 and caspase-3. Caspase-3 can promote activation and cleavage of PARP to subsequently activate the intracellular apoptotic pathway[17]. SAHA has been shown to induce transcription of CDK inhibitor p21/wafl in T24 bladder cancer cells, reducing proliferation and increasing apoptosis[14].

Abnormality of cell cycle regulation is one of intrinsic factors for tumor occurrence. HDACIs can arrest tumor cell cycle, inhibiting growth. Results of this study show that, after 24 h of treatment with 5 μmoL/L SAHA, the proportion of G_0/G_1 phase cells increases from 50.3% to 65.3%, and the proportion of S phase cells decreases from 34.9% to 20.6%. Most cells were arrested in the G_0/G_1 phase and induced to undergo apoptosis. This may be related to increased expression of CDK inhibitor p21/waf1, which is induced by SAHA treatment. Nearly all HDACIs can induce expression of p21/waf1 to inhibit the activities of cyclin and CDK, resulting in cell cycle arrest and inhibition of differentiation. In addition, SAHA has been shown to influence expression of p27. After SAHA treatment, the degree of histone acetylation is elevated, stabilizing the activity of p53 (an important intracellular tumor suppressor protein) and leading to cell cycle arrest[18-20]. The Ras-Raf-MEK-ERK pathway is closely related with tumor cell proliferation. ERK can be activated by various growth factors, leading to interaction with transcription factors (mitogen, c-Jun, c-fos, c-Myc, cERK1) and nuclear proteins to promote the transcription and expression of a variety of oncogenes and genes related to cell cycle regulation, thus promoting cell proliferation and inhibiting apoptosis[21-23].

HBV is a risk factor for development of HCC. An epidemiological survey demonstrated that the carrying rate of HBsAg in China is 7.18%. HBV can be actively

replicated in patients with HCC, causing further liver damage[24-27]. HepG2.2.15 cells can continuously excrete intact HBV Dane particles into culture media. Upon treatment with SAHA, HBsAg and HBeAg content were 2.06 and 1.75 times greater than the control group, respectively, and HBV DNA content was 1.83 times greater than the control group. Taken together, SAHA stimulated replication of HBV. Histone acetylation is involved in regulation of gene transcription. After treatment with SAHA, the level of histone acetylation in HBV DNA is increased, and chromosome structure became incompact. This facilitates the combination of transcription factor with HBV DNA polymerase, thus stimulating HBV replication. However, this mechanism needs further validation. SAHA is an effective drug for HBV-negative HCC patients, but should be cautiously used in HBV-positive HCC patients in combination with anti-HBV drugs.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the world. Its occurrence is related to the multiple-step development process of different genetic alterations. At present, there is no effective treatment method. Suberoylanilide hydroxamic acid (SAHA) is a newly discovered anti-tumor drug which has broad application prospect. It exhibits inhibitory effect of tumor growth, which is been further confirmed in clinical trials.

Research frontiers

Histone deacetylase inhibitors (HDACIs) are a class of new anticancer drugs emerging in recent years, which has attracted widespread attention. Previous clinical trials find that, SAHA has broad-spectrum anti-hematological and solid tumor activities, with good tolerance. However, the effect of SAHA on hepatitis B virus (HBV) replication has not been reported.

Innovations and breakthroughs

SAHA inhibits HepG2.2.15 cell proliferation, promotes apoptosis, and stimulates HBV replication. In combination with anti-HBV drugs, SAHA may potentially be used cautiously for treatment of hepatocellular carcinoma.

Applications

SAHA has been applied in previous clinical trials. Results show that, it has broad-spectrum anti-hematological and solid tumor activities. SAHA can inhibit HepG2.2.15 cell proliferation, deduce the differentiation, and promote the apoptosis. At the same time, it can stimulate the replication of HBV. Therefore, SAHA should be cautiously used for treatment of HCC, and be combined with anti-HBV drugs if necessary. It can be used in the treatment of HBV-negative HCC patients.

Peer review

HCC is one of the most common malignant tumors, and HDACIs are a series of new anticancer drugs with a wide scope of application. In this manuscript, the authors investigated the effects of suberoylanilide hydroxamic acid, a potent HDACI on proliferation and apoptosis of a human hepatocellular carcinoma cell line (HepG2.2.15) and HBV replication. The manuscript is very well written.

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META-ANALYSIS

Single-incision laparoscopic appendectomy *vs* conventional laparoscopic appendectomy: Systematic review and meta-analysis

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Abstract

AIM: To assess the differences in clinical benefits and disadvantages of single-incision laparoscopic appendectomy (SILA) and conventional laparoscopic appendectomy (CLA).

METHODS: The Cochrane Library, MEDLINE, Embase, Science Citation Index Expanded, and Chinese Biomedical Literature Database were electronically searched up through January 2013 to identify randomized controlled trails (RCTs) comparing SILA with CLA. Data was extracted from eligible studies to evaluate the pooled outcome effects for the total of 1068 patients. The meta-analysis was performed using Review Manager 5.2.0. For dichotomous data and continuous data, the risk ratio (RR) and the mean difference (MD) were calculated, respectively, with 95%CI for both. For continuous outcomes with different measurement scales in different RCTs, the standardized mean difference (SMD) was calculated with 95%CI. Sensitivity and subgroup analyses were performed when necessary.

RESULTS: Six RCTs were identified that compared SILA (n = 535) with CLA (n = 533). Five RCTs had a high risk of bias and one RCT had a low risk of bias. SILA was associated with longer operative time (MD = 5.68, 95%CI: 3.91-7.46, P < 0.00001), higher conversion rate (RR = 5.14, 95%CI: 1.25-21.10, P = 0.03) and better cosmetic satisfaction score (MD = 0.52, 95%CI: 0.30-0.73, P < 0.00001) compared with CLA. No significant differences were found for total complications (RR = 1.15, 95%CI: 0.76-1.75, P = 0.51), drain insertion (RR = 0.72, 95%CI: 0.41-1.25, P = 0.24), or length of hospital stay (SMD = 0.04, 95%CI: -0.08-0.16, P = 0.57). Because there was not enough data among the analyzed RCTs, postoperative pain was not calculated.

CONCLUSION: The benefit of SILA is cosmetic satisfaction, while the disadvantages of SILA are longer operative time and higher conversion rate.

Key words: Single incision; Laparoscopic; Appendectomy; Meta-analysis; Systematic review

Core tip: The clinical benefit of single-incision laparoscopic appendectomy (SILA), compared to the conventional three-port laparoscopic appendectomy, has been a controversial issue in recent years. We performed the first systematic review and meta-analysis of randomized controlled trails (RCTs) that have assessed the clinical benefits and disadvantages between SILA and conventional laparoscopic appendectomy (CLA). Six RCTs conducted between 2011 and 2013 were identified and pooled to determine outcomes using meta-analytic methods. From this analysis, we conclude that SILA is as safe as CLA. Although patients receiving SILA had longer operative times and a higher conversion rate, one benefit of SILA is cosmetic satisfaction.

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INTRODUCTION

Appendectomy is one of the most commonly performed surgical procedures of the abdomen in the world. This surgical procedure has been performed for over 100 years, after first being described by McBurney[1]. With rapidly developing, minimally invasive surgery, the laparoscopic appendectomy has become a selectable method for appendectomy. Previous studies have reported that laparoscopic appendectomy has many advantages in comparison to open appendectomy, such as shorter hospital stays, reduced risks of complications, and better cosmetic satisfaction[2,3]. Therefore, the laparoscopic appendectomy, like laparoscopic cholecystectomy, is considered to be a favorable procedure for appendectomy in the future.

In addition, the use of single-incision laparoscopic techniques, which have been described with promising results in multiple studies[4-11], has increased over the past few years. Under such circumstances, surgical appendectomy may be undergoing a transition from the conventional three-port laparoscopic surgery toward the less-invasive, single-incision laparoscopic surgery. With the number of incisions reduced to just one umbilical incision, the potential advantages of single-incision surgery include better cosmetic outcome, less postoperative pain, and faster postoperative recovery. At the same time, this new technique may present potential disadvantages, such as increased operative time, higher conversion rates, and more complications.

Although a number of studies in the last few years have compared the single-incision laparoscopic appendectomy (SILA) with conventional laparoscopic appendectomy (CLA), most only demonstrated the feasibility and safety of SILA. Well-described benefits and disadvantages are still lacking in the literature. To our knowledge, there are no published meta-analyses describing randomized controlled trails (RCTs) comparing SILA with CLA. Therefore, we conducted a systematic review and meta-analysis of RCTs to assess the clinical benefits and disadvantages associated with SILA and CLA.

MATERIALS AND METHODS

Searching strategy

We searched the following databases up through January 2013 to identify RCTs: The Cochrane Library, MEDLINE, EMBASE, Science Citation Index Expanded, and the Chinese Biomedical Literature Database. The search strategies are shown in Table 1. Language was not used as a criterion for selection of studies, and both English and non-English studies were included. Moreover, the citations within the reference lists of the articles were searched manually to identify additional eligible studies. After all searches were completed, the search results were merged using the software package Endnote X6 to remove duplicate records. The title and abstract of every identified record was scanned by two independent authors (Wu SJ and Cheng Y) for the inclusion criteria. If compliance was not clear from the abstract, full-texts were retrieved for further assessment.

Inclusion and exclusion criteria

The aim of this meta-analysis was to specifically compare the benefits and disadvantages of SILA and CLA methods. Therefore, only those studies which provided comparison between those two methods mentioned above were included. Reliability was the most important point considered in this meta-analysis, so only RCTs were included. Prospective non-randomized, retrospective, and improperly performed RCTs were excluded from the analysis.

The definition of SILA was surgery through a single intra-umbilical incision. The included studies used various multiport devices or multiple conventional ports through a single skin incision but with multiple fascial incisions. CLA was defined as surgery with the standard three-port technique *via* a supra-umbilical or infra-umbilical port, a left lower quadrant port, and a right lower quadrant or supra-pubic region port.

Data extraction and validity assessment

Two independent authors (Lu J and Zhang J) extracted and confirmed the data and entered them into an electronic data collection form. Any disagreement in the two reviewers' data collection and quality assessment was discussed until a consensus was reached. For the validity assessment, another two authors (Cai YL and Lin YX) independently assessed the methodological quality of the included trials using the quality checklist recommended by the Cochrane Handbook. The assessment contained six dimensions: (1) random sequence generation; (2) allocation concealment; (3) blinding; (4) addressing of incomplete outcome data; (5) selective reporting; and (6) other bias. Following the evaluation of the above domains, an included trial was judged as having low risk of bias if it was evaluated as "low" in all of the above domains. If the risk of bias was judged as "unclear" or "high", then the trial was listed under the group of trials with "high risk of bias." Otherwise, all disagreements were resolved by discussion and referral to a third author (Xiong XZ) for resolution.

Outcomes

Data for the following outcomes were extracted: total operative time, total complications (wound infection, abscess, ileus, stump leakage, *etc.*), drain insertion, conversion rate, length of hospital stay, postoperative pain as

Table 1 Search strategies

Databases	Period of search	Search strategies
The Cochrane Library	Through January 30, 2013	(1) MeSH descriptor Appendectomy, Laparoscopic explode all trees (2) (laparoscop* or coelioscop* or celioscop* or peritoneoscop*) and appendectom* (3) 1 or 2 (4) "single incision" or "single port" or "single site" or "one port" or "one incision" or "one site" (5) 3 and 4
MEDLINE (Pubmed)	Through January 30, 2013	(1) Appendectomy, laparoscopic [MeSH] (2) (laparoscop* or coelioscop* or celioscop* or peritoneoscop*) and appendectom* (3) 1 or 2 (4) "single incision" or "single port" or "single site" or "one port" or "one incision" or "one site" (5) (randomised controlled trial [pt] or controlled clinical trial [pt] or randomised [tiab] or placebo [tiab] or drug therapy [sh] or randomly [tiab] or trial [tiab] or groups [tiab]) not (animals [mh] not humans [mh]) (6) 3 and 4 and 5
EMBASE (OvidSP)	Through January 30, 2013	(1) (appendectomy.af.) or (exp appendectomy/) (2) ((laparoscop* or coelioscop* or celioscop* or peritoneoscop*).af.) or (exp Laparoscopy/) (3) (single incision or single port or single site or one port or one incision or one site).af. (4) (random* or factorial* or crossover* or placebo*).af. (5) expcrossoverprocedure/or exp double-blind procedure/or exp randomized controlled trial/or single-blind procedure/ (6) 4 or 5 (7) 1 and 2 and 3 and 6
Science Citation Index Expanded	Through January 30, 2013	(1) TS = (appendectom*) (2) TS = (laparoscop* or coelioscop* or celioscop* or peritoneoscop*) (3) TS = ("single incision" or "single port" or "single site" or "one port" or "one incision" or "one site") (4) TS = (random* or blind* or placebo* or meta-analysis) (5) 1 and 2 and 3 and 4
CBM	Through January 30, 2013	Search strategy in was performed in Chinese. Includes search terms similar to the terms used in MEDLINE

MEDLINE: Medical Literature Analysis and Retrieval System Online; EMBASE: Excerpta Medica Database; CBM: Chinese Biomedical Literature Database; MeSH: Medical Subject Heading.

assessed using the visual analogue scale (VAS), and cosmetic satisfaction.

Statistical analysis

We performed all the statistical analyses of the extracted data with Review Manager 5.2.0. For dichotomous data and continuous data, we calculated the risk ratio (RR) and the mean difference (MD) with 95%CIs for both. For continuous outcomes with different measurement scales in different RCTs, we calculated the standardized mean difference (SMD) with 95%CI. Heterogeneity was described with the χ^2 test. A P value less than 0.10 was considered to be significant heterogeneity and the I^2 statistic was used to measure the quantity of heterogeneity. If significant heterogeneity existed, a random-effect model was used. In the absence of significant heterogeneity, a fixed-effect model was adopted.

In the case of missing data, we contacted the original investigators to request further information. If there was no reply, we performed the analysis on an "intention-to-treat" principle, if applicable. Otherwise, we adopted the available-case analysis, also known as the per-protocol analysis. A few published clinical trials reported a median and a range instead of a mean and SD. To adjust this difference, we assumed that the median was equal to the mean, and we estimated the SD as a quarter of the reported range. Funnel plots were used to determine reporting biases. We conducted the meta-analysis and systematic review according to the Cochrane Handbook for Systematic Reviews of Interventions and Preferred Reporting Item for Systematic Reviews and Meta-Analysis.

RESULTS

Search results

We identified a total of 111 records through electronic searches of The Cochrane Library (n = 12), MEDLINE (n = 21), EMBASE (n = 32), Science Citation Index Expanded (n = 44), Chinese Biomedical Literature Database (n = 0), and a manual search of the references in the included RCTs (n = 2). We excluded 39 duplicates and 72 clearly irrelevant records by reading titles and abstracts. Fourteen full-text articles were retrieved for further assessment. We excluded seven articles for the reasons listed in Figure 1.

Description of included trials and risk of bias

Six RCTs published between 2011 and 2013 were identified that fulfilled the inclusion criteria[12-17]. A total of 1068 patients were included. There were 535 patients who received SILA and 533 who received CLA. Two included trials were of pediatric patients[14,15], and the remaining four trials were of adult patients[12,13,16,17]. Details on the included studies are shown in Table 2. The risk of bias is summarized in Table 3. Five RCTs had a high risk of bias[13-17], and one RCT had a low risk of bias[12].

Effect of interventions

Total operative time: All six RCTs reported the operative time to complete appendectomy[12-17]. The operative time

Table 2 Study characteristics

Study	Area	Study design	Participants (SILA/CLA)	Mean age, yr (SILA/CLA)	Male:female ratio (SILA/CLA)
Teoh *et al*[12]	Hong Kong	Multi-center	195 (98/97)	39.2/40.7	58:40/59:38
Lee *et al*[13]	South Korea	Single-center	229 (116/113)	28.4/28.5	64:52/68:45
Perez *et al*[14]	United States	Single-center	50 (25/25)	8.7/8.9	10:15/15:10
St Peter *et al*[15]	United States	Single-center	360 (180/180)	11.1/11.1	99:81/92:88
Sozutek *et al*[16]	Turkey	Single-center	50 (25/25)	30.6/30.0	12:13/7:18
Frutos *et al*[17]	Spain	Single-center	184 (91/93)	28.0/31.0	42:49/47:46

SILA: Single-incision laparoscopic appendectomy; CLA: Conventional laparoscopic appendectomy.

Table 3 Risk of bias assessment

Study	Sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting
Teoh *et al*[12]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Lee *et al*[13]	Low risk	Uncertain	High risk	High risk	Uncertain	Low risk
Perez *et al*[14]	Low risk	Low risk	Low risk	Low risk	Uncertain	High risk
St Peter *et al*[15]	Low risk	Low risk	Low risk	High risk	Low risk	Low risk
Sozutek *et al*[16]	Low risk	Uncertain	High risk	High risk	Uncertain	High risk
Frutos *et al*[17]	Low risk	Uncertain	High risk	High risk	Low risk	High risk

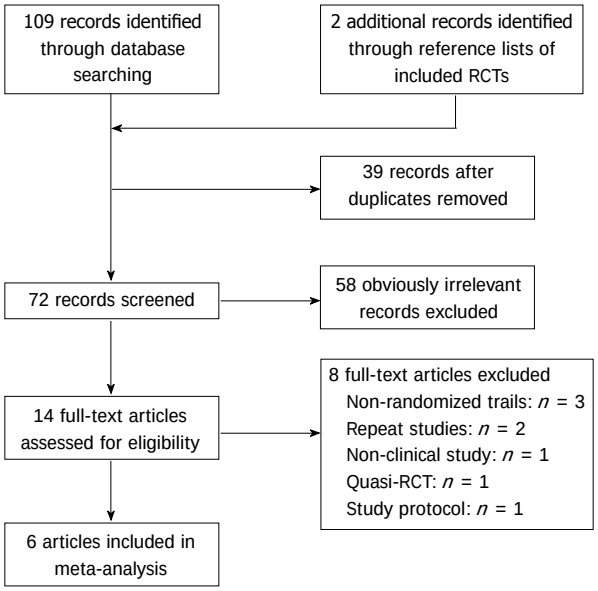


Figure 1 Flow diagram demonstrating the study selection process. RCT: Randomized controlled trial.

was significantly longer in the SILA group than in the CLA group (Figure 2A; MD = 5.68, 95%CI: 3.91-7.46, $P < 0.00001$). There was no evidence of statistical heterogeneity ($\chi^2 = 4.61$, $P = 0.47$, $I^2 = 0\%$).

Total complications: All six RCTs reported the total complications after appendectomy[12-17]. There was no significant difference in the overall incidence of postoperative complications between the two groups (Figure 2B; RR = 1.15, 95%CI: 0.76-1.75, $P = 0.51$). There was no evidence of statistical heterogeneity ($\chi^2 = 2.25$, $P = 0.81$, $I^2 = 0\%$).

Conversion rate: All six RCTs reported the conversion rates during appendectomy[12-17]. This included placement of additional laparoscopic ports for SILA and conversion to open appendectomy from both SILA and CLA .The conversion rate was 7.48% (40 of 535 patients) and 0.75% (4 of 533 patients) in the SILA and CLA groups, respectively. The rate was significantly higher in patients who received SILA than CLA (Figure 2C; RR = 5.14, 95%CI: 1.25-21.10, $P = 0.02$). Significant heterogeneity was present in the trials ($\chi^2 = 6.82$, $P = 0.15$, $I^2 = 41\%$).

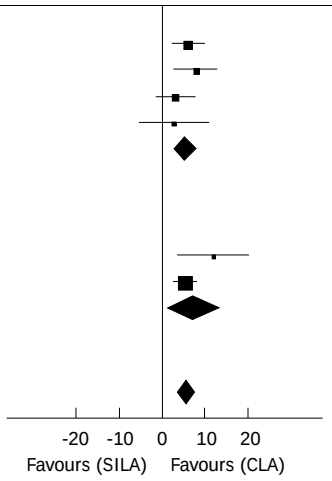
Drain insertion: Only two RCTs reported drain insertion during appendectomy[12,13]. There was no significant difference in the incidence of drain insertion between the two groups (Figure 2D; RR = 0.72, 95%CI: 0.41-1.25, $P = 0.24$). There was no evidence of statistical heterogeneity ($\chi^2 = 0.13$, $P = 0.71$, $I^2 = 0\%$).

Length of hospital stay: The length of hospital stay was evaluated in all studies[12-17], but only three studies reported this data in the form of mean and SD[12,16,17]. By contacting the authors personally by email, we were able to retrieve the mean and SD data for the other two studies[14,15]. Another study provided the mean and range[13]. According to our predefined plan, we equated the SD with a quarter of the reported range. There was no significant difference between the two groups (Figure 2E; SMD = 0.04, 95%CI: -0.08-0.16, $P = 0.57$). There was no evidence of statistical heterogeneity ($\chi^2 = 5.31$, $P = 0.38$, $I^2 = 6\%$).

Postoperative pain: Four of the included trials reported postoperative pain scores using the VAS (10-point or 100-mm) after appendectomy[12,13,16,17]. Teoh *et al*[12] indicated that there were no significant differences in the overall pain scores and the pain scores at rest ($P = 0.109$ and 0.154, respectively), while significantly worse pain was experienced in the SILA group after coughing 10 times and on standing ($P = 0.001$ and 0.038, respectively). Lee *et al*[13] stated that postoperative pain scores were not statistically different between the two groups at 12 h, 24 h, 36 h and 14 d postoperatively ($P = 0.651$, 0.555, 0.570

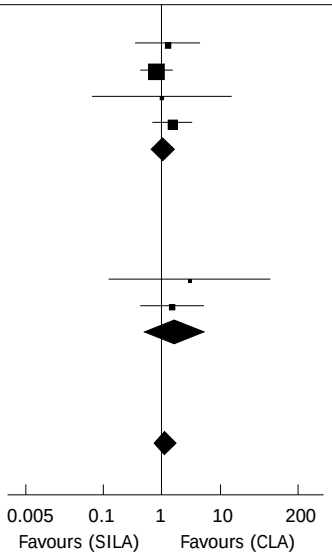
A

Study or subgroup	SILA Mean	SILA SD	SILA Total	CLA Mean	CLA SD	CLA Total	Weight	Mean difference IV, random, 95%CI
1.1.1 Adult								
Frutos *et al*[17]	38.13	13.49	91	32.12	12.44	93	22.4%	6.01 [2.26, 9.76]
Lee *et al*[13]	43.8	21.3	116	35.8	18.9	113	11.6%	8.00 [2.79, 13.21]
Sozutek *et al*[16]	32.6	9.9	25	29.5	6.8	25	14.2%	3.10 [-1.61, 7.81]
Teoh *et al*[12]	63	27.2	98	60.2	31.7	97	4.6%	2.80 [-5.49, 11.09]
Subtotal (95%CI)			330			328	52.8%	5.38 [2.94, 7.83]
Heterogeneity: Tau^2 = 0.00; χ^2 = 2.35, df = 3 (P = 0.50); I^2 = 0%								
Test for overall effect: Z = 4.32 (P < 0.0001)								
1.1.2 Children								
Perez *et al*[14]	46.8	17.5	25	34.8	12.5	25	4.4%	12.00 [3.57, 20.43]
St Peter *et al*[15]	35.2	14.5	180	29.8	11.6	180	42.8%	5.40 [2.69, 8.11]
Subtotal (95%CI)			205			205	47.2%	7.44 [1.46, 13.42]
Heterogeneity: Tau^2 = 11.57; χ^2 = 2.13, df = 1 (P = 0.14); I^2 = 53%								
Test for overall effect: Z = 2.44 (P = 0.01)								
Total (95%CI)			535			533	100.0%	5.68 [3.91, 7.46]
Heterogeneity: Tau^2 = 0.00; χ^2 = 4.61, df = 5 (P = 0.47); I^2 = 0%								
Test for overall effect: Z = 2.44 (P = 0.01)								
Test for subgroup differences: χ^2 = 0.39, df = 1 (P = 0.53); I^2 = 0%								



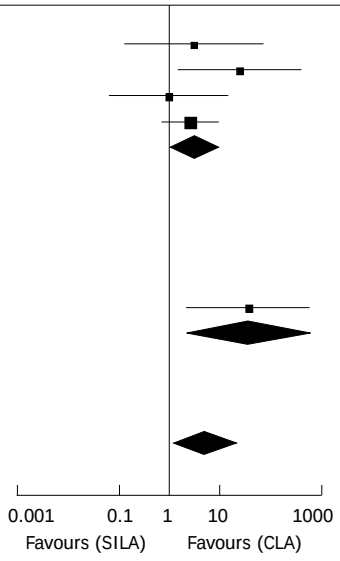
B

Study or subgroup	SILA Events	SILA Total	CLA Events	CLA Total	Weight	Risk ratio M-H, fixed, 95%CI
3.1.1 Adult						
Frutos *et al*[17]	5	91	4	93	10.8%	1.28 [0.35, 4.61]
Lee *et al*[13]	15	116	18	113	49.6%	0.81 [0.43, 1.53]
Sozutek *et al*[16]	1	25	1	25	2.7%	1.00 [0.07, 15.12]
Teoh *et al*[12]	14	98	9	97	24.6%	1.54 [0.70, 3.39]
Subtotal (95%CI)		330		328	87.8%	1.08 [0.69, 1.69]
Total events	35		32			
Heterogeneity: χ^2 = 1.62, df = 3 (P = 0.65); I^2 = 0%						
Test for overall effect: Z = 0.33 (P = 0.74)						
3.1.2 Children						
Perez *et al*[14]	1	25	0	25	1.4%	3.00 [0.13, 70.30]
St Peter *et al*[15]	6	180	4	180	10.9%	1.50 [0.43, 5.23]
Subtotal (95%CI)		205		205	12.2%	1.67 [0.53, 5.28]
Total events	7		4			
Heterogeneity: χ^2 = 0.16, df = 1 (P = 0.69); I^2 = 0%						
Test for overall effect: Z = 0.87 (P = 0.39)						
Total (95%CI)		535		533	100.0%	1.15 [0.76, 1.75]
Total events	42		36			
Heterogeneity: χ^2 = 2.25, df = 5 (P = 0.81); I^2 = 0%						
Test for overall effect: Z = 0.66 (P = 0.51)						
Test for subgroup differences: χ^2 = 0.47, df = 1 (P = 0.49); I^2 = 0%						



C

Study or subgroup	SILA Events	SILA Total	CLA Events	CLA Total	Weight	Risk ratio M-H, random, 95%CI
5.1.1 Adult						
Frutos *et al*[17]	1	91	0	93	14.1%	3.07 [0.13, 74.28]
Lee *et al*[13]	12	116	0	113	16.7%	24.36 [1.46, 406.59]
Sozutek *et al*[16]	1	25	1	25	17.5%	1.00 [0.07, 15.12]
Teoh *et al*[12]	8	98	3	97	34.9%	2.64 [0.72, 9.65]
Subtotal (95%CI)		330		328	83.2%	3.21 [1.04, 9.92]
Total events	22		4			
Heterogeneity: Tau^2 = 0.14; χ^2 = 3.30, df = 3 (P = 0.35); I^2 = 9%						
Test for overall effect: Z = 2.03 (P = 0.04)						
5.1.2 Children						
Perez *et al*[14]	0	25	0	25		Not estimable
St Peter *et al*[15]	18	180	0	180	16.8%	37.00 [2.25, 609.33]
Subtotal (95%CI)		205		205	16.8%	37.00 [2.25, 609.33]
Total events	18		0			
Heterogeneity: Not applicable						
Test for overall effect: Z = 2.53 (P = 0.01)						
Total (95%CI)		535		533	100.0%	5.14 [1.25, 21.10]
Total events	40		4			
Heterogeneity: Tau^2 = 1.05; χ^2 = 6.82, df = 4 (P = 0.15); I^2 = 41%						
Test for overall effect: Z = 2.27 (P = 0.02)						
Test for subgroup differences: χ^2 = 2.52, df = 1 (P = 0.11); I^2 = 60.3%						



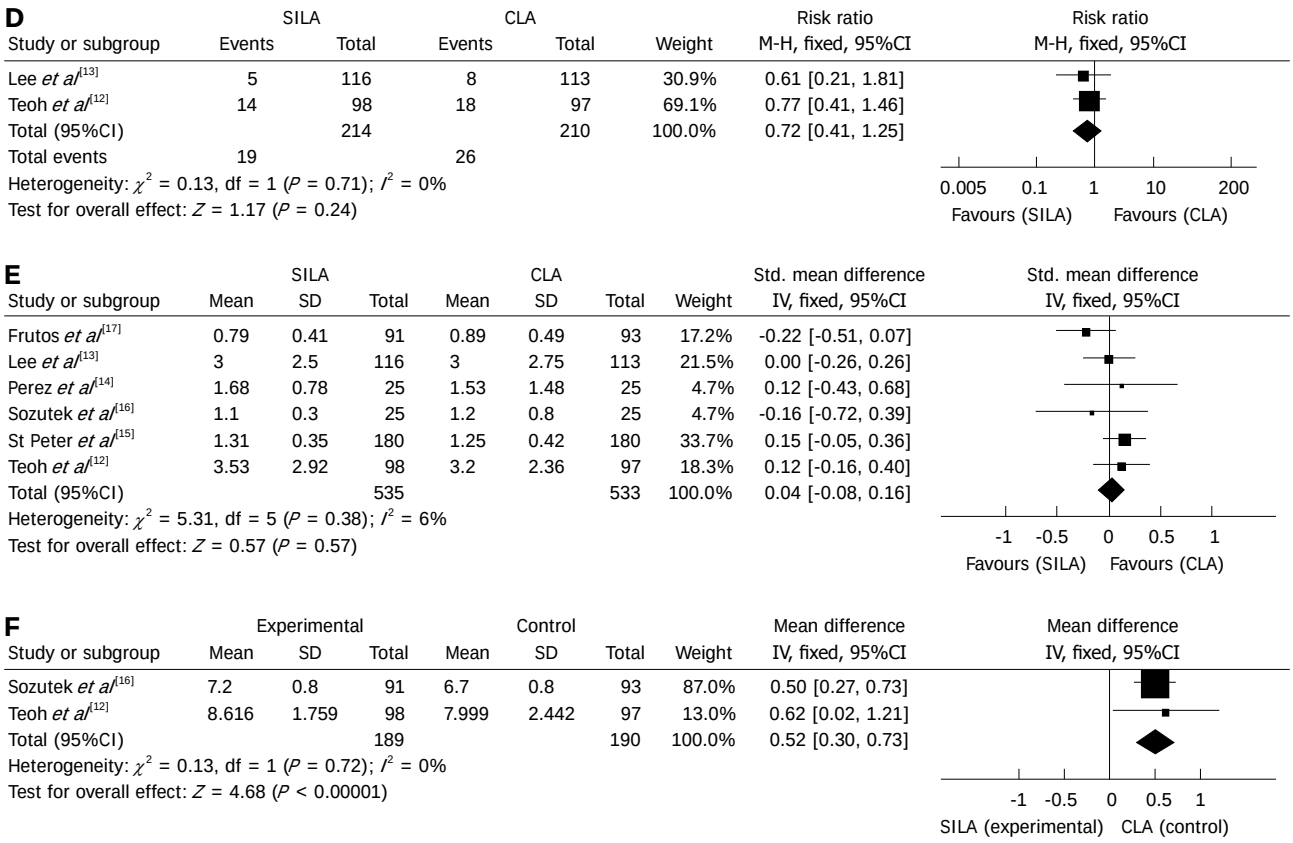


Figure 2 Forest plots of the meta-analysis. A: Comparisons of single-incision laparoscopic appendectomy (SILA) *vs* conventional laparoscopic appendectomy (CLA) in total operative time; B: Total complications; C: Conversion rate; D: Drain insertion; E: Length of hospital stay; F: Cosmetic satisfaction.

and 0.631, respectively). Likewise, Sozutek *et al*[16] stated that no difference was detected in terms of postoperative pain ($P = 0.991$). However, in Frutos' trial, less pain was found in SILA group (SILA/CLA: 2.76 ± 1.64/3.78 ± 1.76, $P < 0.001$). Only one study provided the mean and SD, so those values were not calculated in this analysis.

Cosmetic satisfaction: Three studies reported cosmetic satisfaction scores[12,13,16]. The cosmetic score was also measured by a 5-point VAS with a higher score indicating better satisfaction. The meta-analysis of two studies[12,16], which provided the mean and SD, reported that the cosmetic scores were significantly higher in the SILA group than in the CLA group (Figure 2F; MD = 0.52, 95%CI: 0.30-0.73, $P < 0.00001$). There was no evidence of statistical heterogeneity ($\chi^2 = 0.13$, $P = 0.72$, $I^2 = 0\%$). However, the remaining trial reported no significant difference between the two groups with VAS scores of 4.0 and 3.3 for SILA and CLA, respectively ($P = 0.128$)[13].

Subgroup analysis: Because the age of the patients may have influenced the eventual outcome, we performed a subgroup analysis for operative time, total complications, and conversion rate. In the subgroup analysis of age, the outcomes were also equivalent.

DISCUSSION

The single-incision method of laparoscopic appendectomy, compared to the conventional three-port method, has been a controversial issue in recent years. Numerous studies have been performed to evaluate the differences; however, most of them were non-RCT studies. Fortunately, six new RCTs published between 2011 and 2013[12-17] evaluated the benefits and disadvantages of SILA and CLA in a quantitative manner and provided the basis of this study. This meta-analysis and systematic review of those six RCTs indicated that although SILA was associated with a longer operative time and a higher conversion rate, patients had better cosmetic satisfaction compared with CLA. No significant differences were found in total complications, drain insertion, length of hospital stay, and postoperative pain between the two procedures.

Regarding operative time, a meta-analysis of non-RCTs concluded that there was no difference between the two groups[18]. Those results were inconsistent with the results of this analysis, which determined that the SILA operative time was longer by 5.68 min. This discrepancy may have been due to the lack of surgical experience using the new technique. Performing SILA requires experience in laparoscopic surgery, and a certain number of cases must be performed to overcome the learning curve. A retrospective study by Lee *et al*[19] reported that the operation time tended to shorten when the surgeon gained more experience and accumulated cases. This finding is in agreement with a separate report by Perez *et al*[14], which reported that in the first 25 patients enrolled, the differ-

ence in operative time was significantly greater (49.31 min *vs* 33.50 min, $P = 0.049$) and that this difference decreased in a subsequent group of 25 patients (44.08 min *vs* 36.00 min, $P = 0.123$). Although one disadvantage of SILA is a longer operative time, we believe that with increased experience and developed instrumentation SILA will reach equivalent effectiveness to conventional three-port methods.

Conversion rate is another major concern for surgeons. The high conversion rate is an important disadvantage and has considerably limited the widespread use of SILA. In our meta-analysis, we found that the heterogeneity was very high among the analyzed studies. Therefore, in order to assess the reliability and stability of this outcome, we conducted a sensitivity analysis; only two of the evaluated RCTs precisely described the conversion-fulfilled, predefined outcome[13,15]. After this analysis, a significantly higher rate was observed in the SILA group (RR = 30.64, 95%CI: 4.22-222.68, $P = 0.0007$) and no heterogeneity was found ($\chi^2 = 0.04$, $P = 0.84$, $I^2 = 0\%$). Thus, we confirmed that a higher conversion rate was consistent with SILA treatment. Technical difficulty could account for this. Complicated appendicitis exists in 30% of all appendicitis cases[20] and when the operation is difficult, such as with serious adhesion or significant inflammation, the single-incision approach can be somewhat cumbersome.

In such scenarios, extra incision sites or use of surgical instruments may become necessary. In a study by St Peter *et al*[15], surgeons rated the degree of technical difficulty for every case, excluding perforated appendicitis, on a subjective scale from 1 to 5 with 1 indicating an easy case and 5 indicating a difficult case. Higher surgical difficultly ratings were noted for SILA relative to the standard three-port laparoscopic appendectomy (2.3 ± 1.4 *vs* 1.7 ± 1.0, $P < 0.001$). Thus, not only in complicated appendicitis, but also in uncomplicated appendicitis, the decision to add an additional site or use additional instrumentation is dependent on a lower comfort level with single-site procedures. However, Crohn's disease can be performed with a single-site procedure in the presence of significant inflammation[21]. This indicates that if only to promote surgeon comfort level, pure SILA could become easier to complete. Further technical research and developments are needed to reduce the difficulty of SILA and to allow surgeons to comfortably perform this procedure. This may be the only way to reduce the conversion rate when implementing SILA.

Postoperative pain is another controversial topic to be discussed when a single-incision technique is applied. As a result of a reduced trocar use, less surgical pain was postulated in SILA[22]. A small case series and a retrospective analysis reported that reduced pain was found with SILA[23,24]. Conversely, the combined size of the fascial incision at the umbilicus required to accommodate the single-incision port may give rise to more potential pain compared with multiple, smaller fascial incisions in CLA. A 40-patient pilot trial in adults found significantly greater pain scores in the initial 24 h after SILA[25]. Moreover, from an anatomical point of view, the true pelvic peritoneum has less sensitivity to acute pain than the parietal peritoneum in the umbilicus[26]. Thus, the two ports in the lower abdomen in CLA may cause less pain than repositioning them to the umbilicus. Thus, by analyzing previous studies, whether there is less postoperative pain with SILA is uncertain.

In this analysis, three of the included RCTs indicated that the pain scores were comparable between the two groups[12,13,16]. Although, Teoh *et al*[12] concluded that more pain was identified in activity, the overall scores demonstrated no significant difference. This is in agreement with a previous non-RCT meta-analysis[18]. Moreover, the same comparison in cholecystectomy also showed no significant difference in pain scores at 6 and 24 h between single-incision and multiple-incision procedures[27]. Conversely, another RCT showed less pain was found with SILA, although this difference was very small[17]. Thus, we believe that the pain is not much different between SILA and CLA. However, the overall length of incision may be an important factor in this debate. As many discrepancies exist in the analyzed studies, data from future RCTs are anticipated to resolve these potential differences.

This meta-analysis highlighted cosmetic satisfaction as the significant benefit of SILA over CLA. This so-called "scarless" procedure meets the demand of expecting to conceal the surgical history of patients, especially in young females. Although SILA definitely reduces the number of incisions and often results in better cosmetic satisfaction among patients, there was not enough clinical data to support this claim previously. We recognize that some studies showed better scores without significant differences[13,28], possibly due to existing high cosmetic scores with CLA and leaving only slightly more room for improvement with SILA.

Some limitations exist in assessing cosmetic satisfaction. First, a standard tool to assess the appearance of the wound is still lacking. Second, patients rate the score by their own subjective feeling without a more quantitative reference. We speculate that after surgery, patients may be more focused on whether the disease had been cured rather than on a cosmetic score. Third, wound healing is a long-term process, and the cosmetic benefit should be assessed during both short-term and long-term follow-up examinations. Therefore, prospective RCTs with long-term follow-up are needed to confirm the cosmetic benefits of SILA. Establishing a validated scar assessment tool is also necessary for adequate quantitative analysis.

Six RCTs were included in this review. Most included patients with perforated appendicitis, while only one study excluded patients with perforated appendicitis. Thus, our results were relevant to all types of acute appendicitis. However, the quality of these newly analyzed RCTs was low as only one RCT had a low risk of bias[12].

Meta-analysis is an increasingly popular method of data analysis to examine discrepancies in the literature. Nevertheless, there were some limitations in our research.

First, the number of included RCTs was small, and, among those, two RCTs were also of small sample size. Funnel plots were not performed to assess the publication bias due to the small number of included RCTs. Second, the surgical techniques among the studies were varied; thus, there may be variances in operative time, conversion rate, and complications. Third, a cost analysis was not conducted in this research as cost is always higher with the development of a new technique and the instruments varied significantly with each study.

In conclusion, despite the limitations mentioned above, this review currently provides the best available evidence for comparison of single-incision laparoscopic appendectomy *vs* conventional laparoscopic appendectomy. From a curative perspective, SILA is comparable to CLA in terms of total complications, drain insertion, length of hospital stay, and postoperative pain. The disadvantages of SILA are a longer operative time and a higher conversion rate. One benefit of SILA is patient cosmetic satisfaction. Thus, the option of this new treatment alternative should be carefully discussed with patients. More RCTs are needed to clarify the benefits and disadvantages of SILA compared to CLA.

COMMENTS

Background

Appendectomy is one of the most commonly performed surgical procedures of the abdomen in the world. In recent years, minimally invasive surgery has rapidly developed and conventional laparoscopic appendectomy (CLA) has been widely used. In addition, single-incision laparoscopic appendectomy (SILA), as a new technique, has been introduced as an alternative to conventional three-port laparoscopic appendectomy.

Research frontiers

Both SILA and CLA are used for patients undergoing appendectomy. Many studies, including randomized controlled trials (RCTs), have compared SILA with CLA in the last few years. However, most have only demonstrated the feasibility and safety of SILA. The clinical benefits and disadvantages between SILA and CLA are still controversial.

Innovations and breakthroughs

The authors identified all RCTs comparing SILA with CLA. A meta-analysis and systematic review was conducted according to the Cochrane Handbook. From this study, the disadvantages of SILA were determined to be longer operative times and higher conversion rates, while the benefit of SILA was cosmetic satisfaction among patients. This has not been clearly identified in previous studies.

Applications

From a curative perspective, SILA is proven to be a safe and effective treatment that is comparable to CLA. Based on the benefits and disadvantages of SILA, surgeons should carefully assess each patient's situation and discuss surgical options that meet their needs.

Peer review

This article is a good meta-analysis about single-incision laparoscopic appendectomy *vs* conventional laparoscopic appendectomy. The conclusions are unbiased and give good clues to the readers.

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CASE REPORT

Green tea extract: A potential cause of acute liver failure

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Abstract

The use of herbal products has increased significantly in recent years. Because these products are not subject to regulation by the Food and Drug Administration and are often used without supervision by a healthcare provider, the indication for and consumption of these supplements is quite variable. Moreover, their use is generally regarded as safe and natural by the lay-public. Unfortunately, there has been an increase in the number of reported adverse events occurring with the use of herbal products. We present a case of acute impending liver failure in an adolescent male using a weight-loss product containing green tea extract. Our case adds to the growing concern surrounding the ingestion of green tea extract and serves to heighten healthcare provider awareness of a potential green tea extract hepatotoxicity. Despite the generally touted benefits of green tea as a whole, clinical concern regarding its use is emerging and has been linked to its concentration in multiple herbal supplements. Interestingly, the suspected harmful compounds are those previously proposed to be advantageous for weight-loss, cancer remedy, and anti-inflammatory purposes. Yet, we emphasize the need to be aware of not just green tea extract, but the importance of monitoring patient use of all dietary supplements and herbal products.

Key words: Green tea; Plant extract; Dietary supplements; Liver failure; Liver injury; Hepatotoxicity

Core tip: Green tea extract is one of the most common herbal supplements ingested worldwide and is manufactured into more than 100 different over-the-counter products. Although traditionally considered safe, it has been linked to hepatotoxicity and led to acute impending liver failure in our adolescent patient. Eliminating multiple etiologies and with tissue evidence, a weight-loss supplement containing green tea extract was likely to blame. Recovery was over a two-month course. The lack of regulation and provider guidance in the use of this product and dietary supplements in general is significant. We highlight the importance of monitoring patient use of dietary supplements.

Patel SS, Beer S, Kearney DL, Phillips G, Carter BA. Green tea extract: A potential cause of acute liver failure. *World J Gastroenterol* 2013; 19(31): 5174-5177 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i31/5174.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i31.5174

INTRODUCTION

In the United States, herbal products are classified as dietary supplements, and their use has been increasing over recent decades. In fact, the use of herbal medicine increased from 2.5% in the general population in 1990 to

Table 1 Laboratory studies

Days post-hospitalization	Admission day 1	Hospitalized day 15	Discharge day 24	Follow-up day 45	Follow-up day 94	Follow-up day 185
Aspartate aminotransferase (U/L)	2106	958	525	59	33	35
Alanine aminotransferase (U/L)	2984	1169	665	165	44	31
Alkaline phosphatase (U/L)	186	86	137	148	120	94
Gamma glutamyl transferase (U/L)	78	65	104	49	41	28
Conjugated bilirubin (mg/dL)	12.9	14.7	10.3	0.0	0.0	0.0
Unconjugated bilirubin (mg/dL)	1.9	2.0	2.1	0.8	0.2	0.2
Albumin (g/dL)	4.0	2.5	3.2	3.9	4.1	4.1
Protime (s)	15.9	18.2	14.9	-	-	-
International normalised ratio	1.3	1.5	1.2	1.0	1.0	1.0
Factor 7	-	42%	-	102%	-	-

12.1% in 1997, with $5.1 billion spent as out of pocket expenditures for herbal therapies in 1997[1]. Moreover, according to the 2007 National Health Interview Survey, 17.9% of adults reported use of an herbal supplement in the previous year[2]. Yet, as dietary supplements, these products are not subject to the same regulation as drugs approved by the Food and Drug Administration (FDA). Instead, in accordance with the Dietary Supplement Health and Education Act of 1994, dietary supplements do not need approval of their safety or efficacy by the FDA[3].

Green tea has been consumed worldwide for many years and is a popular herbal ingredient that has been manufactured into more than 100 over-the-counter supplements[4]. Green tea's most touted benefits are its antioxidant and weight-loss or thermogenic properties. Nonetheless, there has been increasing concern regarding the potential hepatotoxicity with the use of green tea extract[5-7]. Here, we present a case of acute impending liver failure in an adolescent male occurring with the use of a weight-loss product containing green tea extract.

CASE REPORT

Our patient is a 16 year-old Hispanic male, who presented to our emergency room with new onset jaundice. The patient noticed yellowing of his skin and darkening of his urine six to seven days prior to admission. He denied abdominal pain, changes in his stool, fever, changes in mental status, alcohol consumption, sick contacts or recent travel. He is currently a high school student.

He does have a history of obesity and was taking several dietary supplements as part of an unsupervised weight-loss plan. Specifically, he was taking Applied Nutrition® Green Tea Fat Burner beginning 60 d prior to admission and took 2 pills daily (or 400 mg epigallocatechin-3-gallate, EGCG, daily). He started whey protein 30 d prior to admission and mixed 1 scoop in 16.9 oz of water three times per week. In addition, he used GNC Mega Men® Sport, taken 2 pills three times per week, beginning 30 d prior to admission. And lastly, he was taking Nopal® (Cactus), 1 pill daily, beginning 60 d prior to admission. Over this time period, he lost 56 pounds.

On physical exam, the patient was jaundiced, most evident in the face and sclera, but also present on the chest and upper extremities. Mental status was intact. Abdominal exam was insignificant, with the liver difficult to appreciate given that the patient was still overweight at the time of exam. Initial labs included: aspartate aminotransferase (AST) 2106 U/L (normal range 15-40 U/L), alanine aminotransferase (ALT) 2984 U/L (normal range 10-45 U/L), alkaline phosphatase 186 U/L (normal range 116-483 U/L), gamma glutamyl transferase (GGT) 78 U/L (normal range 12-33 U/L), conjugated bilirubin (CB) 12.9 mg/dL (normal range < 0.3 mg/dL), unconjugated bilirubin (UB) 1.9 mg/dL (normal range < 0.1 mg/dL), albumin 4 g/dL (normal range 3.7-5.5 g/dL), partial thromboplastin time 33.9 s (normal range 25.4-34.9 s), protime 15.9 s (normal range 11.2-15.4 s), international normalised ratio (INR) 1.3 (normal range 0.8-1.2), and glucose 99 (Table 1). Thus, he was admitted for work-up of acute liver injury and possible impending liver failure. During his hospitalization his peak INR and CB were 1.5 and 17.5 mg/dL, respectively. His lowest albumin and factor 7 level was 2.5 g/dL and 39% (normal range 58%-150%), respectively, indicating a decline in liver synthetic function and impending liver failure. Radiological exam was done on admission and consisted of an abdominal ultrasound with Doppler, read as mild hepatomegaly with normal right upper quadrant Doppler evaluation.

Extensive lab work was ordered to determine the etiology of his impending liver failure. Serological markers of autoimmune hepatitis (filamentous actin and Liv/Kid antibodies), infectious hepatitis A, B and C (serologies for infectious hepatitis E were not performed given insignificant incidence in the United States), Wilson's disease (ceruloplasmin), and alpha-1-antitrypsin deficiency were negative. In addition, cytomegalovirus and Epstein-Barr virus immunoglobulin M/immunoglobulin G and adenovirus polymerase chain reaction were negative.

Given lab work as stated, the patient had an ultrasound-guided liver biopsy completed on hospital day 5 (Figure 1). Liver histology was notable for diffuse portal and lobular mixed inflammatory cell infiltrates with acute and chronic inflammation that included scattered eosinophils and interface hepatitis. There was hepatocyte unrest and ballooning degeneration with multifocal individual hepatocyte necrosis and cholestasis. Injury was most

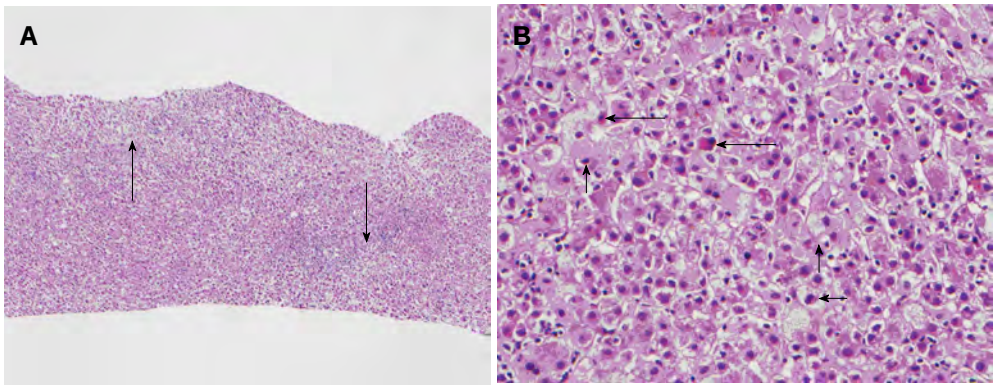

Figure 1 Pathological liver tissue. A: Diffuse portal and lobular inflammatory cell infiltrates (long arrows) [hematoxylin and eosin (HE), × 20]; B: Hepatocytes are reactive with prominent ballooning degeneration (short arrows) and individual cell necrosis (long arrows) (HE, × 200).

prominent in zone 1, but pan-lobular as depicted in Figure 1. He was observed in the hospital until his liver panel began to improve on hospital day twenty-four. Treatment during this admission included initiation of oral vitamin K 5 mg daily on hospital day 2 and ursodiol on hospital day 3. He also received intravenous fluids with a 5% dextrose content, initiated one week after admission and discontinued one week prior to discharge. He was seen again in our clinic at three weeks, ten weeks, and twenty-three weeks after discharge at which time labs (AST, ALT, alkaline phosphatase, GGT, CB, UB, INR and albumin) were repeated. All values continued to improve, along with normalization of both albumin and factor 7 levels, indicating resolution and recovery of his liver function (Table 1).

As several causes of acute liver injury were ruled out, and given his liver histology consistent with previously published reports of toxicity associated with green tea extract, his liver injury can most likely be attributed to his ingestion of this commercially available herbal supplement.

DISCUSSION

As with our patient, many patients using herbal supplements use a combination of products. Again the use is commonly unsupervised and a deviation from the products' user instructions. We are associating our patient's impending liver failure to his ingestion of green tea extract given the history taken, histological findings, and after literature review of all the products and ingredients ingested. A search of the United States National Library of Medicine Dietary Supplements Labels Database and United States National Library of Medicine Clinical and Research Information on Drug Induced Liver Injury for GNC Mega Men® Sport, Nopal® (Cactus), and Whey Protein returned no warnings[8,9]. A PubMed review was significant for a case of acute cholestatic liver injury following ingestion of Whey protein and Creatine supplements. Yet, there have been no other reports that demonstrate this relationship, and instead there have been studies that suggest the hepatoprotective effect of Whey protein in acute and chronic hepatitis[10]. Further investigation of the individual ingredients within the supplements taken, as listed on their respective supplement labels, was concerning for contribution of both Vitamin A and chromium (both contained in the GNC Mega Men® Sport) in development of the liver injury and failure. However, current evidence suggests that Vitamin A toxicity occurs with ingestion of greater than 40000 IU daily or about 12000 micrograms daily[11]. Our patient was taking 5000 IU three times per week, or only 15000 IU per week. In addition, there is no established upper limit of intake of chromium set forth by the Institute of Medicine as there have been few adverse side effects reported[12]. Although likely multi-factorial in nature, current evidence suggests that our patient's liver outcome is most likely secondary to the green tea extract-containing supplement.

Green tea is made from steaming of the tea plant, *Camellia sinensis*. Polyphenols, including catechins and flavanols make up 30%-40% of the extractable solid of dried green tea leaves. The main catechins consist of epicatechin, epicatechin-3-gallate, epigallocatechin, and EGCG. It is proposed that these compounds or extracts give green tea its anticarcinogenic, antioxidant, probiotic, and thermogenic properties[13].

Despite studies that show the benefits of green tea, there have been several recent reports that demonstrate hepatotoxicity following the consumption of concentrated green tea extract. Much interest in green tea hepatotoxicity came after the discontinuation of Exolise, a weight-loss product containing a hydroalcoholic extract of green tea, in France and Spain following the report of acute liver injury with the use of this product. The United States Pharmacopeia subsequently reviewed the safety information for green tea products. They found 34 reports of liver damage, ranging from acute hepatitis to fulminant liver failure requiring transplant, following the use of multiple green tea extract preparations[5]. As a result, the United States Pharmacopeia have suggested, but not mandated, a warning, stating symptoms of liver injury be placed on any green tea extract monograph produced[5]. The green tea product ingested by our patient was without such a warning of potential hepatotoxicity.

In reports of green tea extract-associated hepatotoxicity reviewed between 1999 and 2008, histological exam of the livers showed pathology characteristic of inflammatory infiltrates, cholestasis, steatosis, and necrosis[6]. The hepatotoxicity that follows may be attributed to

those same compounds within green tea extract that have previously been described as beneficial, and in particular to the catechins, of which EGCG is the most abundant and may be the most potent. The major cytotoxic mechanisms include destruction of mitochondrial membranes and the induction of reactive oxygen species formation[14]. Liver injury typically occurs within three months of ingestion[4].

Thus, although green tea has traditionally been considered safe, emerging reports linking liver injury, and in some cases liver failure, with the use of green tea extract should not be ignored. Several issues remain unresolved, including determination of the preparation types and amounts that can be considered safe versus harmful. This is a difficult task to achieve given the lack of FDA regulation of herbal products and other dietary supplements. There are many supplements that contain various formulations (hydroalcoholic *vs* aqueous *vs* powder, *etc.*) and concentrations of green tea extract in combination with other potentially harmful ingredients. Moreover, there is often inconsistent information regarding the complete list of ingredients contained within dietary supplements. Yet, investigations regarding safety and efficacy of these products are lacking. Resources including the United States National Library of Medicine and The Drug Induced Liver Injury Network account for these adverse events and have been established to help us better understand supplement-related hepatotoxicity[4,7]. Yet, until appropriate standards are established, it is imperative that physicians monitor the use of green tea extract, recognize that it may be contained in a variety of products, and be cognizant of its hepatotoxic potential.

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CASE REPORT

Total dysphagia after short course of systemic corticotherapy: Herpes simplex virus esophagitis

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Abstract

A 72 year-old female developed a herpetic esophagitis after 3 d of oral corticotherapy for an acute exacerbation of chronic obstructive pulmonary disease, presenting as odynophagia and total dysphagia. Biospies were taken during a first esophagogastroduodenoscopy (EGD) and the patient was referred to the thoracic surgery service with a presumptive diagnosis of esophageal cancer. A second EGD was planned for dilatation, but by that time the stenosis was completely resolved. The biopsies taken during the first EGD revealed multiple herpetic viral inclusions and ulcerations without any dysplasia or neoplasia. In front of a severe esophageal stenosis, one must still exclude the usual differential diagnosis peptic stenosis and cancer. Visualization of endoscopic lesions can suggest the diagnosis but must be promptly confirmed by biopsy, viral culture or polymerase chain reaction. Although immune systemic effects of corticotherapy are well known and herpetic esophagitis occurs most frequently in immunocompromised individuals, this case emphasizes the importance of clinical awareness concerning short courses of corticotherapy for immunocompetent individuals. This article discusses the reactivation process of herpetic infection in this context and addresses its diagnostic and therapeutic issues.

Key words: Herpes simplex; Esophagitis; Dysphagia; Corticosteroids; Immunocompetence

Core tip: This article reports the case of a 72 year-old female who developed a herpetic esophagitis after 3 d of oral corticotherapy for an acute exacerbation of chronic obstructive pulmonary disease, presenting as odynophagia and total dysphagia. Although immune systemic effects of corticotherapy are well known and herpetic esophagitis occurs most frequently in immunocompromised individuals, this case emphasizes the importance of clinical awareness concerning short courses of corticotherapy for immunocompetent individuals.

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INTRODUCTION

The most frequent causes of infectious esophagitis are candida, cytomegalovirus and herpes simplex virus type 1 (HSV-1). Although typically these esophagitis occurs in the immunocompromised patients, it has been seen also

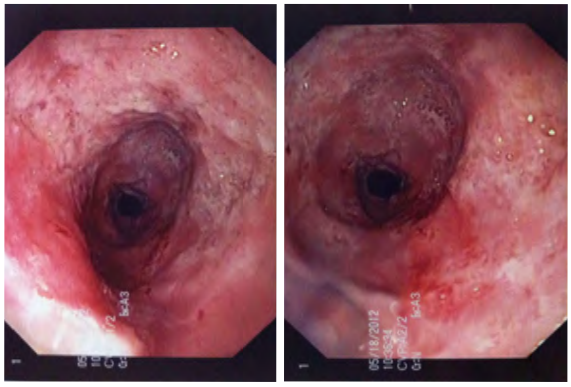

Figure 1 Stenosis of the esophagus, day 4.

in immunocompetent patients. We report a case of a 72 year-old female who developed a herpetic esophagitis presenting as odynophagia and total dysphagia after three days of oral corticotherapy for an acute exacerbation of chronic obstructive pulmonary disease (COPD).

CASE REPORT

A 72 year-old female was referred to the thoracic surgery department following the finding of a impassable stenosis of lower esophagus during an esophagogastroduodenoscopy (EGD). Her pertinent past medical history included repair of a hiatal hernia *via* right thoracotomy several years ago, gastroesophageal reflux and coronary artery disease (CAD) with a spastic component (Takutsubo). The patient also reported a history of caustic injury without subsequent dysphagia in her childhood. She does not recall having had oral or genital herpes, nor cutaneous lesions that would suggest a prior herpetic infection. Three months prior to this event, the patient had received a course of oral prednisone 15 mg daily for ten days, without any complication for her COPD.

The patient had been discharged nine days prior to this event for an acute exacerbation of COPD. During that hospitalization, she was discharged with a prescription of oral prednisone 30 mg daily with standard tapering doses. She decided to interrupt on her own the medication on the third day of this regimen since she presented sudden retrosternal pain, odynophagia and dysphagia to solids and liquids. She reported a slight tenderness at palpation in the epigastric region, but otherwise her complete physical examination was normal.

An EGD was performed by the gastroenterologist on day 4 of her present hospitalization and an impassable esophageal stenosis was discovered in the mid esophagus at 25 cm EGD (Figure 1). Biospies were taken and the patient was referred to the thoracic surgery service with a presumptive diagnosis of a mid esophageal cancer. We planned to repeat the EGD and proceed to a dilatation. But on day 8, the EGD was repeated by another gastroenterologist and by that time the patient reported a slight improvement of her dysphagic symptoms. Hyperhemia concordant with oesophagitis of the mid oesophagus was found, without any stenosis (Figure 2). At this moment, the patient still had some retrosternal pain but she reported improvement in swallowing liquids.

The biopsies taken during the first EGD revealed multiple herpetic viral inclusions and ulcerations without any dysplasia or neoplasia (Figure 3). A viral isolation on Vero cells and diploid human fibroblast cells demonstrated the presence of HSV-1. Acyclovir (5 mg/kg *iv* each 8 h for a total of 10 d) was started along with xylocaine 2% (15 mL *po qid prn*) for symptomatic relief. The patient rapidly improved and was discharged on day 6 with valacyclovir 1 g *po bid* for a total of 16 d.

DISCUSSION

Herpetic esophagitis occurs mostly in immunocompromised hosts such as organ transplant recipient (solid organ and bone marrow) or patients with human immunodeficiency virus (HIV) infection[1]. The main complaints in immunocompetent patients are fever and retrosternal chest pain[2]. In this case, there is occurrence of a complication not frequently described in the litterature: reversible esophageal stricture associated with herpes simplex esophagitis in immunocompetent host following short-term systemic corticotherapy.

Reflux disease and long-standing inflammation are well known causes of benign esophageal stricture. Malignancy must always be ruled out by endoscopy and biopsy. In the setting of herpetic oesophagitis, stenosis secondary to edema have been described in immunocompromised host with HIV[3]. Early endoscopic findings include vesicles, ulcerations and cobblestoning secondary to cluster lesions. The mucosa is friable and exudates are frequently present. Mucosal necrosis can also be seen in late stages[4].

Diagnosis is made by histopathology and virus isolation. The pathognomonic alterations include multinucleated cells with ground-glass, nuclear inclusions with chromatin margination. The cellular inclusions are surrounded by an inflammatory cell infiltrate. Pathologists use peroxidase-conjugated antibodies against HSV-1 and HSV-2. Virus isolation in tissue culture and nucleic acid amplification techniques are sensitive but may give false positive results from colonize oral mucosal surface.

Triggers known of HSV-1 recurrent labial infection are multiple, from immunosuppression to fatigue and stress[5]. In our case, the patient had an acute exacerbation of COPD and a subsequent short-term corticotherapy at low doses. Both of these events could have triggered HSV-1 reactivation, which will be discussed separately.

First, infections represent a major stress, especially in COPD disease. Viral infections have been identified as a causative factor of COPD exacerbation[6]. Although the role of bacterias during acute exacerbations is still under investigations, studies describe immunomodulatory effect of antibiotics as a mecanism leading to reduction of exacerbation rate and severity, addressing the bacterial chronic infection in COPD[7]. It is also important to mention abnormal Th1 responses among the various dis-

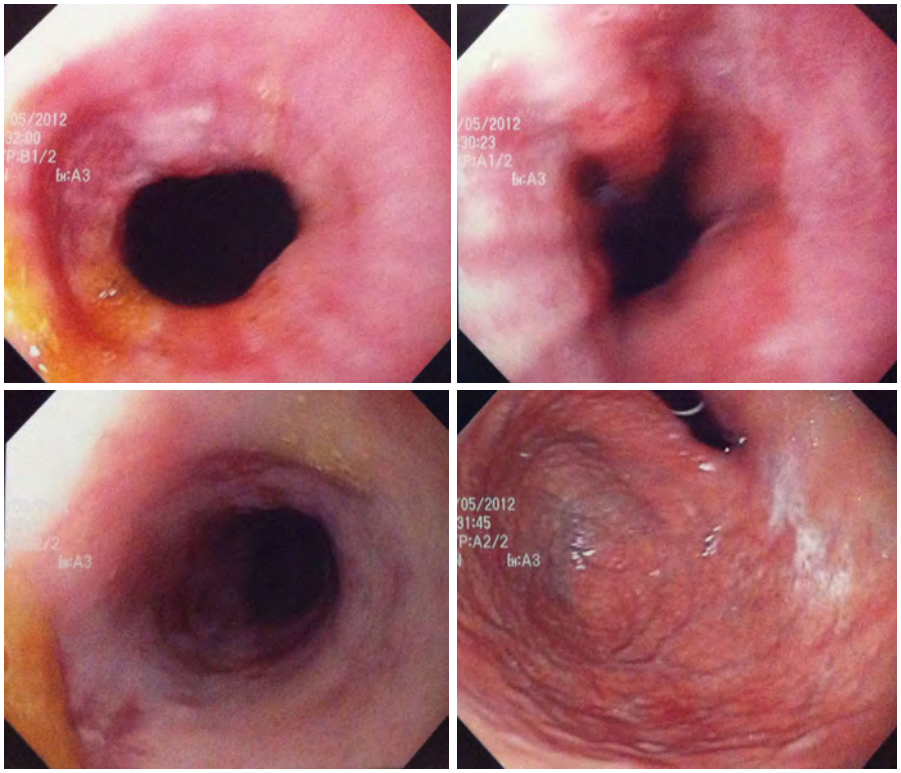

Figure 2 Hyperhemia consistent with esophagitis with complete resolution of the stenosis of the esophagus, day 8.

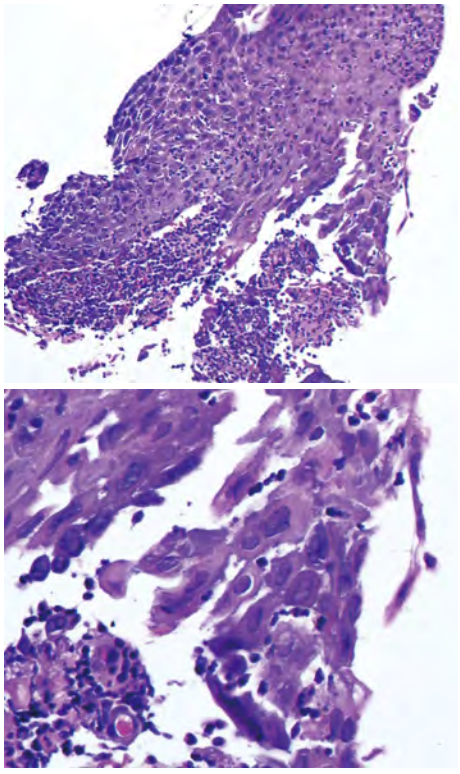

Figure 3 Ulcerated squamous epithelium with herpes virus inclusions easily visible on hematoxylin phloxine saffron coloration.

ruptions of immune system occuring in COPD[8]. Th-1, the main immune response to HSV infection, is then impaired. These evidences could partly explain HSV-1 reactivation in our case.

Second, a common side effect of corticotherapy is secondary infections. Topical corticosteroids have been a proven factor of bovine herpes virus reactivation in intranasal rabbit model[9]. No clinical evidence shows that systemic corticoids reactivate HSV, although topical application on active HSV infection can lead to expansion of lesions. However, glucocorticoids induced a polarization of Th2 over Th1 profile[10]. Th1, the main immune response to HSV infection, is then inhibited. It has not been proven however that reactivation of prior infection can be triggered by these mechanisms. Of interest, it is known that sudden cessation of glucorticoids can be a risk factor of HSV reactivation[11]. Two other case reports of HSV reactivation associated with corticotherapy have been published. One fatal HSV infection under corticotherapy in a patient with Darrier disease[12]. A case of fatal HSV hepatitis following eight days of prednisone 40 mg DIE given for ulcerative colitis has also been reported, in an otherwise immunocompetent patient[13]. In both cases, authors concluded that high clinical suspicion and prompt diagnosis are crucial.

Furthermore, it is interesting to remember that our patient also had a previous COPD exacerbation treated by corticotherapy three months before. HSV-1 esophagitis could have been triggered by the additional burden of the last exacerbation and corticotherapy.

Herpetic esophagitis is usually self-limited in immunocompetent patients, but Acyclovir therapy has been used successfully in many cases. Most authors suggest hasten symptomatic relief and subsequent shortening of clinical course with medical treatment[1,2,4,13-15]. For immunocompetent patients, suggested dosage is 5 mg/kg *iv*

every 8 h for 7-14 d that can be then completed orally as the patient swallowing returns. Oral viscous lidocaine solution (15 mL of 2% solution) can also be administered to lessen the odynophagia.

To conclude, clinicians must be aware that reactivation of herpetic infection can occur following a course of low dose corticotherapy and can cause herpetic esophagitis also in immunocompetent patient. In front of a severe esophageal stenosis, one must still exclude the usual differential diagnosis peptic stenosis and cancer. Visualization of endoscopic lesions can suggest the diagnosis but must be promptly confirmed by biopsy, viral culture or polymerase chain reaction. Acyclovir is considered to be the treatment of choice, with oral xylocaine for pain relief.

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CASE REPORT

Pancreatic duct drainage using EUS-guided rendezvous technique for stenotic pancreaticojejunostomy

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Abstract

The patient was a 30-year-old female who had undergone excision of the extrahepatic bile duct and Roux-en-Y hepaticojejunostomy for congenital biliary dilatation at the age of 7. Thereafter, she suffered from recurrent acute pancreatitis due to pancreaticobiliary maljunction and received subtotal stomach-preserving pancreaticoduodenectomy. She developed a pancreatic fistula and an intra-abdominal abscess after the operation. These complications were improved by percutaneous abscess drainage and antibiotic therapy. However, upper abdominal discomfort and the elevation of serum pancreatic enzymes persisted due to stenosis from the pancreaticojejunostomy. Because we could not accomplish dilation of the stenosis by endoscopic retrograde cholangiopancreatography, we tried an endoscopic ultrasonography (EUS) guided rendezvous technique for pancreatic duct drainage. After transgastric puncture of the pancreatic duct using an EUS-fine needle aspiration needle, the guidewire was inserted into the pancreatic duct and finally reached to the jejunum through the stenotic anastomosis. We changed the echoendoscope to an oblique-viewing endoscope, then grasped the guidewire and withdrew it through the scope. The stenosis of the pancreaticojejunostomy was dilated up to 4 mm, and a pancreatic stent was put in place. Though the pancreatic stent was removed after three months, the patient remained symptom-free. Pancreatic duct drainage using an EUS-guided rendezvous technique was useful for the treatment of a stenotic pancreaticojejunostomy after pancreaticoduodenectomy.

Key words: Balloon dilatation; Endoscopic ultrasound-guided fine needle aspiration; Pancreaticobiliary maljunction; Pancreaticoduodenectomy; Pancreatitis; Postoperative complication

Core tip: The usefulness of pancreatic duct drainage using endoscopic ultrasonography-guided rendezvous technique for stenotic pancreaticojejunostomy after pancreaticoduodenectomy. However, this procedure requires technically skill and the success rate is low. The main reason for failure is the inability to pass through the stenotic anastomosis due to its tightness. In our case, the stenosis was not so tight because the stenosis developed about a month after the operation. A

case of stenotic pancreaticojejunostomy that occurs at any early stage after pancreaticoduodenectomy with a dilated pancreatic duct is possibly a good indication.

Takikawa T, Kanno A, Masamune A, Hamada S, Nakano E, Miura S, Ariga H, Unno J, Kume K, Kikuta K, Hirota M, Yoshida H, Katayose Y, Unno M, Shimosegawa T. Pancreatic duct drainage using EUS-guided rendezvous technique for stenotic pancreaticojejunostomy. *World J Gastroenterol* 2013; 19(31): 5182-5186 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i31/5182.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i31.5182

INTRODUCTION

The surgical mortality rate after pancreaticoduodenectomy (PD) has decreased due to advances in surgical technique, but surgical morbidity has not yet decreased[1]. Stenotic pancreaticoenteric anastomosis is one of the complications after PD[2]. Endoscopic retrograde cholangiopancreatography (ERCP) is performed to treat this complication, but the success rate has been low[3]. Surgeons prefer to avoid re-operation for pancreaticoenteric stenosis due to the operative risks. Recently, interventional endoscopic ultrasonography (EUS) has greatly advanced in terms of the available devices and techniques[4-11]. Pancreaticobiliary cases treated by interventional EUS have been reported, but the usefulness of interventional EUS-fine needle aspiration (FNA) for the treatment of post-operative complications is not yet known. We here report a case with stenotic pancreaticojejunostomy that was efficiently treated by an EUS-guided rendezvous technique.

CASE REPORT

The patient was a 30-year-old female who had undergone excision of the extrahepatic bile duct and Roux-en-Y hepaticojejunostomy for congenital biliary dilatation at the age of 7 (Figure 1A). She was referred to our hospital for upper abdominal and dorsal pain. Pancreatic enzymes and inflammatory reactions (serum amylase: 210 IU/L, serum lipase: 390 IU/L, C-reactive protein: 2.4 mg/dL) were elevated, and abdominal computed tomography (CT) revealed peripancreatic fluid collection and pancreatic swelling. This patient was diagnosed as acute pancreatitis based on these findings and admitted to our hospital in 2007. Magnetic resonance imaging (MRI) and ERCP showed protein plugs in the main pancreatic duct (MPD) and pancreaticobiliary maljunction with a dilated common channel and dilated residual intra-pancreatic bile duct. The pancreaticobiliary maljunction was suspected to be the cause of the protein plugs in the MPD. We performed endoscopic pancreatic sphincterotomy and removed the protein plugs, but she suffered from acute pancreatitis due to recurrent protein plugs until

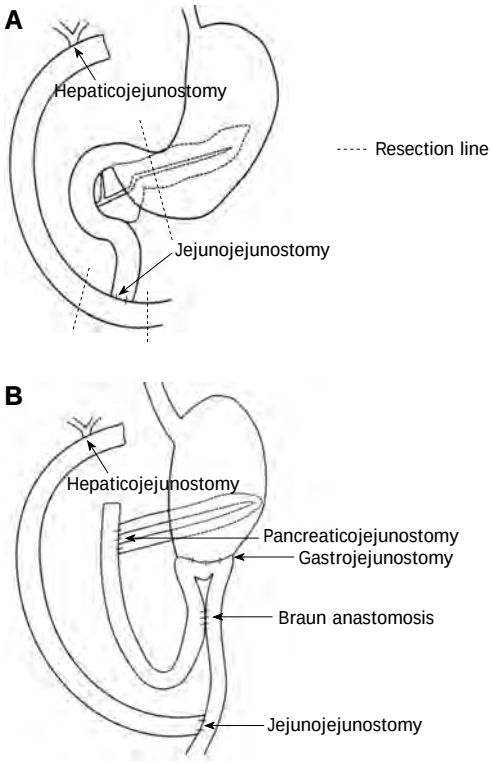

Figure 1 Re-construction after subtotal stomach-preserving pancreaticoduodenectomy. A: Before the operation (after excision of extrahepatic bile duct and Roux-en-Y hepaticojejunostomy; B: After the operation, pancreaticojejunostomy and gastrojejunostomy were performed. Roux-en-Y hepaticojejunostomy was re-established.

2011. PD was thought to be an adequate operation for preventing recurrent acute pancreatitis by protein plugs. After obtaining informed consent, the patient received subtotal stomach-preserving PD. Regarding reconstruction, pancreaticojejunostomy and gastrojejunostomy were performed, and Roux-en-Y hepaticojejunostomy was re-established (Figure 1B).

On the 16th post-operative day, the patient developed a high fever after accidental removal of an external drainage tube placed in the pancreatic duct. Abdominal CT revealed fluid collection and an intra-abdominal abscess near the anastomotic site of the pancreaticojejunostomy. After percutaneous drainage of the abscess and antibiotic therapy, her condition improved. However, upper abdominal discomfort and the elevation of serum pancreatic enzymes persisted. CT and EUS revealed a pancreatic duct dilatation (6 mm) (Figure 2). These symptoms were due to the stenosis of the pancreaticojejunostomy, and we tried ERCP using an oblique-viewing endoscope (GIF-XK240; Olympus, Tokyo, Japan). The anastomotic site of the pancreaticojejunostomy was identified, but we could not perform either pancreatography or a guidewire insertion into the pancreatic duct. We then tried EUS-guided rendezvous technique for drainage of the pancreatic duct on the 53rd post-operative day. We used a convex array echoendoscope (GF-UCT240-AL5; Olympus, Japan) and identified the echo image of the dilated pancreatic duct from the stomach. A vascular structure was confirmed by color

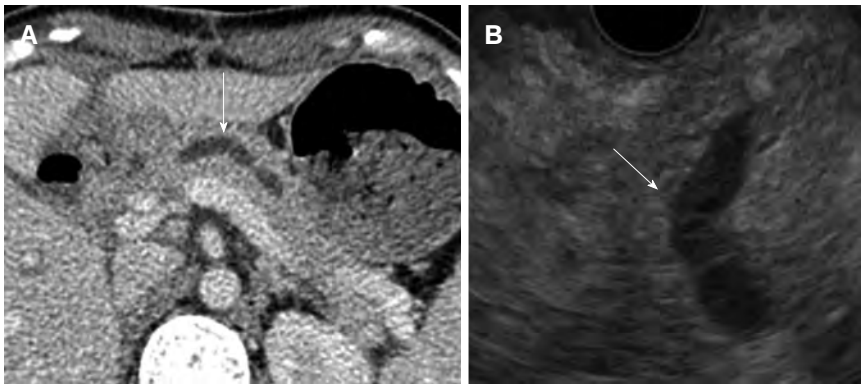

Figure 2 Computed tomography (A) and and endoscopic ultrasonography (B) revealed a dilated pancreatic duct (white arrows).

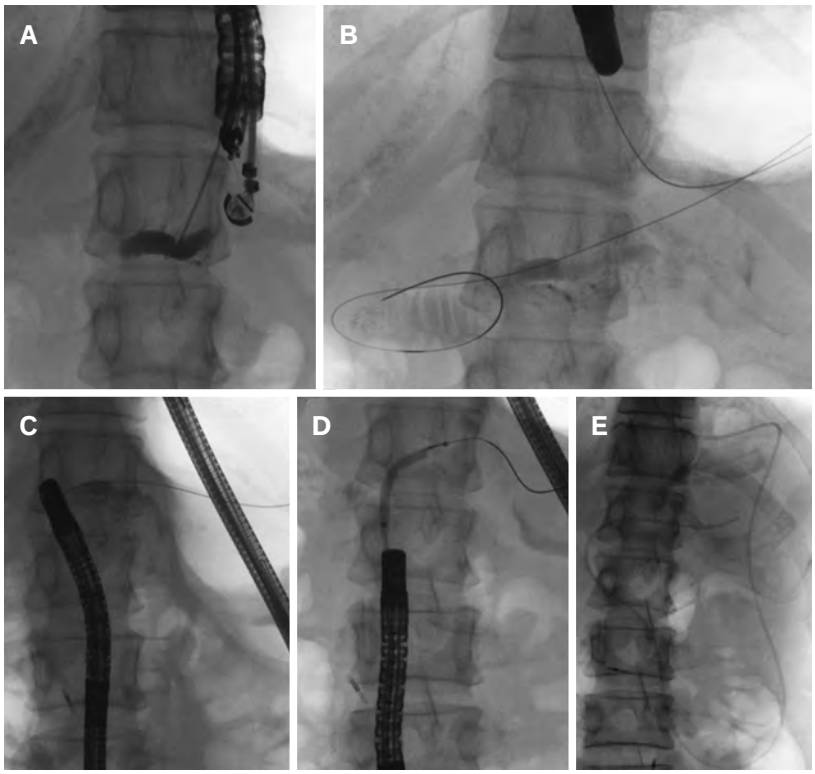

Figure 3 Pancreatic duct drainage procedures using endoscopic ultrasonograph-guided rendezvous technique. A: Pancreatic duct puncture and pancreatography using endoscopic ultrasonography; B: Introducing the guidewire into the jejunum through the pancreatic duct and the stenotic anastomosis; C: After exchanging echoendoscope for oblique-viewing endoscope, the guidewire was withdrawn into the working channel; D: Balloon dilatation of the stenotic anastomosis; E: Placement of an endoscopic naso-pancreatic drainage tube.

Doppler imaging and successfully avoided. The MPD was punctured using a 19-gauge needle (Echo Tip; Cook, Wilston-Salem, NC, United States). Pancreatography was obtained by the injection of contrast medium (Figure 3A), and a 0.025-inch guidewire (VisiGlide; Olympus, Japan) was inserted into the MPD and finally reached to the jejunum through the stenotic anastomosis (Figure 3B). The echoendoscope was removed, leaving the guidewire. After introducing an oblique-viewing endoscope (GIF-XK240; Olympus, Japan) up to the pancreaticojejunostomy, we grasped the guidewire by a snare and withdrew it through the working channel (Figure 3C). The stenosis of the pancreaticojejunostomy was dilated up to 4 mm by a wire-guided balloon catheter (MaxForce; Boston Scientific, Natric, MA, United States) (Figure 3D), and replaced with a 5-Fr endoscopic nasopancreatic drainage (ENPD) tube (GADELIUS, Tokyo, Japan) (Figure 3E). Eight days later, we replaced the ENPD tube with a 7-Fr pancreatic stent (GADELIUS). Her symptom was improved and the serum amylase and lipase values returned to the normal range. When we performed ERCP after 3 mo, we removed the pancreatic stent and confirmed good pancreatic juice drainage. At 1 year after the last endoscopic treatment, she was symptom-free and the blood chemistry test results were normal.

DISCUSSION

Pancreatoenteric anastomotic site stenosis can be a problematic complication after PD. Reid-Lombardo et al. reported that stenotic pancreaticojejunostomy requiring intervention was observed in 4.6% of PD patients[2]. The Patients with stenotic pancreaticojejunostomy after PD tended to be treated with ERCP-related procedures. However, the success rates were not often high due to the inability to reach or to identify the pancreaticojejunostomy through the afferent loop[3]. A long afferent loop and postoperative adhesions might hamper endoscopic

Table 1 Reported cases of pancreatic duct drainage using endoscopic ultrasonography-guided rendezvous technique for stenosis of after pancreticojejunostomy

Ref.	*n*	Age (yr)/sex	Indication	Pancreatic duct	Success	Reasons for failure	Postoperative period (yr)
Kikuyama *et al*[4]	19	72/M	ARP	N/A			N/A
	20	66/M	ARP				
	21	51/M	ARP				
Mallery *et al*[7]	1	35/F	ARP	Non dilated	Yes		4
	2	55/M	Chronic pancreaticocutaneous fistula	Non dilated	No	Failed guidewire passage	1
Kinney *et al*[8]	3		ARP		No		
	4		ARP		No		
	5		ARP, pancreatic stone		Yes	Failed pancreatic	
	6		ARP, pancreatic stone		Yes	duct puncture	
	7	N/A	ARP, chronic pain	N/A	Yes	(in two patients)	N/A
	8		ARP, chronic pain		Yes		
	9		ARP, chronic pain		No	Failed guidewire passage	
	10		Chronic pancreaticocutaneous fistula		No	(in three patients)	
	11		Inwardly migrated surgical stent with recurrent pancreatitis		No		
Barkay *et al*[9]	12	36/F	IPMN	Dilated	No	Failed guidewire passage	
	13	60/M	ARP	Dilated	Yes		
	14	22/F	CP, pancreatic divisum	Dilated	No	Failed guidewire passage	
	15	54/F	CP	Non dilated	No	Failed guidewire passage	N/A
	16	53/M	IPMN	Dilated	No	Failed guidewire passage	
	17	67/M	IPMN	Dilated	No	Failed guidewire passage	
	18	59/F	CP	Dilated	Yes		
DeWitt *et al*[10]	22	25/F	Chronic pain	Dilated	No	Failed guidewire passage	N/A
Itoi *et al*[11]	23	66/M	ARP	Dilated	Yes		12
	24	51/M	ARP	Dilated	Yes		8

F: Female; M: Male; ARP: Acute recurrent pancreatitis; N/A: Not available; IPMN: Intraductal papillary mucinous neoplasm; CP: Chronic pancreatitis.

treatment. Whether lateral-viewing, forward-viewing or oblique-viewing endoscope was chosen, it has been unclear which type is the appropriate choice. Kikuyama *et al*[4] suggested that oblique-viewing endoscope was a good option for ERCP in operated patients, since it was useful for deep cannulation and therapeutic procedures due to the instrument elevator and good angle of view for advancing into the afferent loop. If a conventional endoscope was unable to reach the pancreaticojejunostomy, single or double balloon enteroscopes should be used as an alternative[5]. However, these endoscopes were not appropriate for interventional endoscopic treatment due to the small forceps' channel. In our case, we performed ERCP using an oblique-viewing endoscope and we could reach and identify the pancreaticojejunostomy because the afferent loop was not long (Figure 1B). However, pancreatography and guidewire insertion into the pancreatic duct were not possible.

Recently, interventional EUS has greatly advanced in terms of available devices and techniques. Bataille *et al*[6] first reported pancreatic duct drainage with EUS-guided rendezvous technique in 2002. Thereafter, several reports have described this procedure in post-PD patients (Table 1)[4,7-11]. This procedure is technically challenging and has an approximately 50% success rate. The reasons for failure include the impossibility of puncturing the pancreatic duct without dilatation, and the inability to pass through the stenotic anastomosis due to its tightness and less than ideal orientation of the puncture. Although the early course of this patient was favorable, it is important to follow up this patient carefully due to the risk of restenosis of pancreaticojejunostomy.

Kikuyama *et al*[4] have described difficulty in passing the stenotic anastomosis due to a tight stenosis, since stenosis of the pancreaticoenteric anastomosis usually happens as a late complication[2]. In our case, we expected that the stenosis was not tight since the stenosis developed about a month after PD due to a pancreatic fistula and an intra-abdominal abscess. We supposed that the length of time after the operation and the severity of other complications such as a pancreatic fistula might affect the outcomes, in terms of fibrosis, edema and compression occupying the space around the anastomotic site.

The diameter of the pancreatic duct is an important factor in avoiding complications as well as success. We could achieve successful pancreatic duct drainage in this case since the MPD was dilated enough to puncture (6 mm). Fatal complications have never been reported with this procedure, while a few complications such as abscess, mild pancreatitis or transient fever were reported, and these complications mostly happened to patients with pancreatic ducts of normal diameter[7,9]. Accordingly, for the EUS-guided rendezvous techniques we should select patients who satisfy these conditions.

EUS-guided pancreaticogastrostomy was an option for the treatment of this case. EUS-guided pancreaticogastrostomy has the risk of stent dysfunctions such as obstruction and migration[12], whereas dilatation of the stenotic anastomosis by a balloon catheter has a small risk of restenosis[4]. We therefore selected balloon dilatation for stenotic pan-

creaticojejunostomy using the rendezvous technique. Our case suggests that stenotic pancreaticojejunostomy ocurring at any early stage after PD with a dilated pancreatic duct might be a good indication for this technique.

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CASE REPORT

Complete response to multidisciplinary therapy in a patient with primary gastric choriocarcinoma

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Abstract

Primary gastric choriocarcinoma is a rapidly growing neoplasm with an average survival of several months in untreated patients. Gastrectomy with lymph node dissection followed by chemotherapy is the treatment of choice. Regimens used for gastric adenocarcinoma are usually selected. However, median survival remains less than six months. In this case report, we describe a case of primary gastric choriocarcinoma with a clinical complete response to multidisciplinary treatment including surgery, chemotherapy, and radiofrequency ablation (RFA). The patient was originally referred for general malaise. Esophagogastroduodenoscopy demonstrated a large tumor occupying the fornix, and total gastrectomy with lymph node dissection was performed. Seven days later, multiple liver metastatic recurrences with high serum levels of beta-human chorionic gonadotropin (β-hCG) were recognized. Chemotherapy with a gonadal choriocarcinoma regimen consisting of etoposide, methotrexate, actinomycin D, cyclophosphamide, and vincristine (EMA/CO), was initiated. After three cycles, serum β-hCG decreased markedly and the tumors disappeared. Six months later, multiple lung metastatic recurrences were found. After one cycle of EMA/CO, only one nodule remained. Computed tomography-guided RFA was performed for this oligometastatic tumor. The patient has been alive with no evidence of disease for 10 years after the initial diagnosis. To the best of our knowledge, this patient with recurrent primary gastric choriocarcinoma has achieved the longest survival. The present case is the first report of choriocarcinoma metastatic to the lung successfully treated with RFA. From our retrospective analysis of recurrent or unresectable primary gastric choriocarcinoma, we propose that gonadal choriocarcinoma regimens can be considered as first-line for primary gastric choriocarcinoma.

Key words: Primary gastric choriocarcinoma; Beta-human chorionic gonadotropin; Etoposide, methotrexate, actinomycin D, cyclophosphamide, and vincristine; Oligometastatic; Radiofrequency ablation

Core tip: We described a case of primary gastric choriocarcinoma with a complete response to multidisciplinary treatment including surgery, chemotherapy, and radiofrequency ablation (RFA). The patient has been alive with no evidence of disease for 10 years. To the best of our knowledge, this patient with recurrent primary gastric choriocarcinoma has achieved the longest survival. The present case is the first report of choriocarcinoma metastatic to the lung successfully treated with RFA. From our retrospective analysis of recurrent or unresectable primary gastric choriocarcinoma, we propose that gonadal choriocarcinoma regimens can be considered as first-line for primary gastric choriocarcinoma.

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INTRODUCTION

Choriocarcinoma typically occurs in females at the origin of the chorionic epithelium of the placenta, and is commonly related to gestation. The tumor is rapidly growing, widely metastasizing, and highly invasive of surrounding tissues. Gonadal choriocarcinomas are usually highly sensitive to various types of anti-cancer agents[1].

Primary gastric choriocarcinoma is a type of non-gonadal choriocarcinoma that constitutes less than 1% of all gastric cancers[2]. It was first described by Davidson in 1905, and there are currently approximately 140 reported cases worldwide[3]. Most patients with primary gastric choriocarcinoma do not survive for even one year after surgery[4]. Chemotherapy regimens used successfully for gonadal choriocarcinoma are not as effective for primary gastric choriocarcinoma[5]. The prognosis is considerably worse than gastric adenocarcinoma[4]. Primary gastric choriocarcinoma with liver metastases has the worst prognosis[6].

We report a case of primary gastric choriocarcinoma successfully controlled by multidisciplinary therapy including surgery, chemotherapy, and radiofrequency ablation (RFA). The patient survived for approximately 10 years after initial diagnosis. To the best of our knowledge, the present case has the longest survival of recurrent primary gastric choriocarcinoma in the world.

CASE REPORT

A 65-year-old woman was referred to our clinic for general malaise and dizziness. She had no significant past medical history, and had never been hospitalized. On physical examination, the patient had pale skin due to anemia. Initial laboratory results were normal except for hemoglobin of 7.4 g/dL. Serum carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 19 concentrations were within normal limits. Esophagogastroduodenoscopy demonstrated a large tumor from the fornix to the posterior wall of the upper body of the stomach, with a mixture of protruding and ulcerative lesions, as well as areas of hemorrhage. Biopsied specimens were interpreted as tubular adenocarcinoma with moderate differentiation. Abdominal computed tomography (CT) demonstrated wall thickening at the fornix with disappearance of adipose tissue at the gastrosplenic ligament, suggestive of penetration of the gastric serosa. There was no obvious evidence of metastasis to the lymph nodes or liver, or of peritoneal dissemination. Total gastrectomy with D2 lymphadenectomy was planned.

During laparotomy, the tumor showed invasion to the body of the pancreas, and metastasis to several adjacent lymph nodes was suspected. A 1 cm × 1 cm nodule was detected in the liver. Total gastrectomy with D3 lymph node dissection, distal pancreatectomy, splenectomy, and enucleation of the liver was performed, along with Roux-en-Y reconstruction.

Pathological findings

An elevated 10 cm × 8 cm tumor with surface ulceration and hemorrhage was located at the fornix (Figure 1A). On the cut surface of the specimen, there was a very large lobulated tumor, white to gray in color, with large areas of hemorrhage and necrosis (Figure 1B).

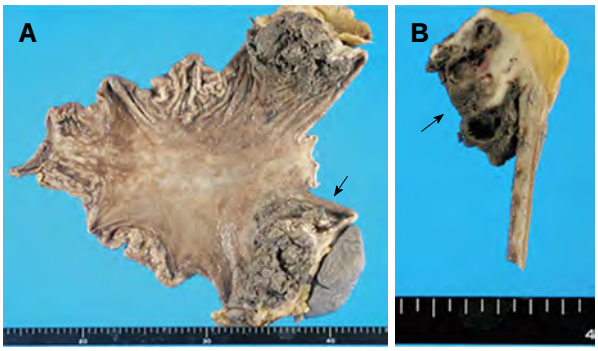

Figure 1 Gross appearance of the resected specimen. A: An elevated tumor with surface ulceration and hemorrhage was located in the fornix (arrow); B: On the cut surface of the specimen, there was a very large lobulated tumor, white to gray in color, with large areas of hemorrhage and necrosis (arrow).

Microscopically, the tumor had two components (Figure 2A). The first component, with histological features suggestive of choriocarcinoma, consisted of unusually-shaped multinucleated giant cells, similar to syncytiotrophoblasts in a characteristic dimorphic plexiform pattern, associated with hemorrhage and necrosis (Figure 2B). The second component consisted of atypical mononucleated cells similar to intermediate trophoblasts in a solid and sheet growth pattern (Figure 2C). Immunohistochemically, the tumor cells in the first component were diffusely positive for β-human chorionic gonadotropin (hCG) (Figure 2D). Immunoreactivity was also seen for human placental lactogen (Figure 2E). The tumor cells in the second component were diffusely positive for β-hCG and placental alkaline phosphatase (Figure 2F and G). Immunoreactivity was also seen for CEA (Figure 2H). These findings are identical to the World Health Organization (WHO) classification of primary gastric choriocarcinoma based on clinicopathological criteria[7]. Histologically, it showed an INF β growth pattern with invasion to the subserosa. Metastasis was detected in the lymph nodes along the short gastric vessels (no. 4SA) and at the right splenic hilum (no. 10). The liver nodule was also identified as a metastasis. The proximal and distal resection margins were clear. Peritoneal cytology was negative. The final classification was T3N1M1, stage Ⅳ, according to the Union for International Cancer Control guidelines.

Postoperative course and follow-up

The postoperative course was unremarkable. Serum β-hCG measured immediately after surgery was 12000 mIU/mL (normal range < 0.7 mIU/mL) (Figure 3). Seven days after surgery, multiple low-density lesions were detected in the liver on abdominal CT (Figure 4A and B). Recurrence was suspected and so systemic chemotherapy consisting of etoposide, methotrexate, actinomycin D, cyclophosphamide, and vincristine (EMA/CO) was initiated. After one cycle, the serum β-hCG concentration

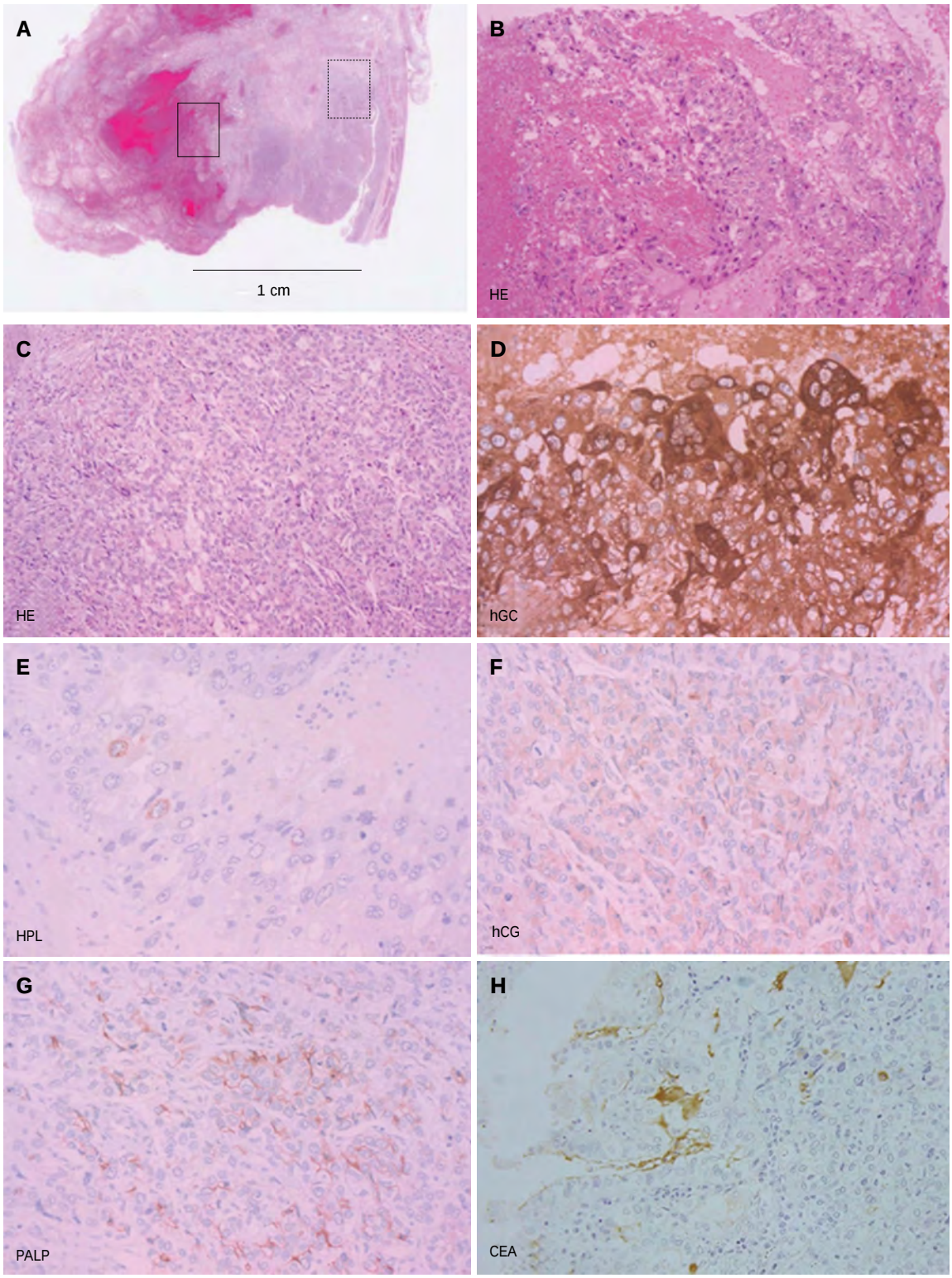

Figure 2 Pathological findings (hematoxylin/eosin staining and immunohistochemical staining). A: The tumor had two components, as indicated by small boxes with solid and dashed lines. Hematoxylin/eosin (HE) × 40; B: In the area marked by a solid line in panel A, unusual multinucleated giant cells in a characteristic dimorphic plexiform pattern associated with hemorrhage and necrosis were observed. HE × 100; C: Atypical mononucleated cells demonstrated a solid and sheet growth pattern in the area marked by a dashed line in panel A. HE × 100; D, E: Tumor cells were diffusely positive for β-human chorionic gonadotropin (hCG) and focally positive for human placental lactogen (HPL) in the area marked by a solid line in panel A. HE × 200; F, G: The tumor cells were positive for beta-human chorionic gonadotropin and placental alkaline phosphatase (PALP) in the area marked by a dashed line in panel A. HE × 200; H: Immunoreactivity was focally positive for carcinoembryonic antigen (CEA). HE × 100.

started to decrease and there was reduction in the size of the tumors on CT. After three cycles, the serum β-hCG level was almost within normal limits and the tumors disappeared with a clinical complete response and no major

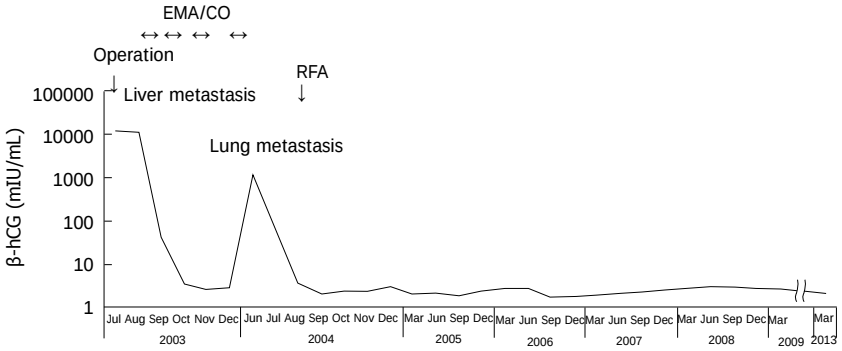

Figure 3 Tumor markers and chemotherapy. Seven days after surgery, metastatic recurrence in the liver was diagnosed. After starting etoposide, methotrexate, actinomycin D, cyclophosphamide, and vincristine (EMA/CO), serum beta-human chorionic gonadotropin (β-hCG) concentrations decreased. After three cycles, serum β-hCG levels decreased markedly to almost within normal limits and clinically the tumors showed a complete response. Six months later, there was a sudden elevation in serum β-hCG levels with the emergence of multiple nodules in both lung fields. Metastatic recurrence in the lung was diagnosed and EMA/CO was restarted. After one cycle, most tumors, except for one nodule in the left lower lobe, disappeared concomitantly with declines in serum β-hCG levels. Computed tomography-guided radiofrequency ablation (RFA) was performed for the oligometastatic tumor. The patient has been alive with no evidence of disease for nine years after RFA.

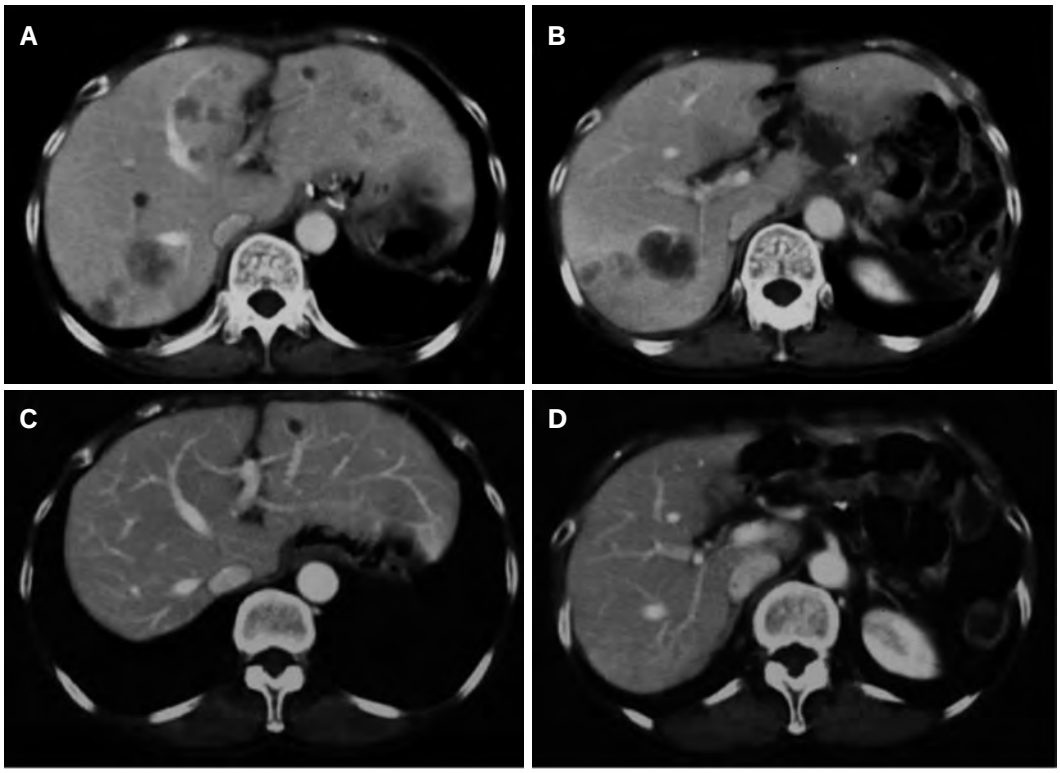

Figure 4 Computed tomography images before and after chemotherapy. A, B: Seven days after surgery, multiple low-density lesions were detected in the liver on abdominal computed tomography; C, D: After three cycles of etoposide, methotrexate, actinomycin D, cyclophosphamide, and vincristine, the tumors disappeared with a clinical complete response.

side effects (Figure 4C and D). Six months after surgery, there were a sudden elevation in the serum β-hCG level (1100 mIU/mL) and the emergence of multiple nodules in both lung fields on CT. Lung metastasis was diagnosed and EMA/CO was restarted. After one cycle, most tumors, except one nodule in the left lower lobe, disappeared along with decreases of serum β-hCG. CT-guided RFA was performed for oligo-recurrence. The patient remains alive with no evidence of disease for nine years after RFA treatment.

Pooled analysis of reported cases of recurrent or unresectable primary gastric choriocarcinoma treated with chemotherapy

We retrospectively collected all reported cases of recurrent or unresectable (including initially unresectable tumors treated with neoadjuvant therapy) primary gastric choriocarcinoma treated with chemotherapy with a clear postoperative prognosis in the English and Japanese literature after 1990 (Table 1)[5,8-31]. Measurement of the overall survival (OS) period began at the time of initial

Table 1 Review of the English and Japanese literature for cases of recurrent or unresectable primary gastric choriocarcinoma treated with chemotherapy after 1990

Cases	Authors	Age (yr)/sex	Type	Site of metastasis	Chemotherapy regimen	Response	Prognosis (mo)
1	Present case	65/F	Recurrent	Liver	EMA/CO	CR	115 NED
2	Waseda *et al*[5]	68/M	Unresectable	Liver	EP	CR	24 NED
3	Shastri *et al*[8]	44/M	Unresectable	Liver	BEP	Size reduction	12 DOD
4	Shimuzu *et al*[9]	43/F	Unresectable	Distant lymph nodes	TS-1/CDDP	Size reduction	7 DOD
5	Yoon *et al*[10]	62/M	Unresectable	Liver	5-FU/USAN/L-OHP	Progression	16 DOD
					PTX/CDDP	Progression	
					5-FU/USAN/CPT-11	Progression	
					BEP	Progression	
					EMA/CO	Progression	
					VIP	Progression	
6	Yoon *et al*[10]	45/M	Unresectable	Liver	BEP	Progression	12 DOD
					VIP	Progression	
					EMA/CO	Progression	
					5-FU/USAN/CPT-11	Progression	
7	Kanemura *et al*[11]	79/M	Recurrent	No data	TS-1/PTX	Progression	5 DOD
8	Yasumoto *et al*[12]	76/F	Unresectable	Liver	5-FU	Progression	1 DOD
9	Mori *et al*[13]	36/F	Recurrent	Brain and lung	EMA/CO	CR	54 NED
10	Enokido *et al*[14]	54/M	Unresectable	Liver	TS-1	Progression	3 DOD
11	Adachi *et al*[15]	78/M	Unresectable	Liver	TS-1	CR	12 NED
12	Kishimoto *et al*[16]	69/M	Recurrent	Liver	Epi-ADM/MMC (TACE)	Progression	17 DOD
					5-FU (HAI)	Progression	
					UFT	Progression	
13	Kawaguchi *et al*[17]	60 M	Recurrent	Liver	MTX/BLM/CDDP/CPA	Progression	5 DOD
14	Liu *et al*[18]	36/F	Unresectable	Colon (infiltration)	BEP	Size reduction	6 DOD
					VBL/IFM/CDDP	Progression	
15	Inaki *et al*[19]	56/M	Recurrent	Liver	MAC (second-line after UFT as adjuvant)	Progression	3 DOD
16	Bayhan *et al*[20]	26/F	Recurrent	Lung	MAC	Size reduction	18 NED
17	Satoh *et al*[21]	58/M	Recurrent	Paraaortic lymph nodes	VP-16/CDDP	Progression	6 DOD
18	Kinoshita *et al*[22]	74/M	Unresectable	Liver	MTX	Progression	3 AWD
19	Imai *et al*[23]	63/F	Recurrent	Liver	MA	Progression	3 DOD
20	Fujimoto *et al*[24]	57/M	Recurrent	Liver	MAC	Progression	6 DOD
21	Ogura *et al*[25]	45/F	Recurrent	Liver	5-FU/MMC/Epi-ADM (HAI)	Size reduction	10 DOD
					VP-16/CDDP	Progression	
22	Kan *et al*[26]	67/M	Unresectable	Liver	5'DFUR/CDDP	Size reduction	10 DOD
					5'DFUR/CDDP/VP-16	Progression	
					MTX	Progression	
23	Saito *et al*[27]	57/M	Recurrent	CEA elevation	5-FU/CDDP (second-line after Tegafur as adjuvant)	SD	12 AWD
24	Imatake *et al*[28]	50/M	Unresectable	Liver	MMC/5-FU/lentinan	Progression	2 DOD
25	Okada *et al*[29]	57/F	Recurrent	hCG elevation	VAC	Progression	7 DOD
26	Kobayashi *et al*[30]	60/M	Unresectable	Liver	MTX/ADM (HAI)	Progression	5 DOD
27	Masuda *et al*[31]	79/M	Unresectable	Liver	UFT	Progression	1.5 DOD

M: Male; F: Female; USAN: Leucovorin; CDDP: Cisplatin; 5-FU: Fluorouracil; L-OHP: Oxaliplatin; CPT-11: Irinotecan; PTX: Paclitaxel; BLM: Bleomycin; CPA: Cyclophosphamide; VBL: Vinblastine; Epi-ADM: Epirubicin; IFM: Ifosfamide; VP-16: Etoposide; 5'DFUR: Doxifluridine; ACT-D: Actinomycin D; ADM: Adriamycin; VCR: Oncovin (vincristine); MMC; Mitomycin C; UFT: Tegafur-uracil; CEA: Carcinoembryonic antigen; β-hCG: β-human chorionic gonadotropin; NED: No evidence of disease; DOD: Died of disease; AWD: Alive with disease; CR: Complete response; SD: Stable disease; TACE: Transcatheter arterial chemoembolization; HAI: Hepatic arterial infusion; EMA/CO: Etoposide, methotrexate, actinomycin D, cyclophosphamide, and vincristine.

diagnosis. Death due to primary gastric choriocarcinoma was the only endpoint considered for the purpose of this study. OS curves were obtained using the Kaplan-Meier method, and differences were compared using the log-rank test. P values < 0.05 were considered significant.

Our search revealed 12 previous cases treated using gonadal choriocarcinoma regimens. 11 patients received first-line chemotherapy, of whom two had a complete response with etoposide and cisplatin (EP)[5] and EMA/CO[13], respectively (Table 1). The median survival of the patients treated with gonadal choriocarcinoma regimens used as the first-line was 9.5 mo compared to 5.0 mo in patients treated with gastric adenocarcinoma regimens. Although the difference was not significant, treatment results showed a favorable prognosis with the gonadal choriocarcinoma regimen ($P = 0.1$) (Figure 5).

DISCUSSION

We report a rare case of primary gastric choriocarcinoma that showed a clinical complete response to multidisciplinary treatment, including surgery, chemotherapy, and RFA. The patient obtained nine years of disease-free survival. The present case represents the first report of cho-

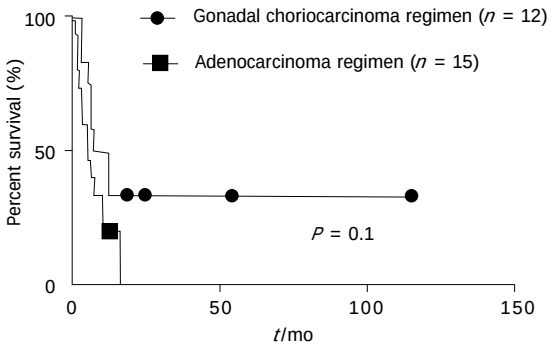

Figure 5 Overall survival with gonadal choriocarcinoma regimen and adenocarcinoma regimen. The median survival of patients treated with gonadal choriocarcinoma regimens used as the first-line was 9.5 mo compared to 5.0 mo in patients treated with gastric carcinoma regimens. Although this difference was not statistically significant, treatment results showed a favorable prognosis with gonadal choriocarcinoma regimens (P = 0.1).

riocarcinoma metastatic to the lung successfully treated with RFA. We propose that EMA/CO is useful as a first-line regimen for primary gastric choriocarcinoma.

Choriocarcinoma has been reported in extragonadal sites such as the lung, liver, breast, prostate, urinary bladder, nose, and gastrointestinal tract[32]. Primary gastric choriocarcinoma is extremely rare. There are several theories on the histopathogenesis of primary gastric choriocarcinoma (*i.e.*, histological resemblance to choriocarcinoma, arising from a gonadal anlage displaced in the abdomen, a long delayed metastasis from a genital primary lesion, arising from gastric teratoma, and retro-differentiation of gastric carcinoma cells to embryonal ectodermal status with the ability to form trophoblasts)[33,34]. In some cases, there is a combination of malignant cytotrophoblasts and syncytiotrophoblasts admixed with areas of typical glandular differentiation, which supports the retro-differentiation hypothesis[35]. In recent years, Okada *et al*[33] described the possibility of normal gastric cells with the ability to produce hCG, which can directly develop into gastric choriocarcinoma. However, most authors favor the concept of retro-differentiation within an area of adenocarcinoma over primary gastric cells developing into choriocarcinoma due to the fact that less than 25% of cases are pure choriocarcinoma[35]. In such cases, the more rapidly growing choriocarcinoma component seems to have replaced the adenomatous elements. In the present case, there was a component of adenocarcinoma, which was indicated by positive CEA immunohistological staining. This finding supports the hypothesis that primary gastric choriocarcinoma originates from pre-existing gastric adenocarcinoma.

Primary gastric choriocarcinoma is a rapidly growing neoplasm that has an average survival of only a few months in untreated patients[6]. Gastrectomy with lymph node dissection followed by chemotherapy is the treatment of choice. Although some case reports and small studies have reported benefits from chemotherapy, a standard treatment has not been established due to the rarity of this tumor. Chemotherapy regimens usually used successfully for gonadal choriocarcinoma, including MAC (methotrexate, actinomycin-D, cyclophosphamide), CHAMOCA (cyclophosphamide, hydroxycarbamide, doxorubicin, actinomycin D, methotrexate, melphalan, and vincristine), and EMA/CO are generally considered to have a lower success rate in the treatment of primary gastric choriocarcinoma[5]. Several studies employed regimens used for gastric adenocarcinoma, such as a combination of fluorouracil and cisplatin or TS-1-based therapy, based on the concept that primary gastric choriocarcinoma develops from the retro-differentiation of gastric adenocarcinoma[4,10,14,36]. However, despite recent advances in combination chemotherapy, median survival is still less than six months with these regimens. In our retrospective analysis, the median survival with gastric carcinoma regimens was 5.0 mo compared to 9.5 mo with gonadal carcinoma regimens. In the present case, we chose EMA/CO because it is the first-line regimen for high-risk gestational trophoblastic neoplasia due to its favorable effectiveness-to-toxicity ratio. EP, BEP (bleomycin, etoposide, cisplatin), or VIP (etoposide, ifosfamide, cisplatin) is used in refractory cases[1]. In fact, the recurrent tumor showed a dramatic response to EMA/CO even with metachronous tumors in the lung. From our analysis and experience, EMA/CO can be considered a candidate for first-line treatment of recurrent or unresectable primary gastric choriocarcinoma.

RFA has been gaining popularity rapidly as a treatment for lung cancer[37]. In recent years, RFA has been used to treat oligometastases and oligo-recurrences of metastatic lung cancer such as colorectal carcinoma, hepatobiliary carcinoma, renal cell carcinoma, and sarcoma[37]. Oligometastasis and oligo-recurrence refer to the presence of one or a few metastatic or recurrent lesions with a controlled primary tumor[38]. The International Registry of Lung Metastasis reported that the five-year OS of patients with complete resection of metastatic lung tumors was 36%, compared with 13% for patients without resection[39]. Furthermore, for patients whose lung metastases were completely resected, survival depended on the number of tumors, *i.e.*, fewer metastatic lesions indicated better survival. Such data may provide the rationale for using local therapy including RFA for oligometastases and oligo-recurrences. In the present case, since the recurrent tumors in the liver and lungs were well-controlled by EMA/CO, we treated the oligometastatic tumor in the lung with RFA. The patient was disease-free for nine years without additional chemotherapy after RFA. The present case is the first successful case report of metastatic choriocarcinoma of the lung treated by RFA in the world.

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CASE REPORT

Radical excision of Barrett's esophagus and complete recovery of normal squamous epithelium

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Abstract

To treat Barrett's esophagus (BE), radiofrequency ablation or cryotherapy are effective treatments for eradicating BE with dysplasia and intestinal metaplasia, and reduce the rates of Barrett's esophageal adenocarcinoma (BAC). However, patients with BE and dysplasia or early cancer who achieved complete eradication of intestinal metaplasia, BE recurred in 5% within a year, requiring expensive endoscopic surveillances. We performed endoscopic submucosal dissection as complete radically curable treatment procedure for BE with dysplasia, intestinal metaplasia and BAC.

Key words: Barrett's esophagus; Radiofrequency ablation; Cryotherapy; Endoscopic submucosal dissection; Radically curable treatment

Core tip: Radiofrequency ablation or cryotherapy is effective for eradicating Barrett's esophagus (BE) with dysplasia; however, it recurs in 5% in a year. Endoscopic submucosal dissection is a complete radically curable treatment procedure for BE with dysplasia and Barrett's esophageal adenocarcinoma.

Mori H, Kobara H, Rafiq K, Nishiyama N, Fujihara S, Ayagi M, Yachida T, Kato K, Masaki T. Radical excision of Barrett's esophagus and complete recovery of normal squamous epithelium. *World J Gastroenterol* 2013; 19(31): 5195-5198 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i31/5195.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i31.5195

INTRODUCTION

Barrett's esophagus (BE) is a condition of the esophageal mucosa where the esophageal squamous epithelium is replaced with columnar epithelium because of prolonged reflux of gastric acid and bile acid into the esophagus. However, the definition of the esophageal gastric junction (EGJ) is different between Japan and other countries and remains controversial. In Japan, EGJ is defined as the distal limit of the lower esophageal palisade vessels, but as the proximal margin of the gastric folds (Prague criteria). The japanese criteria may be more suitable for the definition of EGJ and for the diagnosis of endoscopic BE than other criteria[1]. Moreover, pathologically, the need to identify goblet cells in esophageal biopsies of BE is also controversial. Morphological evaluation of EGJ biopsies cannot distinguish whether the columnar epithelium comes from the distal esophagus or the proximal stomach[2,3]. There is also some controversy with regard to the definition, classification and histological findings and grading of dysplasia on BE[4]. The consensus indication for the treatment of BE are histological findings of

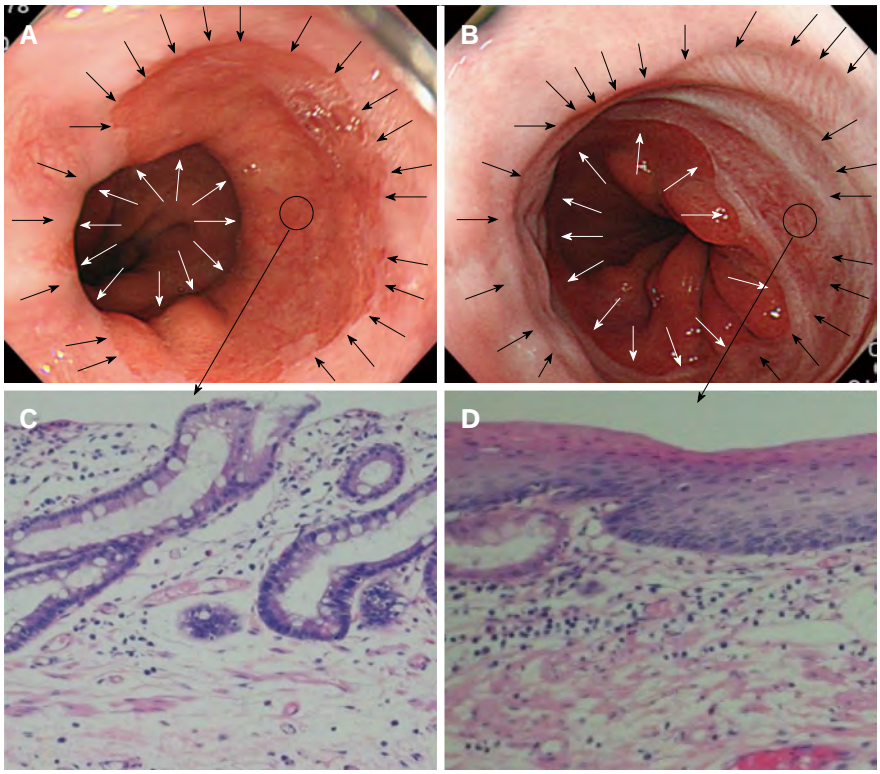

Figure 1 Short segment Barrett's esophagus undergoing endoscopic submucosal dissection. A: Short segment Barrett's esophagus (SSBE) predominantly of the right sidewall. The white arrows indicate the distal side, the black arrows indicate the proximal side, and the black encircled area indicates the preoperative biopsy site; B: After circumferential resection of SSBE with endoscopic submucosal dissection: regenerating squamous epithelium is seen between the distal side (white arrows) and the proximal side (black arrows). The black encircled area indicates the postoperative biopsy site; C: Specialized columnar epithelium with the preoperative intestinal metaplasia (hematoxylin and eosin stain, × 200); D: Postoperative regenerating squamous epithelium. Specialized columnar epithelium is not seen (hematoxylin and eosin stain, × 200).

high-grade dysplasia (HGD) by biopsies. After diagnosis of intestinal metaplasia of specific columnar epithelium and HGD, radio frequency ablation (RFA) or cryotherapy is performed because of the increased risk of Barrett's adenocarcinoma (BAC) associated with HGD[5]; however, the recurrence rate of BE is 5% within a year[6-9]. This indicates that even patients who underwent RFA require closer endoscopic surveillance. As a complete radically curable treatment, we performed entire circumference resection by endoscopic submucosal dissection (ESD) and subsequent steroid treatment. The application and permeation with balloon dilatation were performed to prevent stenosis[10]. We report two cases of en bloc resection of BE and BAC with ESD, in which the patients showed a complete recovery of normal squamous epithelium without recurrence.

CASE REPORT

Case 1

A 40-year-old man, whose mother died of BAC associated with BE, underwent screening with esophagogastroduodenoscopy and was diagnosed with circumferential short segment BE (SSBE), which was the same condition that caused his mother's death (Figure 1A and C). We could not detect severe dysplasia by biopsies before ESD. However, the patient insisted upon radical resection of SSBE. Moreover, narrow band imaging magnified endoscopy revealed irregular microvascular and microsurface patterns, which prompted us to recommend ESD. The patient underwent *en bloc* resection with ESD because he chose to undergo excision of SSBE with ESD to prevent BAC. For the resection line, the distal side was the border of the palisade vessel and the adoral fold, and the proximal side was 10 mm proximal to the columnar epithelium. Circumferential resection was performed with ESD. On days 5, 8, 12, 15 and 20 after ESD, steroid application and permeation with balloon dilatation were performed to prevent stenosis. We used triamcinolone acetonide (TA) gel as the steroid application. The TA gel was made and applied as follows: total of 10 mL TA (100 mg) was mixed with 7.5 mL Endolubri jelly (Olympus Medical Systems, Tokyo, Japan) to make 17.5 mL of gel. Beginning at the distal side of the artificial ulcer, steroid gel was applied to the ulcer floor while pulling the scope out spirally to the proximal side of the ulcer, using a spraying tube to apply the steroid gel precisely. Subsequently, 5 min of balloon dilatation (12-15 mm in diameter) was performed immediately to allow the steroid gel to permeate into the artificial ulcer. Three months after ESD and steroid treatment, squamous epithelium without stenosis was recovered at the excision site. The site returned to normal stratified squamous epithelium, which was confirmed by a biopsy of the regenerating squamous epithelium after resection

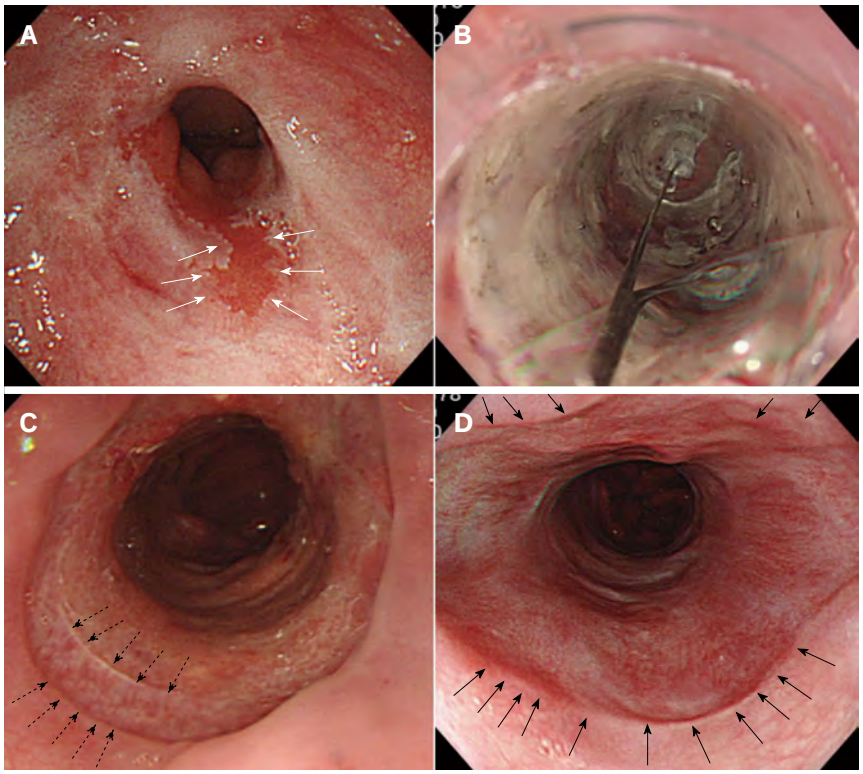

Figure 2 Barrett's adenocarcinoma with high-grade dysplasia and severe stenosis. A: Barrett's adenocarcinoma (white arrows) with high-grade dysplasia and severe stenosis of the lower esophagus; B: On days 5, 8, 12, 15 and 20 after endoscopic submucosal dissection, steroid application and balloon dilatation were performed to prevent stenosis; C: The base of the artificial ulcer on day 12 after surgery. Regenerating squamous epithelium can be seen from the resection margins of the proximal side indicated by the dotted arrows; D: On day 60, the base of the artificial ulcer is thoroughly covered with regenerating squamous epithelium (black arrows). No recurrence has been observed for 5 years.

(Figure 1B and D). This patient received only a proton pump inhibitor and has not relapsed for 4 years.

Case 2

A 65-year-old woman presented with a 20-year history of dysphagia associated with reflux esophagitis. BAC with HGD and severe stenosis of the lower esophagus was diagnosed (Figure 2A). The patient underwent ESD resection of BE and BAC and circumferential resection of the site of the stenosis. After ESD, steroid application and balloon dilatation were performed to prevent stenosis (Figure 2B). BAC was excised and BE was replaced with squamous epithelium (Figure 2C). The normal stratified squamous epithelium was observed at the excision site 6 mo after ESD, which was confirmed by a biopsy (Figure 2D). Narrow band imaging magnified endoscopy also showed a normal intraepithelial papillary capillary loop (IPCL type I). The patient has had no recurrence for 5 years. In addition, high-grade stenosis of the lower esophagus almost disappeared.

DISCUSSION

The cancerization rate and therapy of BE are still controversial[6,7]. Some researchers have reported lower incidences of dysplasia and BAC among patients with non-dysplasic BE, and most patients were cancer free after a long-term follow-up. Therefore, endoscopic surveillance intervals should be lengthened[8]. Others reported that the incidence of BAC in patients with BE was 30 times more frequent than in the general population[9]. Although closed endoscopic random biopsy is recommended for BE including HGD to detect BAC at an early stage, cancer detection is impossible unless BAC is at the site of biopsy[10,11]. RFA is an established treatment for BE with dysplasia. Although the short-term results of RFA have been determined, there have been concerns about recurrence of BE after RFA. It is reported that for BE treated by RFA, 56% were in complete remission after 24 mo. However, 33% of these patients had disease recurrence within the next 2 years[12,13], which is a very high recurrence rate. Therefore, we hypothesized that radical excision by ESD without recurrence and stenosis is best and most complete radically curative treatment procedure for BE with dysplasia, intestinal metaplasia and BAC. In Japan, ESD is recommend for Barrett's esophageal cancer after accurate diagnosis using narrow band imaging with magnifying endoscopy because of its high curative rate. However, the esophagus is a narrow organ and healing of an artificial ulcer that occupies two thirds or more of the circumference of the esophagus may result in the formation of a significant stricture. Recently, several studies have demonstrated the effectiveness of local injection or oral administration of steroids for preventing strictures[14,15]. We developed and reported a new method for preventing post-ESD stricture by steroid application and

permeation with balloon dilatation[7]. The proximal side of the lower esophageal sphincter has a lumen with a relatively high expansion ability, and is resectable circumferentially without stenosis by steroid application and permeation with balloon dilatation, even if BE with HGD is subjected to circumferential resection with ESD. In the ESD procedure, recurrence does not occur because the procedure involves en bloc resection and the regenerating epithelium returns to normal squamous epithelium. Thus, ESD seems to be effective in the treatment of BE associated with HGD.

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CASE REPORT

Isolated splenic metastases from gastric carcinoma: A case report and literature review

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Abstract

Isolated metastases to the spleen from gastric carcinoma is very rare. Only a few cases have been reported in the literature. We herein present a case of isolated splenic metastases in a 62-year-old man, occurring 12 mo after total gastrectomy for gastric carcinoma. The patient underwent a laparoscopic exploration, during which two lesions were found at the upper pole of the spleen, without involvement of other organs. A laparoscopic splenectomy was performed. Histological examination confirmed that the splenic tumor was a poorly differentiated adenocarcinoma similar to the primary gastric lesion. The postoperative course was uneventful and the patient has been well for 9 mo, with no tumor recurrence. The clinical data of 18 cases of isolated splenic metastases from gastric carcinoma treated by splenectomy were summarized after a literature review. To our knowledge, this is the first reported case of isolated splenic metastases undergoing laparoscopic splenectomy.

Key words: Metastasis; Splenic neoplasms; Stomach neoplasms; Laparoscopy; Splenectomy

Core tip: Isolated metastases to the spleen from gastric carcinoma is very rare. We report a case of isolated splenic metastases in a 62-year-old man, occurring 12 mo after total gastrectomy for gastric carcinoma who underwent laparoscopic splenectomy. The patient has been well for 9 mo after surgery with no tumor recurrence. The clinical data of 18 cases of isolated splenic metastases from gastric carcinoma treated by splenectomy were summarized after a literature review. To our knowledge, this is the first reported case of isolated splenic metastases undergoing laparoscopic splenectomy.

Zhu YP, Mou YP, Ni JJ, Zhou YC, Jiang JW, Jiang ZN, Wang GY. Isolated splenic metastases from gastric carcinoma: A case report and literature review. *World J Gastroenterol* 2013; 19(31): 5199-5203 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i31/5199.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i31.5199

INTRODUCTION

Splenic metastases from gastric carcinoma are uncommon and are generally detected as part of multi-organ metastases. Isolated splenic metastases from gastric carcinoma are exceedingly rare with only a few cases having been documented in the literature. Here, we report a case of isolated splenic metastases, occurring 12 mo after eradication of gastric carcinoma, which was successfully

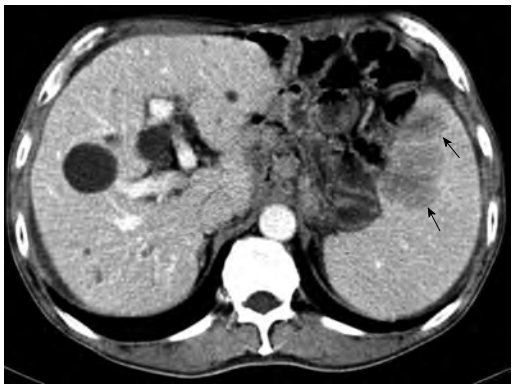

Figure 1 Computed tomography showed two low-density lesions (arrows), 4.5 cm × 3.5 cm and 2.5 cm × 2.0 cm in size respectively, at the upper pole of the spleen.

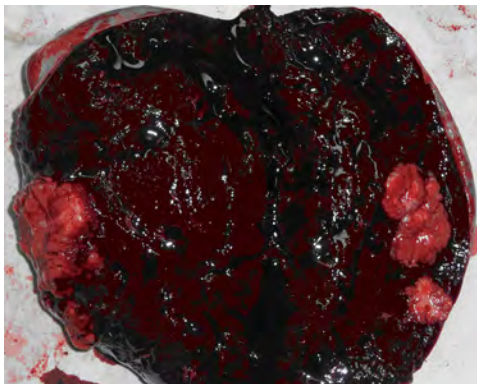

Figure 2 Cross section of the spleen showing two yellowish-white lesions at the upper pole.

treated by laparoscopic splenectomy. To the best of our knowledge, this is the first report documenting metachronous splenic metastases treated by laparoscopic surgery. In order to better understand the clinical behavior of isolated splenic metastases with gastric carcinoma origin, we reviewed a total of 18 such cases from the literature. The detailed features and prognoses of these cases were summarized in this study.

CASE REPORT

A 62-year-old man was diagnosed with gastric carcinoma located in the M and L portion of the stomach. The tumor was of ulcerative type in gross appearance (Borrmann Ⅲ type) and was 9 cm × 8 cm in size. The patient underwent a total gastrectomy with a standard D2 lymph node dissection in February 2011. Histology of the resected specimen revealed a poorly differentiated adenocarcinoma infiltrating the serosa with nodal involvement (12 of 25 nodes were positive for metastases), fulfilling the criteria of stage ⅢB according to the American Joint Committee on Cancer TNM staging classification for carcinoma of the stomach (7th ed, 2012)[1]. The patient received six cycles of intravenous chemotherapy consisting of 5-fluorouracil, leucovorin and oxaliplatin after surgery. Ultrasonography and abdominal computed tomography

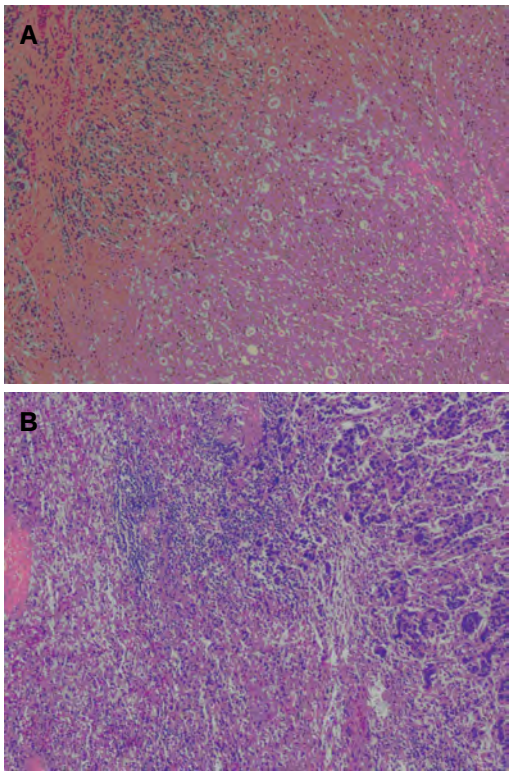

Figure 3 Histological findings of the primary gastric carcinoma (A) and the splenic metastatic tumor (B) (hematoxylin-eosin stain, × 40).

(CT) scan did not reveal any remarkable metastatic lesions during the postoperative follow-up.

In February 2012, an abdominal CT scan showed two low-density lesions, 4.5 cm × 3.5 cm and 2.5 cm × 2.0 cm, respectively, at the upper pole of the spleen without obvious contrast enhancement (Figure 1). Two hypoechoic lesions in the corresponding location of the spleen were revealed by ultrasonography. The previous history of gastric carcinoma contributed to a presumptive diagnosis of metachronous splenic metastases. The patient was given two cycles of chemotherapy consisting of intravenous 5-fluorouracil, leucovorin and oxaliplatin. A thorough diagnostic workup, including gastroscopy, CT scan of chest and abdomen, and ^{18}F-fluorodeoxyglucose (^{18}F-FDG) positron emission tomography scan, was negative for extra-splenic tumor dissemination. However, ultrasound as well as CT scan revealed enlargement of the splenic lesions which indicated their poor responsiveness to the chemotherapeutics. The patient underwent a laparoscopic exploration since his splenic metastases were revealed to be isolated and resectable. The splenic lesions were confirmed during the procedure, while no other intra-abdominal organ metastasis or peritoneal dissemination was seen. A laparoscopic splenectomy was performed in April 2012. The specimen showed two lesions, measuring 4.5 cm × 4.0 cm and 3.0 cm × 2.0 cm respectively, occupying the upper pole of the spleen. The tumors were yellowish-white in color, which demarcated them quite clearly from the adjacent splenic parenchyma, and showed no bleeding or necrosis (Figure 2). Histological examination showed that the lesions were metastatic adenocarcinoma consistent with the features of the primary gastric carcinoma (Figure 3) with no lymph

Table 1 Summary of patients with isolated splenic metastasis from gastric carcinoma treated by splenectomy

No.	Source	Gender /age (yr)	Primary gastric carcinoma		Splenic metastasis			CT appearance	Survival time (mo)/status/ tumor dissemination
			Location[1]	Histology[2]	Interval[3] (mo)	Other involvement	Suggested route		
1	Takebayashi *et al*[10]	F/64	U, M	Por, T3, n1, M1	0	LNs	Lymphatic	NS	3/dead/lung
2	Fujita *et al*[11]	F/75	R	Tub, T4, n3, M1	0	LNs	Hematogenous	NS	NS
3	Mori *et al*[12]	M/49	R	Tub, T3, n3, M1	0	LNs	Hematogenous	NS	12/alive/NS
4	Okuno *et al*[13]	M/56	U, M	Por, T3, n2, M1	0	LNs and peritoneum	Lymphatic	NS	1/dead/peritoneum, bone, pancreas, adrenal, liver
5	Sakamoto *et al*[14]	M/67	U, M	Tub, T4, n2, M1	0	LNs	Hematogenous	NS	8/dead/liver
6	Ishida *et al*[15]	M/65	U	Por, T2, n2, M1	0	LNs	NS	NS	5/dead/tumor embolism of portal vein
7	Lu *et al*[16]	M/59	U	Hepatoid AC, T4	0	LNs	NS	Heterogenous low-density lesions	18/alive/liver
8	Ikeda *et al*[17]	M/57	U	Por, T3, n1, M0	17	None	NS	NS	15/alive/ NS
9	Shirai *et al*[18]	M/63	M	Pap, T1, n0, M0	33	None	Hematogenous	NS	20/alive/ NS
10	Tatsusawa *et al*[19]	M/54	M	Tub, T2, n2, M0	102	None	Hematogenous	NS	NS
11	Takahashi *et al*[20]	M/64	L	Tub, T2, n3, M0	16	None	Hematogenous	NS	7/dead/liver, lung
12	Opocher *et al*[4]	F/76	L	Tub, T2, N0, M0	57	None	Hematogenous	Round cystic area with fluid content	13/alive/none
13	Opocher *et al*[4]	M/66	NS	Por, T2, N1, M0	36	None	Hematogenous	NS	14/alive/none
14	Williams *et al*[5]	M/69	U	AC	43	None	NS	Soft tissue mass with cacification	NS
15	Yamanouchi *et al*[8]	M/69	L	Tub, T2, N1, M0	48	None	Hematogenous?	Low-density area	40/dead/liver, peritoneum
16	Sunitsch *et al*[21]	F/80	L, R	Por, T1, N0, M0; tp, T1, N0, M0	37	None	NS	NS	NS
17	Kawasaki *et al*[22]	M/76	U	Pap, T1, N1, M0	12	None	Hematogenous?	Low-density lesion	24/alive/none
18	Zhou *et al*[23]	M/76	U	Tub	36	None	NS	NS	30/alive/none

[1]U, M, and L: Indicate the upper, middle, and lower thirds of the stomach, respectively; R: Residual stomach; [2]Por: Poorly differentiated adenocarcinoma; tub: Tubular adenocarcinoma; pap: Papillary adenocarcinoma; tp: Tubulopapillary adenocarcinoma; AC: Adenocarcinoma; T1: Tumor invades lamina propria, muscularis mucosae or submucosa; T2: Tumor invades muscularis propria; T3: Tumor penetrates subserosal connective tissue without invasion of visceral peritoneum or adjacent structures; T4: Tumor invades serosa (visceral peritoneum) or adjacent structures[1]; n: n0 indicates no evidence of lymph node metastasis; n1, n2, and n3 indicate metastasis to the groups 1, 2, and 3 lymph nodes, respectively, according to the Japanese Classification of Gastric Carcinoma[24]; N: N0: No regional lymph node metastasis; N1, N2 and N3 indicate metastasis in 1-2, 3-6, 7 or more regional lymph nodes; M: M0: No distant metastasis; M1: Distant metastasis; [3]Interval: Interval from the detection of primary gastric carcinoma to the detection of the splenic secondary tumor. 0 indicates the the splenic secondary tumor detected at the same time as the primary tumor. LNs: Lymph nodes; CT: Computed tomography; NS: Not specified.

node involvement at the splenic hilum.

The patient recovered uneventfully and was discharged eight days after surgery. He recovered well and showed no evidence of tumor recurrence at the last follow-up in December 2012.

DISCUSSION

Splenic metastases from non-hematologic malignancies are infrequent, with an incidence of 0.6%-1.1% in populations with carcinoma according to a large clinicopathologic study[2]. The rarity of splenic metastases might be explained by the following reasons: (1) the poorly developed lymphoid system of the spleen, especially the lack of afferent lymphatic vessels, prevents the spleen from receiving metastatic tumor cells *via* the lymphatic route; (2) the sharp angle of splenic artery branching from the celiac trunk inhibits large clumps of tumor cells from passing through; and (3) the microenvironment of the spleen may hinder the growth of micrometastatic foci[3].

Most splenic metastases are accompanied by multivisceral tumor dissemination. Skin melanoma and carcinomas of the breast, lung, ovary, colorectum and stomach are the major primary sources of splenic metastases, and gastric carcinoma accounts for 6.9%[2]. Very few splenic metastases occur as isolated splenic lesions, synchronous or metachronous to the primary tumor. To our knowledge, only seven cases of synchronous splenic metastases and 11 cases of metachronous splenic metastases from gastric carcinoma have been treated by splenectomy to date. A summary of these cases is shown in Table 1.

Isolated splenic metastases are often first identified by ultrasonography or CT scan as most of them are asymptomatic. However, some patients harboring splenic me-

tastases complain of fatigue, weight loss, fever, abdominal pain, splenomegaly, anemia, or thrombocytopenia[3]. Generally, when an isolated splenic lesion is found during the oncologic follow-up, a metastatic origin should be suspected. Serum levels of carcinoembryonic antigen and carbohydrate antigen 19-9 have been reported to be of predictive value in detecting the appearance of isolated splenic metastases in advance of imaging identification[4]. In our review, the splenic metastases presented variously on CT scan, and ranged from a cystic lesion, low-density occupying lesion to a solid mass, and showed different patterns of enhancement. A calcified splenic mass, which is a common feature of metastases from primary mucinous adenocarcinoma, was also described[5]. In this regard, it is sometimes difficult to distinguish the suspected splenic metastases from primary splenic lesions such as lymphoma, vascular tumor, or infectious disorder. It has been reported that ^{18}F-FDG positron emission tomography was of value in distinguishing benign from malignant masses of the spleen[6]. As a highly vascular organ, the spleen is often considered an inappropriate target for fine-needle aspiration (FNA) due to the potential risk of bleeding. Nevertheless, Cavanna *et al*[7] reported a series of 160 patients who underwent biopsy of the splenic mass by FNA with an overall accuracy rate of 98.1% and with no complications, which demonstrated that the technique is safe and effective.

In the present case, two masses were detected in the spleen by imaging techniques 12 mo after a radical gastrectomy. The previous history of gastric carcinoma indicated the diagnosis of a splenic metastasis which was subsequently supported by an ^{18}F-FDG positron emission tomography scan. Ultimately, the pathological study of the surgical specimen, which showed a papillary adenocarcinoma with a high morphological resemblance to the primary gastric carcinoma, confirmed our clinical diagnosis.

Gastric carcinoma in the U or M portion is likely to invade the spleen directly because of their anatomical contiguity. In such cases, the metastatic lesions are always detected synchronously or very shortly after a radical gastrectomy. In contrast, splenic metastases are often detected later when they are caused by the hematogenous route. In our literature review, the mean time from the diagnosis of primary stomach carcinoma to the development of secondary splenic metastases was 39.7 mo with the longest time being 102 mo[8]. The late occurrence of blood-borne isolated splenic metastases could be explained by some recent advances in the knowledge of the metastatic mechanism: it may develop from an early micro-metastasis within the spleen and progress to an observable lesion after a period of clinical latency[9]. We also noted that the mean duration from the detection of the primary gastric tumor to the detection of the splenic secondary tumor was shorter in Lam and Tang's review (3-36 mo, mean 8 mo)[2]. We speculate that there might have been a proportion of the splenic metastases in their analysis which were caused by direct invasion from primary gastric tumors, whereas there was not a single case in our review which was reported to be caused by direct invasion. This might explain the time difference between the two reviews.

As isolated splenic metastases from gastric carcinoma are rarely documented in the literature, it is still difficult to predict the clinical behavior of this disease. However, the existing preliminary records show a tendency that long-term remission could be expected after splenectomy in a metachronous splenic metastasis, while a synchronous splenic metastasis always indicates early tumor progression and a worse outcome. In our review, patients with gastric carcinoma and synchronous splenic metastases had a mean post-operative survival time of seven months, apparently shorter than that of the patients with metachronous splenic metastases (mean 20.3 mo after splenectomy).

Although splenectomy provides a possible means of radical treatment in patients with isolated splenic metastases, it should be decided with caution as a splenic metastatic lesion which is supposed to be "isolated" sometimes may represent an initial clinical manifestation of systemic metastases at multiple sites. Under such circumstances, surgical stress from splenectomy might cause adverse clinical effects in the patient. With this in mind, following the discovery of the splenic lesions in the present patient, rather than performing splenectomy immediately, he was given two cycles of chemotherapy and this time span was used for observation as well. As no new metastatic lesions emerged, a laparoscopic exploration and splenectomy was performed.

As a result of previous surgery, there was severe adhesion in the upper quadrant of the abdomen, especially between the transverse colon and the spleen, which needed careful separation. Conversely, the isolation of the splenic pedicle was not as difficult, as the connective tissue in this region had been removed and all the short gastric vessels had been severed during the previous operation of total gastrectomy. To the best of our knowledge, this is the first report documenting metachronous splenic metastases treated by laparoscopic surgery. The patient's postoperative course was uneventful and he has been well with no evidence of tumor recurrence for nine months.

According to our experience, laparoscopic splenectomy seems to be a promising approach in achieving long-term survival in patients with metachronous isolated splenic metastases after eradication of gastric carcinoma.

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Chronic pancreatitis as presentation of Crohn's disease in a child

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Abstract

It is reported that a pancreatic disease may precede the diagnosis of inflammatory bowel disease (IBD) both in children and in adults. Idiopathic chronic pancreatitis, however, occasionally co-exists with the IBD, mainly at pediatric age. We report a case of a patient who progressively developed the features of a chronic pancreatitis, before the diagnosis of Crohn's Disease (CD). Ten months after the onset of the first episode of pancreatitis the patient developed bloody diarrhea, mucus stools and biochemical findings of inflammation. The colonoscopy revealed a diffuse colitis without involvement of the last loop and the gastroscopy showed inflammation of the iuxta-papillary area. The histological findings confirmed the diagnosis of CD that involved the colon and the duodenum. In conclusion, in children the idiopathic chronic pancreatitis may be an unusual presentation of CD. Thus, if other known cause of chronic pancreatitis are not found, a not invasive work up to exclude the IBD should be warranted. An early coincidental diagnosis of the IBD may delay the progression of the pancreatic disease.

Key words: Inflammatory bowel disease; Crohn's Disease; Pediatric age; Bloody diarrhea; Pancreatic disease

Core tip: We report a cases of chronic pancreatitis associated with Crohn's Disease (CD). We have not been able to find reports of this association in the pediatric medical literature. The present case suggests that in children the idiopathic chronic pancreatitis may be an unusual presentation of CD. Thus, if other known cause of chronic pancreatitis are not found, a not invasive work up to exclude the inflammatory bowel disease should be warranted. The early recognition of the CD, indeed, may help in delay the progression of the pancreatic disease.

Knafelz D, Panetta F, Monti L, Bracci F, Papadatou B, Torre G, Dall'Oglio L, Diamanti A. Chronic pancreatitis as presentation of Crohn's disease in a child. *World J Gastroenterol* 2013; 19(31): 5204-5206 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i31/5204.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i31.5204

TO THE EDITOR

It is well known that pancreas can be involved in the course of inflammatory bowel diseases (IBD)[1]. The pancreatic disease can occur in cases of biliary lithiasis or of the administration of 5-aminosalicylates (5-ASA), corticosteroids, and azathioprine (AZA) or 6-mercaptopurine[1,2]. Most cases of pancreatitis are clinically silent

and the frequency of clinical pancreatitis is markedly lower than that of asymptomatic hyperamylasemia or of evidence of exocrine pancreas insufficiency[1]. The potential association of the IBD with the pancreatic diseases makes therefore a periodic assessment of the pancreatic function in all patients affected by IBD advisable. A pancreatic disease may also precede the onset of the IBD. It is shown that an acute pancreatitis may represent the picture of presentation of the IBD in children such as in adults[3]. As recently reported by Broide *et al*[3] the prevalence of acute pancreatitis as symptom of the onset of the IBD is 2.17% in children and 0.06 in adults[3]. Two previous reports found a prevalence of acute pancreatitis preceding IBD in 27%[4] and in about 5%[5] of the cases. Therefore it was suggested[3] that in children, after an episode of acute pancreatitis, specific attention should be paid to other IBD susceptibility factors, that could indicate investigations by colonoscopy and gastroscopy. Idiopathic chronic pancreatitis, on the other hand, was occasionally reported in association with the IBD, mainly at pediatric age[6-8]. We report a case of a patient who progressively developed the features of a chronic pancreatitis, before the diagnosis of Crohn's disease (CD). When she was 4 years old, she was admitted with abdominal pain, slightly raised C-reactive protein and of erithro-sedimentation rate and high serum level of amylase and lipase. No gallstones were found, but only edema and enlargement of the pancreatic gland were reported on abdominal ultrasound. She received the treatment of the acute pancreatitis (intra-venous fluids, bowel rest, antibiotic, protease and gastric acid inhibitors), with beneficial effects on the pain and of the biochemical indices. Thereafter, she developed numerous episodes of acute pancreatitis. Therefore we planned the imaging and blood examinations to exclude all the causes of the chronic and recurrent pancreatitis. Serological tests for Cytomegalovirus, Epstein-Barr virus, Echovirus, Rubella, Adenovirus, Coxsackie virus, *Legionella* and *Mycoplasma* resulted negative. Immunoglobulin G serum level was into the normal range, so to exclude an autoimmune pancreatic process. The sweet test was negative. Genetic analysis for the mutations of cystic fibrosis transmembrane conductor regulator gene, for the cationic trypsinogen (*PRSS1*) gene, and serine protease inhibitor Kazal type 1 (*SPINK1*) were all negative. The magnetic resonance cholangiopancreatography showed a picture of chronic pancreatitis. This result along with the persistence of severe abdominal pain led us to schedule an endoscopic retrograde cholangiopancreatography to have a clear pancreatogram and therefore to define the chronic process but also to perform the pancreatic sphinterectomy[9]. The sphinterectomy was followed by partial extraction of the pancreatic concretions and by the placement of a plastic stent into the main pancreatic duct for transpapillary drainage. The pancreatogram showed pancreatic calcifications and the distortion of the main pancreatic duct, both findings consistent with established chronic pancreatitis. The sphinterectomy and the placement of the stent reduced the abdominal pain but did not impede the course of the pancreatic failure that required the enzymes replacement. Ten months after the onset of the first episode of pancreatitis the patient developed bloody diarrhea, mucus stools and relevant increase of inflammation indices. The following not invasive work up to define the suspected IBD showed positive anti-saccaromyces cerevisiae antibodies and fecal calprotectin. Stool cultures and stool test for *Clostridium difficile* toxins A and B were all negative. Upper and lower endoscopies were therefore scheduled. Colonoscopy revealed a diffuse colitis without involvement of the last loop. The gastroscopy revealed a duodenal involvement, with inflammation of the iuxta-papillary area. Then the histological examination confirmed the diagnosis of CD localized in the colon and in the duodenum. Furthermore the nutritional treatment with an amino-acid based formula by naso-gastric tube induced a partial regression of the intestinal symptoms that relapsed when she restarted a diversified oral nutrition. The use of 5-ASA and of AZA determined an immediate increase of the pancreatic enzymes; thus the patient received corticoid alone, that improved the intestinal symptoms, without influencing the pancreatic function. The clinical phenotype of the CD in this patient was very severe, with several relapses requiring repeated courses of steroid treatment and therefore we began the biological drugs. We started with infliximab but, after the second administration that induced a severe anaphylactic reaction, we temporary suspended this treatment and we started a period of bowel rest by home parenteral nutrition. The following re-exacerbations were treated by short courses of steroids that also showed beneficial effects on the pancreatic exacerbations, with immediate regression of the pancreatic pain and of the inflammatory indices. When she was 13 years old she begun the adalimumab that determined a prolonged period of remission. The girl is now 16 years old, she reached the pubertal development and she is treated by adalimumab, without severe re-exacerbations of the intestinal disease. The pancreatic function is supported by the pancreatic enzymes. To our knowledge in literature are reported 16 cases of chronic pancreatitis associated with CD[5-8] and none of them occurred at pediatric age. Therefore we report the first pediatric case of chronic pancreatitis as picture of presentation CD. In our experience this is the first case of CD presenting as chronic pancreatitis and therefore we may consider this association very rare according with the literature data. It' s not clear if the pancreatic inflammation may be a metastatic presentation of CD or the complication of the duodenal involvement[1]. In our patients the CD-associated pancreatitis was due to the duodenal and to the iuxta-papillary area involvement, causing duodenal reflux and papilla obstruction. In this case the diagnosis of IBD was made only 10 mo from the onset of the pancreatic complaints, when the patient had already developed the intestinal signs suggestive of intestinal inflammation. When the CD was recognized the pancreatic disease had already progressed towards a chronic relapsing process

with intractable pain and exocrine pancreas insufficiency. We may speculate that in this case the earlier diagnosis of CD might reduce the severity of the pancreatic disease, delaying the course of the pancreatic failure. Both pancreatic pain and biochemical inflammation recovered indeed after short treatments with steroids, so confirming that the progression of the pancreatic disease might be influenced by a timely diagnosis of CD. In conclusion in children the idiopathic chronic pancreatitis may be an unusual presentation of CD. Thus, if other known cause of chronic pancreatitis are not found, a not invasive work up to exclude the IBD should be warranted. An early coincidental diagnosis of the IBD may delay the progression of the pancreatic disease.

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EDITORIAL

Bile duct cyst in adults: Interventional treatment, resection, or transplantation?

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Abstract

Cystic dilatations of the bile ducts may be found along the extrahepatic biliary tree, within the liver, or in both of these locations simultaneously. Presentation in adults is often associated with complications. The therapeutic possibilities have changed considerably over the last few decades. If possible, complete resection of the cyst(s) can cure the symptoms and avoid the risk of malignancy. According to the type of bile duct cyst, surgical procedures include the Roux-en-Y hepaticojejunostomy and variable types of hepatic resection. However, the diffuse forms of Todani type Ⅴ cysts (Caroli disease and Caroli syndrome) in particular remain a therapeutic problem, and liver transplantation has become an important option. The mainstay of interventional treatment for Todani type Ⅲ bile duct cysts is *via* endoscopic retrograde cholangiopancreatography. The diagnostic term "bile duct cyst" comprises quite different pathological and clinical entities. Interventional therapy, hepatic resection, and liver transplantation all have their place in the treatment of this heterogeneous disease group. They should not be seen as competitive treatment modalities, but as complementary options. Each patient should receive individualized treatment after all of the clinical findings have been considered by an interdisciplinary team.

Key words: Bile duct cyst; Caroli syndrome; Caroli disease; Hepatic resection; Liver transplantation; Interventional treatment

Core tip: This is an invited editorial on the role of different treatment options for patients with bile duct cysts. It is not meant to be a thorough review on the numerous aspects of this disease, but instead intends to provide critical insights into current developments in interventional and surgical therapies, defining their potential and indicating that they should not be seen as competitive but as complementary options. The diagnostic term "bile duct cyst" comprises quite different pathological and clinical entities. Interventional therapy, hepatic resection, and liver transplantation all have their place in the treatment of this heterogeneous disease group.

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INTRODUCTION

The modified Todani[1,2] classification that is widely used for bile duct cysts has several drawbacks. In particular, it combines different disease processes and does not account for the risk of malignant transformation or differences in epidemiology, pathogenesis, complications, and treatment. As a result, its clinical significance has been the subject of critical discussion and further modifications have been proposed. Moreover, there is uncertainty on the categorization of some variants.

Type I bile duct cysts comprise the subtypes I A (cystic dilatation of the common bile duct), I B (segmental dilatation of the common bile duct), and I C (fusiform dilatation extending to the common hepatic duct). Type

Ⅱ is a true diverticulum in the extrahepatic duct (supraduodenal). Type Ⅲ is a choledochocele confined to the common bile duct within the duodenal wall. An important differential diagnosis for a type Ⅲ cyst is a juxtapapillary duodenal diverticulum. Type Ⅳ is divided into type Ⅳa (multiple intra- and extrahepatic bile duct cysts) and type Ⅳb (multiple extrahepatic bile duct cysts, which are less common than type Ⅳa). Finally, type Ⅴ corresponds to Caroli disease (single or multiple intrahepatic bile duct cysts). When associated with congenital hepatic fibrosis, type Ⅴ is termed Caroli syndrome and is inherited as an autosomal recessive trait[3-5].

Caroli disease (CD) and Caroli syndrome (CS) are part of a broader spectrum of diseases with ductal plate malformations. These diseases have a close relationship with congenital kidney disorders, notably autosomal recessive polycystic kidney disease[6]. Generally, Caroli syndrome is characterized by early onset, with rapid disease progression due to the combination of cholangitis and portal hypertension[7-9]. Ductal plate malformations affect different levels of the intrahepatic biliary tree, including the large and proximal ducts in CD, the smaller ducts in CS and congenital hepatic fibrosis, and the more peripheral interlobular ducts in polycystic liver disease and von Meyenburg complexes[10,11]. Type Ⅴ cysts can be considered as a distinct disease entity from types I-Ⅳ, and type Ⅲ might be an anatomical variation rather than a true dilatation of the common bile duct[12]. Indeed, Ziegler *et al*[13] suggested that the classification of bile duct cysts should not include choledochoceles (type Ⅲ), as they differ from choledochal cysts with respect to age, gender, presentation, pancreatic duct anatomy, and their management.

Michaelides *et al*[14] described a dilatation of the central portion of the cystic duct apart from the dilatation of the common hepatic and common bile duct, giving the cyst a bicornal configuration. They suggested classifying this finding as a further subtype of Todani I cysts, namely Todani ID. However, Calvo-Ponce *et al*[15] have already proposed Todani ID as a cyst above the junction of the common hepatic duct and the cystic duct.

Okada *et al*[16] described a "common channel syndrome" and Lilly *et al*[17] used the term "forme fruste choledochal cyst" for a long common channel secondary to a proximal junction of the common bile duct and pancreatic duct with stenosis of the distal common bile duct, combined with cholecystitis and the classical pathological features of a choledochal cyst in the wall of the common bile duct. Forme fruste choledochal cysts are associated with only minimal or no dilatation of the extrahepatic bile duct, pancreaticobiliary maljunction, and protein plugs or debris in the common channel[18-20]. The cut-off diameter of the extrahepatic bile duct above which the diagnosis of a forme fruste is unacceptable has been arbitrarily described as 10 mm[20].

Kaneyama *et al*[21] reported variants with a type Ⅱ diverticulum arising from a type IC bile duct cyst, resulting in "mixed type I and Ⅱ" cysts. Visser *et al*[22] argued that all varieties of type I bile duct cysts have some element of intrahepatic dilatation. They concluded that type I and Ⅳa cysts only differ in terms of the extent of intrahepatic dilation, which makes discriminating between these two types rather arbitrary. Furthermore, an additional category, termed type Ⅵ, has been used for cystic malformations of the cystic duct[23,24]. However, these cysts could also be classified as a subtype of type Ⅱ[25]. Loke *et al*[26] described diverticular cysts of the cystic duct, whereas Wang *et al*[27] reported a patient with type I and type Ⅲ choledochal cysts that occurred simultaneously.

Notwithstanding these drawbacks, the crucial advantages of the Todani classification are its widespread use and reproducibility, which allow comparisons among the various published studies that have been built upon it. Therefore, its further use is to be advocated, despite its shortcomings. For this reason, the Todani system is also used in this editorial as a background for the therapeutic considerations.

INTERVENTIONAL TREATMENT

For type Ⅲ bile duct cysts, endoscopic retrograde cholangiopancreatography (ERCP) is an important diagnostic tool, and interventional therapy *via* ERCP is the mainstay of treatment. For the diagnosis of the other types, the less-invasive magnetic resonance cholangiopancreatography (MRCP) has widely replaced ERCP and represents the "gold standard" approach. However, ERCP remains the imaging modality of choice for type Ⅲ cysts because it also enables a simultaneous therapeutic sphincterotomy to be performed. Originally, type Ⅲ cysts were treated by transduodenal excision and sphincteroplasty; however, endoscopic sphincterotomy is now considered to be sufficient. Ohtsuka *et al*[28] reported malignancies in 3 out of 11 patients with type Ⅲ cysts. Therefore, endoscopic surveillance is recommended. Interventional therapy with ERC and shock-wave lithotripsy, in addition to antibiotics and bile acid treatment (ursodeoxycholic acid, UDCA)[29], is also used for type Ⅴ cysts, but cannot be expected to be curative. Its success is usually only temporary, and recurrent episodes of cholangitis cannot be prevented[30]. Internal biliary stents have also been described as a therapeutic option in anecdotal case reports[31,32].

A crucial argument against long-term treatment with interventional techniques and for removal of the cyst is the increased risk of malignancy. About 2.5%-17.5% of patients with bile duct cysts develop malignancies, and this incidence increases with age[33]. In a series of 38 adult patients published by Visser *et al*[22], 21% developed malignancies. The underlying mechanisms that cause carcinogenesis may be complex, as a consequence of chronic inflammation, cell regeneration, and DNA breaks leading to dysplasia. Pancreatic reflux might also play a role in causing K-ras mutations, cellular atypia, and the overexpression of p53[34]. Malignancy in Caroli disease has been reported to occur in 7%-15% of patients[33], underlining the need for surgery and surveillance.

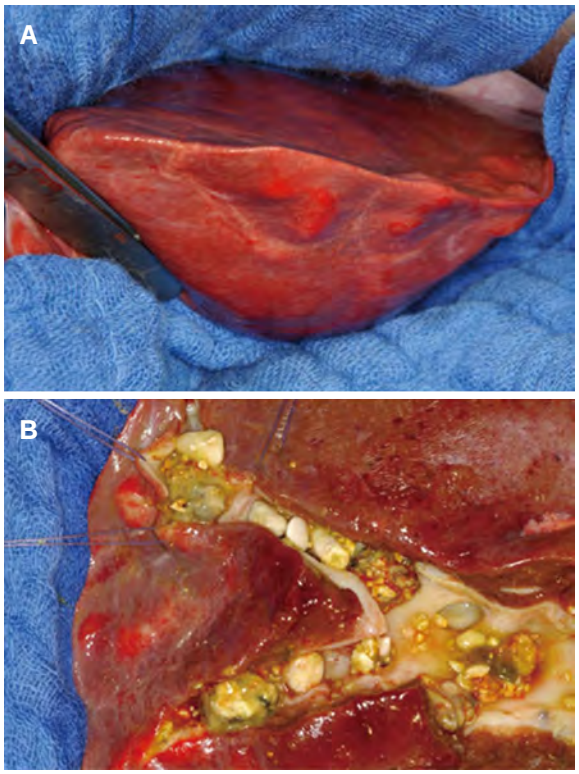

Figure 1 Intraoperative picture (A) and a detailed view (B) of the operative specimen (left hepatic lobe) in a patient with Todani type V bile duct cysts.

RESECTION

Complete resection of cysts and Roux-en-Y hepaticojejunostomy (RYHJ) is the procedure of choice for type I and IVb bile duct cysts. The success rate of this approach has been reported as 92%[35]. If adequate, the procedure may be performed laparoscopically. The benefits of the laparoscopic approach are also underlined in the reports by Gander *et al*[36] and by Palanivelu *et al*[37]. Potential complications of RYHJ include cholangitis, pancreatitis, biliary calculi, and, rarely, malignancy. Watanabe *et al*[38] reported that malignancy occurred in less than 1% of patients after a previous cyst excision, but higher incidences have been observed that may be due to incomplete cyst removal. Postoperative complications are usually seen in patients with inflammation and fibrosis at the time of surgery.

For forme fruste choledochal cysts, excision of the malformed ductal tissue with biliary reconstruction is required, as cholecystectomy alone is an inadequate treatment[39].

In all cases, complete removal of the cyst makes a decisive difference. With incomplete removal, Liu *et al*[40] reported a malignancy rate of 33.3%, compared to 6% after complete resection. Simple excision may be feasible for type II cysts, with ligation at the neck and without the need for biliary reconstruction. If possible, a laparoscopic approach may be advantageous[41].

For type IVa cysts, a tailored approach is necessary. Visser *et al*[22] suggested that excision of the extrahepatic component with hepaticojejunostomy should be performed; however, in cases with symptomatic intrahepatic affections (with stones, cholangitis, or biliary cirrhosis), treatment should correspond to that used for type V cysts, with hepatic resection for localized disease and transplantation for diffuse forms.

Although reported numbers in the literature are low, there is broad consensus that hepatic resection is the therapy of choice in patients with localized forms of type V cysts (Figure 1). If patients with monolobar disease remain asymptomatic, they will only require supportive dissolution therapy and surveillance; however, the risk of malignancy also has to be considered. For example, Kassahun *et al*[42] determined a cholangiocarcinoma incidence of 9.7%, whereas Ulrich *et al*[30] reported 9.1% and Bockhorn *et al*[43] reported 25%.

In symptomatic patients with acute cholangitis, a variable symptom-free interval may be achieved by using antibiotic treatment with or without endoscopic sphincterotomy and calculi removal or lithotripsy. However, most of these patients will develop recurrent cholangitis, chronic inflammation, and an increased risk of cholangiocarcinoma. These patients are best treated by liver resection if their hepatic function is preserved and there are no contraindications to liver surgery.

In earlier publications, a rather small proportion of monolobar disease (20%-25%) was described, with almost 90% of these cases located in the left hepatic lobe. Due to small patient numbers, the prevalence of localized CD may have been underestimated. Recent studies indicate higher percentages (80%) of patients with localized disease[30,42] and a more variable distribution of disease between the left and right lobes.

TRANSPLANTATION

Diffuse forms of type V cysts (CD and CS) remain a therapeutic problem. In patients with these cysts, combined procedures with partial hepatectomy and biliodigestive anastomoses[44,45] have been described, but transplantation offers the only curative option. The progression of congenital hepatic fibrosis in CS and the development of secondary biliary cirrhosis in patients with CD may also lead to portal hypertension that is not treatable by conservative means. De Kerckhove *et al*[46] reported congenital hepatic fibrosis in 27% of their patients and the primary indication for orthotopic liver transplantation was recurrent cholangitis (90%).

An important argument for liver transplantation is the avoidance of cholangiocarcinoma development[47]. Concerns include the choice of an appropriate time point for transplantation, procedural risks, and the use of immunosuppression in young and otherwise healthy individuals. Potential postoperative complications include rejection and vascular thrombosis[46]. Pre-transplant workup of these patients for occult cholangiocarcinoma is crucial.

In patients with associated polycystic kidneys and renal failure, immunosuppression after kidney transplantation may predispose to severe cholangitis, and therefore, combined liver/kidney transplantation should be considered. The results of liver transplantation for diffuse forms of type V cysts compare favorably with those of transplantations for other indications. In the review carried out by Millwala *et al*[48], the overall graft and patient survival rates at 1, 3, and 5 years were 79.9%, 72.4% and 72.4%, and 86.3%, 78.4% and 77%, respectively; living donor transplantation was performed in 3.8% of cases. In the single-center study by Habib *et al*[49], overall graft and patient survival rates at 1, 5, and, 10 years were reported as 73%, 62%, and 53%, and 76%, 65% and 56%, respectively.

In conclusion, the diagnostic term "bile duct cyst" comprises quite different pathological and clinical entities. Interventional therapy, hepatic resection, and transplantation all have their place in the treatment of this heterogeneous disease group. They should not be seen as competitive, but complementary options. In spite of several shortcomings, the modified Todani classification offers a basis for treatment planning and for comparing results reported in the literature. Each patient, however, should receive tailored individual treatment after all findings have been discussed by an interdisciplinary team.

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REVIEW

MicroRNAs and liver cancer associated with iron overload: Therapeutic targets unravelled

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Abstract

Primary liver cancer is a global disease that is on the increase. Hepatocellular carcinoma (HCC) accounts for most primary liver cancers and has a notably low survival rate, largely attributable to late diagnosis, resistance to treatment, tumour recurrence and metastasis. MicroRNAs (miRNAs/miRs) are regulatory RNAs that modulate protein synthesis. miRNAs are involved in several biological and pathological processes including the development and progression of HCC. Given the poor outcomes with current HCC treatments, miRNAs represent an important new target for therapeutic intervention. Several studies have demonstrated their role in HCC development and progression. While many risk factors underlie the development of HCC, one process commonly altered is iron homeostasis. Iron overload occurs in several liver diseases associated with the development of HCC including Hepatitis C infection and the importance of miRNAs in iron homeostasis and hepatic iron overload is well characterised. Aberrant miRNA expression in hepatic fibrosis and injury response have been reported, as have dysregulated miRNA expression patterns affecting cell cycle progression, evasion of apoptosis, invasion and metastasis. In 2009, miR-26a delivery was shown to prevent HCC progression, highlighting its therapeutic potential. Several studies have since investigated the clinical potential of other miRNAs with one drug, Miravirsen, currently in phase Ⅱ clinical trials. miRNAs also have potential as biomarkers for the diagnosis of HCC and to evaluate treatment efficacy. Ongoing studies and clinical trials suggest miRNA-based treatments and diagnostic methods will have novel clinical applications for HCC in the coming years, yielding improved HCC survival rates and patient outcomes.

Key words: MicroRNAs; Liver cancer; Iron regulation; Hepatitis C; Therapeutic targets

Core tip: Hepatocellular carcinoma (HCC) has a high incidence and low survival rate, largely attributable to late diagnosis, resistance to treatment, tumour recurrence and metastasis. MicroRNAs (miRNAs) are regulatory RNAs that modulate protein synthesis and are involved in several biological and pathological processes including the development and progression of HCC. miRNAs represent important new targets for therapeutic intervention for HCC and have potential as diagnostic and prognostic HCC biomarkers. Ongoing studies and clinical trials suggest miRNA-based treatments and diagnostic methods will have clinical applications for HCC in the coming years, yielding improved HCC survival rates and patient outcomes.

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INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for 85%-90% of primary liver cancers; it ranks as the fifth most common cancer worldwide and the third leading cause of death from malignancy[1]. The development and progression of HCC is a multistage process, with transformation typically beginning in hepatocytes of livers undergoing chronic hepatitis or cirrhosis[2]. The major risk factor for HCC is chronic hepatitis due to infection with the hepatitis B or hepatitis C virus (HBV/HCV) accounting for 80%-90% of all HCC cases worldwide[3]. The other most important risk factors for hepatocarcinogenesis are alcoholic and non-alcoholic steatohepatitis-associated cirrhosis; less common risk factors include genetic conditions such as hereditary haemochromatosis (HH), alpha-1 antitrypsin deficiency[4,5] and aflatoxin B1 intake. Regardless of the underlying risk factor, hepatocytes progress through several hyperplastic and dysplastic stages before eventually acquiring a malignant phenotype, with subsequent intrahepatic metastasis and distant spread of HCC cells[6]. The 5-year survival rate of patients with HCC remains quite low, between 6%-11%. This is attributable to late diagnosis, resistance to treatment, tumour recurrence and metastasis[2].

Previously, studies investigating HCC development and progression have focused on the therapeutic potential of targeting various genes and proteins[7]. However, a new group of regulatory RNA molecules has more recently been identified, called microRNAs (miRNAs). Involvement of miRNAs in HCC development and progression has been demonstrated; as such miRNAs have considerable diagnostic and therapeutic potential for HCC. Here, the role of miRNAs in the pathogenesis of HCC is reviewed with a focus on their regulation of iron homeostasis and in the setting of iron overload, a common pathological event observed in several liver diseases associated with HCC development. The relevance of miRNAs to HCC progression with regard to hepatic fibrosis and response to injury, as well as their contribution to cell cycle progression, evasion of apoptosis and metastasis is explored. Finally, the potential diagnostic and therapeutic value of miRNAs in HCC is discussed.

miRNAs

miRNAs are endogenous single stranded RNAs, approximately 22 nucleotides in length. They are non-coding but are important post-transcriptional regulators of gene expression. miRNAs were first discovered in 1993, and since then the considerable extent of the gene regulatory capacity of miRNAs has been investigated. These investigations have demonstrated that specific miRNAs have central roles in critical biological processes such as development, cell proliferation, apoptosis and oncogenesis. The mechanisms of action and biogenesis of miRNAs have been reviewed in detail[8,9].

Mature miRNAs enter the RNA-induced silencing complex (RISC) in the cytosol. In this complex miRNA can post-transcriptionally regulate gene expression. Their mechanism of action is determined by the level of complementarity between the miRNA and the 3'-untranslated region (UTR) target on the mRNA. In perfect complementarity, miRNA-mRNA binding induces mRNA cleavage and degradation by RISC. In imperfect complementarity, miRNA-mRNA binding represses target mRNA translation[10]. Occassionally, miRNAs can upregulate translation even in conditions of growth arrest[11]. However translation is more commonly inhibited and the target mRNAs are eventually degraded in cytoplasmic processing bodies[12].

Functional target sites on mRNAs usually consist of a 6-8-nt long sequence complementary to the miRNA sequence (followed by an adenosine), this is termed the miRNA "seed" sequence and is located at the 5' end of the miRNA[13]. The complementary sequence commonly referred to as a miRNA recognition element (MRE) is usually located in the 3'-UTR of the target mRNA. Some recent studies have shown miRNAs can also bind to MREs located in the 5'-UTR or the open reading frame[14-17]. Unusually miRNAs can act as decoys and bind to ribonucleoproteins independent of a seed sequence and RISC, thus interfering with roles requiring mRNA binding[18].

Given the considerable potential for variety in miRNA-mRNA interaction, it is not surprising that a single miRNA can target several genes[19-22]. In addition, approximately 60% of mRNAs carry at least one evolutionarily conserved MRE. Bioinformatic analysis predicts that the 3'-UTR of a single transcript is often targeted by several miRNAs, a prediction that has been validated experimentally for many genes[22]. The complex, widespread and cooperative regulation of gene expression by miRNAs is an important consideration when studying normal and pathological processes in terms of understanding the processes themselves and identifying potential biomarkers. Recently investigators have begun to study the role of miRNAs in the pathogenesis of HCC. In particular, several studies have demonstrated a role for miRNAs in HCC development and progression, wherein the importance of miRNAs in iron homeostasis and hepatic iron overload were highlighted.

Many risk factors underlie the development of HCC and one process commonly altered is iron homeostasis. Iron overload in the liver occurs in several liver diseases associated with the development of HCC, including chronic hepatitis due to HCV infection and also due to genetic conditions such as HH. Hepatic iron overload is an independent risk factor for the development of HCC[23] and emerging evidence points towards miRNAs as central regulators of iron homeostasis

miRNAs, HCC AND IRON OVERLOAD

Hepatic iron overload and HCC

Hepatocytes act as the principal site of iron storage in the body, storing iron as ferric oxyhydroxyapatite in the core of ferritin. During iron overload, the ability of hepatocytes to safely sequester iron is exceeded, denaturation of

ferritin subunits occurs leading to ionic iron release into the hepatocyte cytoplasm[24]. The effects of hepatic iron overload have been particularly well studied in patients with the inherited iron metabolism disorder, HH and in Africans with dietary iron overload.

Patients with HH, without timely appropriate treatment, almost always develop hepatic fibrosis and cirrhosis due to hepatic iron accumulation[25]. Similarly patients with African dietary iron overload can develop cirrhosis, albeit less often[26,27]. HCC is a potential complication in untreated HH patients associated with premature death[28,29]. Comparison studies have showed that cirrhosis plays a role in the development of HCC in HH[30,31] however, HCC can also develop in HH patients without cirrhosis, albeit rarely[32-37]. Together this suggests that hepatic iron storage could directly contribute to HCC development[38,39], in addition to its indirect effect as a cause of cirrhosis. This concept is in keeping with a study comparing cirrhosis incidence in HH and non-iron related liver diseases, where the risk of HCC was greater in HH[40]. Interestingly, despite HCC initially being thought not to occur in dietary iron overload, three case/control studies have demonstrated a causal association between African dietary iron overload and HCC, even after allowing for the confounding effects of cirrhosis, chronic HBV and HCV infection and prolonged aflatoxin B1 exposure[41-43]. Dietary iron overload resulting in HCC has also been reported in animal models[44,45] supporting the directly hepatocarcinogenic effects of hepatic iron accumulation.

HCC can also develop with other causes of hepatic iron accumulation namely, thalassaemia major, sideroblastic anaemia and hereditary spherocytosis[46-48]. Lesser degrees of hepatic iron accumulation are seen in other liver diseases, such as chronic HCV hepatitis and alcoholic liver disease. Nonetheless, it is thought to have an important role in these diseases[24]. One area of recent interest is hepatic iron accumulation with HCV infection. As the main risk factor for HCC development, HCV is particularly relevant to HCC. Iron promotes the initiation of HCV translation by increasing expression of eukaryotic initiation factor 3a and La protein, whereas inhibiting expression of these proteins suppresses HCV translation[49,50]. Interestingly the expression of the chief iron regulatory hormone, hepcidin, is suppressed in chronic HCV infected patients. Given that hepcidin expression has direct anti-viral activity against HCV in cell culture[51] this represents an exciting area of ongoing research.

Hepatic iron accumulation has also been implicated in non-alcoholic fatty liver disease (NAFLD). Hyperferritinemia is associated with higher hepatic iron and fat content in NAFLD[52], and is also an independent predictor of liver damage in NAFLD patients[53]. As altered iron trafficking is frequent in patients with NAFLD, one recent study investigated the role of the Ala736Val polymorphism of TMPESS6 (an inhibitor of hepcidin expression) in NALFD-associated hepatic iron accumulation[54]. Homozygosity for this polymorphism was associated with low hepatic iron stores and was negatively associated with hepatic iron accumulation independent of age, gender, human haemochromatosis (HFE) genotype and beta thalassaemia trait.

Pathogenesis of HCC in hepatic iron overload

A recent animal study examined the long-term effects of iron overload in HCC[44]. A high-iron diet was given to Wistar albino rats over 16 mo to induce hepatic iron overload. Altered hepatic foci developed in many animals by 20 mo. By 28 mo, these foci were more numerous and had become identical to the iron-free preneoplastic nodules seen in HH patients who develop HCC[55]. HCC was evident at 32 mo in the absence of portal fibrosis or cirrhosis. The mechanisms by which free iron induces hepatocarcinogenesis are not yet fully characterised but are likely due to the generation of reactive oxygen intermediates (ROI) and oxidative stress which damages DNA, lipids, and proteins resulting in both necrosis and apoptosis within hepatocytes[56-60]. Oxidative DNA damage correlates with cell immortalisation in HCC through induction of telomerase activity. This process has been associated with miR-92 over expression, a miRNA affecting specific cell proliferation and apoptosis pathways[61]. Iron overload leading to lipid peroxidation is also thought to contribute to HCC development[62-66]. Moreover, excess hepatic iron may induce immunologic alterations, leading to impaired immune surveillance of malignant transformation. Nontransferrin-bound iron can markedly suppress lymphocyte proliferation[67]. The same study showed that ferritin can inhibit lymphocyte proliferation. Indeed, the presence of both iron and ferritin were found to significantly reduce the tumouricidal function of macrophages[68]. In addition to its solitary effects, iron overload can act in tandem with other HCC risk factors to produce hepatocarcinogenesis. For example, dietary iron overload and aflatoxin B1 exposure have superadditive effects on mutagenesis rates[69]. Furthermore ROI generation and mutagenesis are synergistically increased in animal models with both risk factors, leading to greater DNA damage[70-73].

Control of cellular iron uptake by miRNAs: Most cells obtain iron from plasma *via* iron-bound transferrin (Tf-Fe2) uptake. Tf-Fe2 binds to TfR1 on the cell surface and the complex is internalised by clathrin-dependent endocytosis. Acidification of early endosomes aids iron release from transferrin[74], so that it can be reduced to Fe^{2+} by metalloreductases[75]. Transport into the cytoplasm occurs *via* endosomally-expressed Divalent metal transporter 1 (DMT1). Cell surface TfR1 levels reflect cellular iron requirements, with regulation of TfR1 expression mainly achieved by the IRE/IRP regulatory system[76]. However, recent studies have shown that the transferrin cycle is also controlled by miRNAs, at two separate steps (Figure 1A).

Cancerous cells have elevated TfR1 expression to meet the increased iron requirements of rapid cellular proliferation[77,78]. Conversely, differentiation of a human

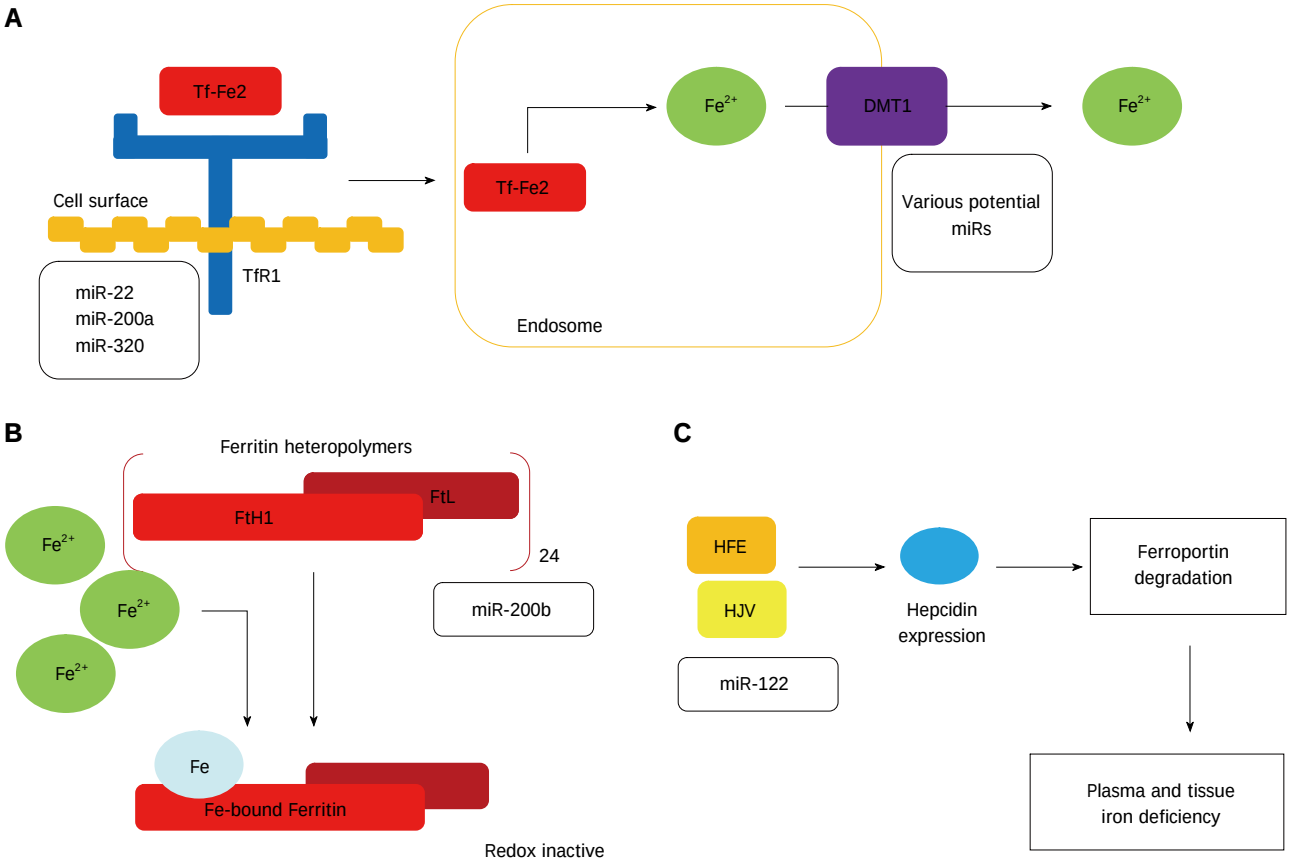

Figure 1 Effect of microRNAs on iron uptake, storage, and systemic regulation. A: Iron-bound transferrin (Tf-Fe2) binds to the transferrin receptor TfR1 which is regulated by microRNA (miR)-22, miR-200a and miR-320. The complex is endocytosed leading to release of iron, its reduction to Fe^{2+} and transport to the cytoplasm *via* DMT1 which may be regulated by various miRNAs; B: miR-200b regulates ferritin heavy (FtH1) and light (FtL) chains. Ferritin polymers containing 24 subunits detoxify excess iron *via* FtH1's ferroxidase activity and store intracellular iron; C: Levels of human haemochromatosis (HFE) protein and hemojuvelin (HJV) are regulated by miR-122, the levels of which are decreased in hereditary haemochromatosis. Reciprocal increases in HFE and HJV, in turn, enhance expression of hepcidin leading to decreased iron absorption due to degradation of ferroportin. DMT: Divalent metal transporter.

leukaemia cell line decreases TfR1 expression[79]; this is accompanied by reciprocal increases in miRNAs predicted to bind to the TfR1 3'-UTR (miR-22, miR-200a and miR-320). Of these, miR-320 was demonstrated to suppress the activity of a luciferase reporter vector under the control of the TfR1 3'-UTR[80]. Similarly, enforced miR-320 expression in a lung carcinoma cell line can reduce TfR1 expression and slow cell cycle progression and cell growth. This growth inhibitory effect can be reversed by treatment with a soluble iron solution suggesting that reduced TfR1 expression in miR-320-overexpressing cells lowers iron availability and reduces cell proliferation[81]. Currently, it is unknown whether miR-320-mediated TfR1 regulation is limited to cancer cells or whether it has a role under normal physiological conditions.

In addition to miRNA-dependent TfR1 regulation, miRNAs control the transferrin cycle at the release of iron from the endosome *via* DMT1. The gene coding for DMT1 (*SLC11A2*) produces four variant mRNA transcripts. These differ either at their 5' end due to alternative promoter usage (DMT1A and 1B isoforms), or at the 3' end, due to alternative splicing determining the presence or absence of an IRE sequence motif[82]; only the IRE-containing isoforms are controlled in response to cellular iron levels by IRP binding[83]. All DMT1 isoforms can transport iron and, with the exception of the duodenal 1A isoform, are ubiquitously expressed[84]. Of note, miRNA-controlled DMT1 expression by let-7d can contribute to the uptake of non-transferrin bound iron[85]. Further studies are needed to determine how miRNA-dependent control of DMT-1 expression is integrated with additional DMT-1 control mechanisms.

Importantly, as miRNA maturation requires iron in the form of heme[86], the finding that miRNAs control cellular iron uptake suggests a possible regulatory loop in which iron is needed for the efficient synthesis of mature miRNAs, while certain mature miRNAs control cellular iron uptake.

Control of cellular iron storage by miR-200b: Ferritin heteropolymers consist of 24 subunits of heavy (FtH1) and light (FtL) chains that bind iron from the cytoplasmic "labile iron pool"[87]. The FtH1 subunit has ferroxidase activity necessary for iron deposition in ferritin. Ferritin detoxifies excess iron into a redox-inactive form, preventing chronic oxidative stress and subsequent cell and

tissue damage. Ferritin also acts as an intracellular iron store mobilised *via* proteasomal and lysosomal degradation. One recent study showed that human breast cancer cells with an aggressive mesenchymal phenotype express significantly higher FtH1 and FtL mRNA and protein levels and have a smaller labile iron pool compared to breast cancer cells with a less aggressive epithelial phenotype[88]. High FtH1 concentrations correlated with low miR-200b expression, a miRNA that binds both FtH1 and FtL 3'UTRs (Figure 1B). Of clinical relevance, miR-200b transfection improved sensitivity of breast cancer cells to doxorubicin. Additionally, patients with higher plasma ferritin levels showed worse treatment outcomes, emphasising the clinical significance of this facet of iron regulation. These findings suggest that down regulation of miR-200b in human breast cancer contributes to increased cancer aggressiveness. Whether FtH1 and FtL are regulated by miR-200b in hepatocytes and if this has implications for HCC remains to be determined[89,90].

Control of systemic iron regulation by miR-122: The liver regulates systemic iron homeostasis *via* hepcidin and monitors systemic iron availability through genes involved in HH (*e.g.*, HFE, hemojuvelin and TfR2), the bone morphogenetic protein (Bmp) 6 and the Smad4 protein. These all function in the regulation of hepcidin transcription. Low hepcidin activity due to mutations in HFE, hemojuvelin, TfR2 or hepcidin itself lead to the development of HH which is associated with increased iron uptake from the diet and increased iron release from macrophages.

miR-122 is selectively expressed in the liver. One recent study demonstrated that miR-122 expression is reduced in a mouse model of HFE-mutated HH[91]. Depletion of miR-122 in wild type mice led to low systemic iron levels, decreased plasma iron levels and lower transferrin iron binding capacity. These events in turn resulted in an insufficient iron supply to erythroid cells and a mild impairment of haematopoiesis[91]. Furthermore, the iron contents of the liver and spleen were also reduced. Interestingly, miR-122 depletion altered systemic iron homeostasis through changes in the level of expression of genes involved in the sensing of systemic iron levels (*i.e.*, HFE, Hemojuvelin, and Bmpr1a), as well as genes that transmit signals *via* the Bmp/Smad signalling pathway, to regulate hepcidin transcription[91]. This study also validated HFE and hemojuvelin as direct targets of miR-122 (Figure 1C).

This suggests a miR-122-dependent regulatory loop that controls systemic iron homeostasis whereby depletion of miR-122 derepressed HFE and hemojuvelin expression, in turn increasing hepcidin transcription. As a result, high circulating hepcidin levels can enhance the degradation of ferroportin on target cells, leading to lower iron absorption from the diet and iron release from macrophages. This likely leads to plasma and tissue iron deficiency, with mild impairment of erythropoiesis. miR-122 levels are not regulated as a result of iron accumulation in the liver of HH patients, but more likely as a consequence of the signalling activities reduced by a lack of HFE which is known to attenuate BMP/Smad signalling in HH patients and its respective murine disease model[92].

The finding that miR-122 regulates systemic iron homeostasis is one of a growing number of functions known for this liver-specific miRNA. For example, miR-122 is necessary for HCV infection and replication, as well as for responsiveness to interferon therapy[93-95], all processes involving alterations in iron homeostasis[96]. miR-122 levels are reduced in cirrhosis[97] and HCC[98,99], two pathologies known to be exacerbated by increased liver iron levels[24]. Evidently, miRNAs have an important role in the maintenance of iron homeostasis, given their roles in controlling the level of cellular uptake of iron-bound transferrin, iron storage by ferritin, and hepatic control of systemic iron levels *via* hepcidin (Figure 1). Furthermore, tissue iron overload causes oxidative stress that itself has been shown to alter miRNA expression[100,101].

Overall, these findings suggest that miRNAs control large regulatory networks that link microenvironmental stress, such as oxidative stress and hypoxia to the regulation of iron metabolism. As the maintenance of iron homeostasis is critical for many essential cellular functions, it is expected that several more miRNAs that directly or indirectly control iron-related genes will be discovered. Given the role of miRNAs in regulating iron homeostasis and the significance of iron overload to the development of HCC, miRNAs likely play an important role in the pathogenesis of HCC (Table 1). However, further studies elucidating the full extent of miRNAs' functions in iron homeostasis under normal conditions are needed to improve our understanding of the role of miRNAs in pathologies such as HCC.

miRNAs AND HCC PROGRESSION

In HCC miRNAs can act as oncogenes, promoting hepatocyte progression to HCC, or as tumour suppressors, preventing this process[2]. Increased oncogenic miRNA levels result in reduced translation of their gene targets, contributing to HCC development and progression. By contrast, miRNAs acting as tumour suppressors prevent the expression of their oncogenic targets and hence the downregulation of such miRNAs permits greater expression of these oncogenic genes, again contributing to HCC development and progression. Progression from normal hepatocytes to HCC is a multistage process. Several changes in the liver structure and in normal cell processes must occur for this progression to continue, mediated in part by altered miRNA expression profiles. These include liver fibrosis and hepatic stellate cell-mediated liver regeneration, while at the molecular level changes in cell cycle progression, susceptibility to apoptosis and capacity for invasion and metastasis are needed.

Liver fibrosis, hepatic stellate cells and liver regeneration

miRNA expression profiles show considerable overlap

Table 1 MicroRNAs with a role in hepatocellular carcinoma

miRNAs	Function	Outcomes
miR-22 miR-200a miR-320	Predicted to bind iron-bound transferrin receptor (TfR1)	Targets TfR1, DMT1 expression thereby inhibiting cell cycle progression and growth
miR-200b	Targets ferritin heteropolymers (FtH1, FtL)	Decreased miR-200b linked with enhanced cancer aggressiveness *via* increased iron indices
miR-122	Targets HFE, hemojuvelin, BMPr1a, BMP/SMAD signalling, hepcidin	Control of systemic iron homeostasis. Decreased miR-122 corresponds to decreased HFE and hemojuvelin expression. This correlates with increased hepcidin expression

HCC: Hepatocellular carcinoma; miR/miRNA: MicroRNA; DMT: Divalent metal transporter; HFE: Human haemochromatosis; BMP: Bone morphogenetic protein; FtH: Ferritin heavy; FtL: Ferritin light.

in fibrotic disorders. The most significant mediators are the miR-29 family, important in regulating translation of extracellular matrix components and effectors of cellular differentiation[102]. Also important are miRs affecting translation of proteins involved in the pro-fibrotic transforming growth factor (TGF)-β/SMAD signalling pathway. Microarray analyses in a CCl_4 rodent model of hepatic fibrosis have shown 31 differentially expressed miRs, 10 of which are over expressed in fibrotic tissue including miR-125-p, -199b, -221 and -302c[103]. This same study revealed a significant down regulation in 21 miRs, most notably the miR-29 family. Down regulation of miR-29b and miR-29c was independently confirmed in a bile duct ligation model and similar observations for miRs-29a/b/c have been reported in humans liver tissue samples of patients with a Desmet fibrosis score of 2-4[104].

Hepatic fibrosis is also affected by miR-132 levels. In two different models of hepatic fibrosis (BDL and CCl_4), where a significant reduction in miR-132 levels was observed, this down regulation was found to alter the activity of hepatic stellate cells (HSCs). HSCs are the main effector cells of hepatic fibrosis, acting as the primary source for type I collagen deposition following liver injury. HSC activation occurs in response to hepatic insults including viral infection, alcohol consumption and obesity. During their activation, quiescent lipid-rich cells are transdifferentiated into fully activated myofibroblasts. The activated cells can secrete pro-fibrogenic mediators such as TGF-β, and produce extracellular matrix components[105]. Involvement of miRNAs in the process of HSC activation has been demonstrated. For example, let-7 family members are significantly up regulated in HSCs of BDL animals whereas miR-150, -187, -194 and -207 are down regulated[106]: over expression of miR-150 and miR-194 in human HSCs can inhibit HSC proliferation and prevent HSC transdifferentiation[106]. miR-150 together with another miR, miR-94, inhibits c-Myb and Rac-1, two proteins involved in pathways contributing to hepatic fibrosis development and progression. Further studies investigating differential miRNA expression in quiescent and activated rat HSCs showed that miR-15b and miR-16 are also implicated in HSC activation[107,108]. This process is also regulated by miR-27a and b which are up regulated and in turn repress RXRα[109]. Of interest miR-132 activates the methylCpG binding protein MeCP2 and components of the polycomb repressive process. Down regulation of miR-132, as seen in hepatic fibrosis, permits MeCP2 translation. This protein is subsequently recruited to the 5'UTR of PPARγ mRNA and through alteration of methylation patterns suppresses the quiescent profile of HSCs[110] - this is an example of a miRNA acting as an activator rather than an inhibitor of gene expression. Thus as our understanding of the role of miRNAs in the regulation of HSC differentiation improves so will the understanding of liver pathology and hepatic responsiveness to injury.

Cell cycle progression

Aberrant cell cycle control is necessary for the development and progression of all human cancers, including HCC. Cell cycle regulation by oncoproteins and tumour suppressors is often defective resulting in increased cell proliferation. miRNAs targeting the main proliferation pathways have been identified in HCC. These miRNAs exert their effects through an interaction with essential regulators of the cell cycle, including cyclin-dependent kinase enzyme (CDK) complexes, Cip/Kip family proteins which act as cell cycle inhibitors, and the phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway, among others.

Cyclins are positive cell cycle regulators, controlling cell cycle stage advancement *via* activation of CDKs. Cyclin D2 and E2, mediators of cell cycle arrest, are directly targeted by miR-26a; low miR-26a levels are frequently found in HCC[111]. Modulation of cyclin G1 affects transcriptional activity and p53 protein stability, resulting in reduced G2-M phase and lower invasive capacity of HCC cells[112]. miR-122 inhibits hepatocyte growth by targeting cyclin G1 expression, however it is barely detectable in primary human HCC[113]. Levels of miR-122 are determined by several key regulatory molecules, including the transcription factors HNF1A, HNF3A and HNF3B[114]. Low miR-122 correlates with high serum response factor, a validated miR-122 target and important promoter of tumour development[115]. Expression of miR-195 is also reduced in HCC. Normally it regulates expression of cyclin D1, CDK6 and EnF3 however in its absence there is a failure to induce cell cycle arrest at the G1-S checkpoint[116]. CDK6 is also targeted by miR-124, a miRNA which blocks G1-S transition. miR-124 is silenced in HCC by CpG methylation, as is miR-203[117].

Another method by which oncogenic miRNAs contribute to cell cycle progression is *via* inhibition of cyclin-dependent kinase inhibitors (CDKIs), most notably the members of the Cip/Kip family. Both miR-106b and miR-93 are overexpressed in HCC and directly target

p21 and promote cell cycle progression[118]. miR-221 and miR-222 both inhibit expression of p27 mRNA, another member of the Cip/Kip family[119] whilst miR-221 also regulates the CDKI p57[120]. Direct targeting of these two CDKIs leads to greater numbers of HCC cells in the S-phase thus promoting cell growth.

PI3K has an important role in balancing cell survival and apoptosis. Its activation leads to increased cell growth *via* phosphorylation of mTOR by AKT kinase, an effect that is inhibited by PTEN. mTOR is a target of miR-199a-3p; restoring normal levels of miR-199a-3p can cause cell cycle arrest in HCC by blocking the G1-S transition, sensitising cells to doxorubicin[121]. miR-221 and miR-222, in addition to their effect on p27 also target DNA damage-inducible transcription factor 4 (DDIT4), a modulator of mTOR signalling[122]. PTEN is directly targeted by miR-21, -221 and -222; all three are often found to be overexpressed in HCC[123,124]. As such, suppression of PTEN resulting in increased PI3K/AKT pathway activation is an important mediator of HCC cell survival.

Other important cell cycle regulators are known targets of aberrantly-expressed miRNAs in HCC. Let-7g down regulates c-Myc, an oncogenic transcription factor. This suppresses HCC cell proliferation through reduced c-Myc-induced miR-17-92 transcription, a tumour-promoting miR[125,126]. Others such as miR-1[127] and miR-375[128] suppress HCC cell proliferation whereas miR-18a stimulates proliferation *via* targeting the ESR1 gene thereby preventing oestrogen's protective effects against HCC in females[129].

These studies emphasise the important role that miRNAs have in the progression of HCC by regulating oncogenes and tumour suppressors, and a number of miRNAs have now been identified in this context.

miRNAs: INVASION, METASTASIS AND APOPTOSIS

Evasion of apoptosis

Evasion of apoptosis is another key step in malignant transformation and tumour progression. This allows cells to escape normal surveillance mechanisms, enabling continued survival in the tumour microenvironment. The tumour suppressor gene *p53* increases miR-34 expression leading to cell cycle arrest and apoptosis, whereas low miR-34 levels, as are frequently seen in HCC, are believed to contribute to apoptosis evasion[130-133]. miRNAs directly target the Bcl-2 family of genes, their proteins being either pro-apoptotic (Bim, Bmf, Bax, Bak, Bid) or anti-apoptotic (Bcl-2, Bcl-W, Bcl-XL, Mcl-1)[134]. miR-122 and let-7b regulate Bcl-w and Bcl-XL, respectively, whilst Mcl-1 is regulated by miR-101 and miR-29[104,135-137]. Reduced levels of all of these miRNAs are often seen in HCC thus increasing resistance to apoptosis. Bcl-2 is also targeted by miR-29[104]; increasing miR-29 levels can sensitise HCC cells to pro-apoptotic signals, a finding of great therapeutic application potential. With respect to miRNA regulation of pro-apoptotic Bcl-2 family members, miR-221 and miR-25 are commonly over expressed in HCC and target Bmf and Bim, respectively[138,139]. miRNAs can also target other apoptosis-related genes. miR-602 is increased in HBV-related HCC, it targets RASSF1A to exert an anti-apoptotic effect[140].

Invasion and metastasis

Invasion and metastasis are two hallmarks of cancers and the leading causes of cancer-related mortality. Survival rates after curative resection of HCC are still poor due to high recurrence secondary to intrahepatic metastasis. Given this, a better understanding of the mechanisms underlying invasion and metastasis is critical to improvements in patient survival. Several metastasis-related genes important in HCC have been identified, and with them, several miRNAs promoting and preventing metastasis in HCC.

miRNAs promoting metastasis: As mentioned, levels of miR-21, -221 and -222 are increased in HCC[124]. These miRNAs directly target PTEN, contributing to cell growth but also mediating cell invasion. miR-221 and miR-222 also modulate the expression of TIMP3 and phosphatase 2A subunit B (PPP2R2A), thereby preventing inactivation of metalloproteases, important enzymes involved in cell migration and invasion, and activating the PI3K pathway[124,141]. miR-181b is induced by TGF-β and also targets TIMP3 on a functional level, increasing MMP2 and MMP9 activity[142]. The TGF-β-mediated metastasis pathway is well characterised, and this TGF-β/miR-181/TIMP3 axis may be an important component. One study has also shown a novel miRNA, miR-143 is induced by NFκB, promoting metastasis of HBV-related HCC by inhibiting expression of fibronectin[143]. High miR-17-5p levels are often found in HCC. This miRNA activates p38 mitogen-activated protein kinase and leads to greater heat shock protein 27 phosphorylation thereby promoting HCC invasion[144].

The chromosomal region 8q24 is implicated in metastasis in HCC. Two frequently amplified miRNAs contained within, miRNA-30d and miRNA-151, are involved in HCC invasion and metastasis[145,146]. An increased miR-30d expression is frequently seen in HCC enhancing metastasis through repression of G-αi2. This can contribute to metastasis both within the liver and to the lung. RhoGDIA, thought to be a suppressor of HCC metastasis is targeted by miR-151; with subsequent activation of Rac1, Cdc42 and Rho GTPases enhancing cell migration and invasion[134]. Moreover, this miRNA is often co-expressed with host gene focal adhesion kinase (FAK); it can function synergistically with FAK to increase HCC cell motility and spread[134].

miRNAs preventing metastasis: ADAM10 (a distintegrin and metalloprotease family 10), serum response factor (SRF), and insulin-like growth factor 1 receptor (Igf1R) promote tumorigenesis. These are validated targets of

Table 2 MicroRNAs as potential diagnostics and therapeutics for hepatocellular carcinoma

miRNAs	Detail	Relevance
miR-221	4.8 fold higher in HCC patients, positively correlates with cirrhosis, tumour size and stage. Negatively correlates with overall survival	Potential circulating biomarker
miR-199a	Reduced and significantly associated with HCC	Potential circulating biomarker
miR-16	Reduced and significantly associated with HCC	Potential circulating biomarker
miR-26	Low levels associated with high IL-6 and shorter survival	Potential biomarker to assess prognosis of HCC
miR-375	Lower than normal levels associated with β-catenin mutation	Potential for HCC classification system, determine treatment allocation
miR-107	Reduced levels associated with HFN 1α	Potential for HCC classification system, use to determine treatment allocation
miR-122	Expression inhibited using Miravirsen LNA-modified oligonucleotides	Direct effect in chimpanzee model in reducing HCV replication and viraemia
miR-196	Selective target for intervention	Implications for treatment
miR-26a	Deliverable to HCC sites using adeno-associated virus serotype 8	Decreased proliferation and induced tumour-specific apoptosis
miR-124	Induces tumour-specific apoptosis	Prevents and suppresses HCV development in murine model

HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; miR/miRNA: MicroRNA; IL: Interleukin.

miR-122 and their expression is up regulated in primary human HCC due to decreased miR-122 levels[115,147]. Metastatic HCCs also show significantly lower let-7g levels, a miRNA that targets type I collagen a2 and when present at normal levels should prevent HCC spread[148].

The hepatocyte growth factor (HGF)/c-Met signalling cascade is considered a key pathway in HCC metastasis[134]. HGF interacts with the c-Met receptor tyrosine kinase to increase cell motility and invasion, while also conferring apoptotic protection. c-Met is associated with aggressive HCC and poor outcomes, and is regulated by miR-1, -34a, -23b and -199-3p levels of which are low in HCC[134]. Silencing of miR-1 inhibits HCC cell growth, and increases cell invasion, through c-Met down regulation[127]. Ectopic expression of miR-34a prevents HCC invasion and migration by reducing c-Met-induced phosphorylation of extracellular signal-related kinases 1 and 2 in HepG2 cells[149]. Likewise, over expression of miR-23b reduces levels of c-Met and urokinase-type plasminogen activator, a downstream target of HGF/c-Met signalling; this inhibits HCC proliferation and migration[150]. Regulation of cell cycle progression by restoring miR-199-3p levels to normal leads to induction of G1-phase cell cycle arrest (miR-199-3p targets c-Met and mTOR) thereby decreasing HCC cells' invasive ability[121]. Finally, miR-101 is also downregulated in HCC and reduces HGF-induced cell invasion and migration *via* inhibition of FOS oncogene expression[151]. Taken together these studies highlight how miRNAs control the central processes of invasion, metastasis and apoptosis that contribute to malignant transformation and tumour progression.

miRNAs AS DIAGNOSTICS FOR HCC

miRNAs are predominantly down regulated in tumour tissues[152], a pattern also seen in HCC. Several issues affect the identification and quantification of aberrantly expressed miRNAs in clinical samples confounding their potential as biomarkers. Despite these issues, several consistently dysregulated miRNAs have been identified in HCC (Table 2). Numerous studies have shown that circulating miRNA levels are altered in HCC progression. For example, serum miR-221 concentrations are 4.8-fold higher in HCC patients; high miR-221 levels correlate positively with cirrhosis, tumour size and tumour stage, and negatively correlate with overall survival[153]. Currently, there are few clinically useful serum HCC markers; α-fetoprotein (AFP), Lens culinaris agglutinin-reactive AFP (AFP-L3) and des-γ-carboxyprothrombin (DCP) are of limited use[154]. The American Association for the Study of Liver Diseases discarded AFP as a marker for HCC surveillance and diagnosis in its July 2010 Practice Guidelines, highlighting the need for new biomarkers. miRNAs may have this potential. However, their use is complicated by the need for appropriate controls, as HCC usually develops from an underlying liver condition. For example, one study compared the miRNA expression profiles of three patient groups: one with HCC, one with chronic liver disease and one consisting of normal controls[155]. This study also showed that serum miR-16 and miR-199a concentrations were reduced and significantly associated with HCC[155]; of potential clinical relevance, miR-16 was more sensitive for detection of HCC than the three currently used biomarkers. Overall, these findings show the feasibility of miRNAs as serum markers for diagnosis of HCC. Should they continue to outperform current HCC markers in further studies, circulating miRNAs could be used in first-line testing of HCC patients. However, the study of circulating miRNAs as HCC biomarkers is a relatively recent concept, with further studies and validation of results in larger patient cohorts needed before miRNAs are used in the clinical setting. In particular, the discovery of a miRNA which sensitively and reliably diagnose early stage HCC would greatly enhance their potential for clinical use.

miRNA expression profiles can also be used to assess prognosis. For example, low miR-26 expression is associated with high interleukin-6 expression and shorter survival[156]; better response to interferon treatment also occurs in patients with low miR-26 levels. Furthermore, a 20-miRNA signature which accurately predicts survival and recurrence of HCC has been developed[157]. These studies suggest that miRNA profiling may play an impor-

tant role in HCC management in the clinic, both for classification of HCC into subtypes determining treatment and in assessment of prognosis. Patterns of dysregulated miRNAs distinguish tumours based on molecular characteristics. For example, β-catenin mutation is associated with reduced miR-375 levels, and reduced miR-107 levels with HNF1α[158]. Such findings led to the proposal of a miRNA-based HCC classification system[159]; this could be used to determine treatment allocation, based on molecular pathology.

miRNAs AS THERAPEUTICS FOR HCC

Efficacy of miRNA-based gene therapy in HCC treatment has been demonstrated (Table 2). In one study, miR-122 expression was inhibited in chimpanzees using SPC3649 LNA-modified oligonucleotides. As miR-122 up regulates HCV replication in infected hepatocytes, its inhibition reduced HCV RNA production and decreased viraemia[160]. A phase I trial for SPC3649 (Miravirsen) resulted, becoming the first miRNA-targeted drug to enter human clinical trials. Miravirsen was well-tolerated and is currently undergoing phase Ⅱ trials in HCV null responders to pegylated interferon-α and ribavirin. However, issues regarding possible viral escape are arising, with one study showing that mutations in the miR-122 binding site in HCV 5'-UTR decreases Miravirsen efficacy[161]. Similarly, therapeutic miR-196 targeting has been investigated, with the results of these and similar studies likely to have significant implications for future treatment of HCV infection and HCC[162]. Recently it was demonstrated that HNF4α, a key regulator of hepatocellular carcinogenesis, becomes stably inhibited during hepatocellular transformation. Perturbation of this event through miR-124 systemic administration can prevent and suppress HCC development in a murine liver cancer model by inducing tumour-specific apoptosis without toxic side effects[163]. Thus miR-124 has therapeutic potential for treating liver cancer.

Several virally-delivered "classical" gene therapy products developed for HCC are currently progressing through clinical trial phases; however, virus-delivered miRNA-based gene therapies have yet to be tested in clinical trials[2]. Accurate assessment of this method's potential risks must be performed before further progress can be achieved. Nevertheless, early results from studies investigating the therapeutic delivery of miRNAs are showing promise. One such study in mice used self-complementary AAV serotype 8 (scAAV8) to deliver miR-26a to the HCC site; this delivery restored miR-26a expression in HCC cells, specifically decreasing cancer cell proliferation, inducing tumour-specific apoptosis, and protecting from HCC progression without toxicity[112]. 80% of treated mice had no or small tumours at 3 wk post-transduction, while most liver tissue in the untreated control group was replaced with HCC tumours. This study is of critical importance to the future of HCC treatment in that it was the first to demonstrate the therapeutic potential of restoration of expression of a dysregulated miRNA in the liver. Despite this, the relevance of therapeutic miRNA delivery to human HCC patients remains to be determined, emphasising the considerable amount of research needed in this field before clinical applications can be made. Nevertheless, the early successes of RNA-based therapies in clinical trials demonstrate that miRNAs and their inhibitors show great therapeutic promise for HCC. Future studies will no doubt shed light on how best miRNAs have the potential to alter survival rates of HCC patients.

Findings have also pointed towards long non-coding RNAs (lncRNA) as important tumorigenic candidates actively involved in gene regulation, with lncRNAs suggested as a link in carcinogenesis. Morover, lncRNAs can act as negative regulators of miRNAs and therefore may become important factors to consider when developing miRNA therapeutics. Several reports demonstrate an association of lncRNA with the development, progression, metastasis and poor prognosis in HCC patients[164-168].

CONCLUSION

In summary, studies have demonstrated unequivocally that miRNAs are important modulators of mRNA and protein expression. They are known to be involved in a variety of biological and pathological processes, such as the regulation of iron homeostasis and in HCC development and progression. As predicted by bioinformatic analysis and confirmed by numerous studies, some miRNAs target multiple genes involved in HCC progression. Similarly, several miRNAs often regulate a single aberrantly expressed gene. From these findings, we see that HCC progression is determined by a complex interaction of dysregulated miRNAs and their target mRNAs. This must be kept in mind when investigating the therapeutic potential of miRNAs, as changing the expression of a single miRNA may not be adequate to alter expression of the target gene.

Investigations into the potential clinical uses of miRNAs are ongoing, most notably in the early diagnosis and treatment of HCC. In addition, using miRNAs to subdivide HCC cases based on molecular pathology has been proposed; this system could also determine treatment allocation and aid in prognostic assessment. Overall, it seems likely that miRNAs will play an increasingly important role in the diagnosis and treatment of liver diseases associated with HCC over the coming years, leading to improved patient survival rates and better patient outcomes.

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REVIEW

Risk factors for local recurrence following neoadjuvant chemoradiotherapy for rectal cancers

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Abstract

Local recurrence (LR) has an adverse impact on rectal cancer treatment. Neoadjuvant chemoradiotherapy (nCRT) is increasingly administered to patients with progressive cancers to improve the prognosis. However, LR still remains a problem and its pattern can alter. Correspondingly, new risk factors have emerged in the context of nCRT in addition to the traditional risk factors in patients receiving non-neoadjuvant therapies. These risk factors are decisive when reviewing treatment options. This review aims to elucidate the distinctive risk factors related to LR of rectal cancers in patients receiving nCRT and to clarify their clinical significance. A search was conducted on PubMed to identify original studies investigating patients with rectal cancer receiving nCRT. Outcomes of interest, especially potential risk factors for LR in patients with nCRT, were then analyzed. The clinical importance of these risk factors is discussed. Remnant cancer cells, lymph-nodes and tumor response were found to be major risk factors. Remnant cancer cells decide the status of resection margins. Local excision following nCRT is promising in ypT0-1N0M0 cases. Dissection of lateral lymph nodes should be considered in advanced low-lying cancers. Although better tumor response resulted in a relatively lower recurrence rate, the evidence available is insufficient to justify a non-operative approach in clinical complete responders to nCRT. LR cannot be totally avoided by current multidisciplinary approaches. The related risk factors resulting from nCRT should be considered when making decisions regarding treatment selection.

Key words: Local recurrence; Rectal cancer; Neoadjuvant chemoradiotherapy

Core tip: This review identifies the distinctive risk factors associated with local recurrence (LR) in patients with rectal cancer receiving neoadjuvant therapy. These factors are different from the traditional risk factors seen in patients treated with surgery and/or adjuvant therapy alone. The clinical significance of these risk factors is clarified in detail. To our knowledge, no reviews concerning this topic have been published. The present manuscript might help to understand the origin of LR following neoadjuvant chemoradiotherapy and may receive attention from investigators devoted to improving the prognosis of rectal cancer.

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INTRODUCTION

Local recurrence (LR) is a major problem and threatens the prognosis of rectal cancer patients. For locally pro-

gressive tumors, LR can not be prevented just by improving surgical techniques. Therefore, preoperative, also known as neoadjuvant, therapy has been advocated due to its ability to down-stage tumors and thus increase resectability. Multidisciplinary neoadjuvant approaches have been proven to effectively control LR[1,2] and improve overall survival[3,4]. However, LR still occurs[5,6] and its pattern can change[7,8] with regard to time and location. For example, the time from operation to LR is prolonged[9]. Most importantly, neoadjuvant therapy and its downsizing effects on tumors have resulted in the emergence of some LR-associated risk factors unlike those related with only surgery plus adjuvant chemoradiotherapy, such as vascular invasion or tumor differentiation[8,10]. These distinctive risk factors, consisting of isolated remnant cancer cells and tumor response to neoadjuvant chemoradiotherapy (nCRT), have been reported to be associated with the prognosis of patients[11]. Therefore, determination of the characteristics of these factors and their clinical significance would provide very helpful data for clinical practice.

The aim of the present review was to characterize the risk factors in patients receiving neoadjuvant therapy, mainly nCRT. Moreover, the clinical implications of these risk factors in treatment decision-making following nCRT were also explored.

SEARCHING STRATEGIES AND SELECTING CRITERIA

A systematic review was performed in order to explore potential risk factors for LR following nCRT. A literature search was performed in PubMed and EMBASE databases for English-language papers published over the last 10 years, with outcome data limited to humans. The search terms used included "rectal cancer" or "rectal neoplasm"; "neoadjuvant" or "preoperative"; "radiotherapy" or "chemotherapy" or "chemoradiotherapy"; "recurrence" or "local recurrence" or "local control" or "local relapse" or "local failure" or "prognosis".

The criteria for including potential studies in the systematic review were: (1) randomized clinical trials (RCTs) or cohort studies investigating patients with rectal cancer receiving nCRT; (2) retrospective studies of LR in patients with rectal cancer who were treated with nCRT; and (3) studies evaluating parameters (risk factors) that may influence the outcome in terms of LR in patients with rectal cancer who were treated with nCRT. Articles that did not show LR or investigate the causes of LR were excluded. Furthermore, abstract-only publications and chapters from books were excluded. When the same series of patients were reported by the same authors in different articles, only the series with the longest follow-up was included in the review.

Two reviewers independently reviewed each article, and discrepancies were resolved by discussion and consensus. All data were extracted from the main text, tables, and figures of the articles. Traditional risk factors such as differentiation, vascular invasion, TNM staging and circumferential resection margin status were excluded. Risk factors related to the downsizing effect of nCRT were included.

Analysis of the data from the included studies was carried out. Descriptive statistics (simple counts, means, and medians) were either directly derived from the article or calculated based on the data presented in the article, and used to report studies, patients, and treatment-level data. Outcomes of interest, especially potential risk factors for LR in patients who received nCRT were synthesized by pooling relevant data, and then analyzed. Due to high heterogeneity among the studies and lack of RCTs, a meta-analysis was not deemed appropriate.

PATTERNS OF LR FOLLOWING nCRT

Time and location of LR

To better understand the risk factors, a deep insight into the patterns of LR is required. The patterns of LR can be described by two aspects, namely timing and location. The first aspect is the time interval to development of LR. Habr-Gama *et al*[9] found that the mean recurrence interval was 52 mo (18-79 mo) in 6 cases with sustained complete clinical response to nCRT. However, Coco *et al*[6] reported that the time to development of LR was longer than 5 years in approximately one third of cases treated with nCRT (4 of 14 cases). Similar results were observed in studies[12,13], in which only neoadjuvant radiotherapy (nRT) was administered. However, in a study which included patients receiving surgery alone or associated with postoperative chemoradiotherapy (pCRT) with an average follow-up of 10 years, LR occurred in 72% of patients within 18 mo of surgery[14]. These data suggest that neoadjuvant therapy may have an ongoing impact, different from that of pCRT, on the natural history of rectal cancer. This may be the reason why a better response can be induced by nCRT over time[15,16].

The second pattern is the subtle alteration concerning subsites of LR. It has been shown that the incidence of anastomotic recurrence is declining[12,17]. The two most common sites of LR in nCRT cases are the lower pelvis (56%) and presacral region (22%)[18,19]. Syk *et al*[20] indicated that the majority of LRs in patients receiving nRT were located anatomically below the S1-S2 interspace. The higher frequency of LR within the presacral area in patients undergoing nRT may be explained by the unique anatomical locations of the mesorectum and lateral lymph nodes (LLNs). The mesorectum is defined as the fatty and fibrous tissues surrounding the rectum. Most mesorectal tissues are located at the dorsal side of the rectum and include lymphatic and vascular vessels to which cancer may disseminate. Furthermore, a recent anatomical study revealed the presence of an alternative lymphatic drainage pathway from mesorectal LNs to LLNs[21] using three-dimensional reconstruction and histological section. This connection may provide a pathway for the cancer cells to spread or escape and LLNs may

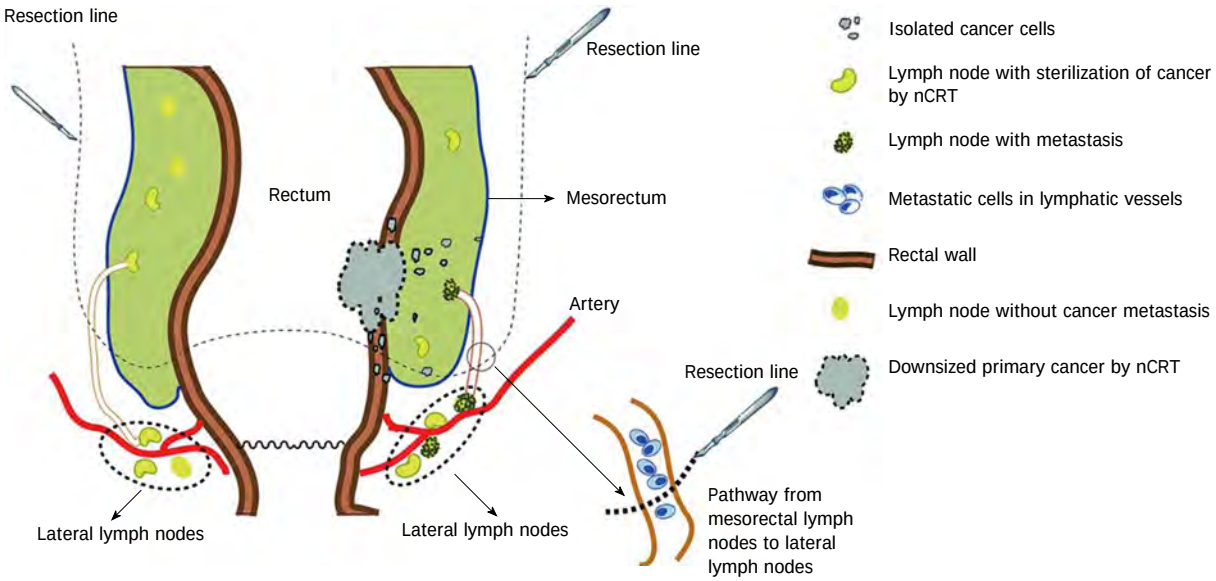

Figure 1 A diagram of risk factors for local recurrence in cases treated with neoadjuvant chemoradiotherapy. Resection line marks the resection range of a standard total mesorectum resection. nCRT: Neoadjuvant chemoradiotherapy.

serve as a harbor for these cells[22,23]. Some isolated cancer cells in the mesorectum or lymphatic tissues (see "Isolated tumor cells") serve as seeds for LR following nCRT. These cells are inhibited, but not killed, by nRT and rest in the G0 phase[24]. During surgery, cells may be spilled and implanted in the lower pelvis and presacral region resulting in LR.

We hypothesize that the seeds of LR may be the cancer cells at the margin of the mesorectum or within the lymphatic pathway from the mesorectum to LLNs. During a standard total mesorectum resection (TME), these cells may "leak" following complete resection of the mesorectum, implant in the presacral space due to the force of gravity and trigger subsequent LR (Figure 1). This hypothesis may be further confirmed if the tumor cells can be separated from post-operative lymph fluid drainage.

Clinical importance of follow-up

Understanding the altered LR patterns in patients with different neoadjuvant and intraoperative therapies has practical implications. On the one hand, delayed LR occurs in patients receiving nCRT, and thus, the standard 5-year follow-up currently recommended by the European Society for Medical Oncology[25] should be extended to at least 7-8 years and intensified monitoring is required in selected cases[26]. In addition, if delayed LR is expected to occur in a proportion of patients, the observational period in prospective and randomized trials[4,27] should be prolonged in order to draw more definitive conclusions. On the other hand, attention should be paid to common regions involved in LR in patients receiving neoadjuvant therapies which may help us accurately select the area at high risk for radiotherapy and avoid unnecessary irradiation.

ISOLATED REMNANT CANCER CELLS

As mentioned above, nCRT may be "suppressive" rather than "destructive" for a certain proportion of cancer cells. Thus, the surviving cells, if not removed by surgery, may restore their viability and evolve into seed cells for LR (Figure 1). These seed cells can be divided into two groups, extranodal and intranodal seed cells, according to their relationship with lymph nodes (LNs). Furthermore, two major types of LR derived from extranodal seed cells, tumor budding (inside the bowel wall) and mesorectal microfoci (MMF), have been reported, according to their locations.

TUMOR BUDDING

Relationship with LR

Tumor budding is described as a subset of isolated cancer cells located at the invasive front and extending from the neoplastic gland structures to the adjacent stroma[28]. Tumor budding has been reported to be an independent factor predicting prognosis[29,30]. Research on nCRT cases has shown that tumor budding is always described as isolated or small clusters of remnant cancer cells resulting from tumor regression. A control-case study[24] showed that nRT increased the frequency of budding cells compared with surgery without nRT (mean 54 *vs* 38, $P = 0.03$). These cells are always surrounded by fibrosis or an inflammatory reaction induced by nCRT. NCRT-induced tumor budding can be classified into two grades: high grade (clusters of budding cells easily observed by pathological examination) and low grade (minimal or isolated budding barely detected by pathological examination). According to Gavioli *et al*[31] study of 139 patients with nCRT, LR did not appear in the low grade budding group, while 8.8% of the high grade budding patients developed LR. In a more recent study, patients with low grade budding also had better 5-year disease-free survival than those with high grade budding (87.5% *vs* 55.6%, $P < 0.0001$).

Table 1 Intramural spreading distance after neoadjuvant therapy

Ref.	No. of patients	Neoadjuvant therapy regimen		Intramural spreading distance		
		Radiotherapy (Gy)	Chemotherapy	0-5 mm	6-10 mm	> 10 mm
Chmielik *et al*[32]	106	5 × 5	None	93	9	4
Chmielik *et al*[32]	86	50.4	5-Fu + LV	78	8	0
Mezhir *et al*[37]	20	50.4	5-Fu + LV	12	7	1
Guillem *et al*[36]	109	50.4	5-Fu + LV	108	1	0

5-Fu: 5-fluorouracil; LV: Leucovorin.

Clinical significance: decide the status of distal resection margin

It has been shown that the distal intramural spread of tumor budding is discontinuous in 57% of patients receiving nCRT[32]. The nature of this discontinuity is of special clinical importance; the supposed "clean" distal resection margin (DRM) in sphincter-sparing resection may not necessarily be free of cancer cells and longer a DRM may be required in a proportion of patients due to the possible existence of tumor budding. Thus, the focus is now "How far does tumor budding go?" Two studies demonstrated that DRMs less than 10 mm did not compromise LR[33,34]. In contrast, a study with a longer follow-up (5.6 years) demonstrated that a DRM less than 8 mm was associated with increased LR[35]. Why was there discrepancy between these two studies? First, the average period of follow-up may have had an influence. The follow-up time in these two studies may have been too short to draw definite conclusions (both were less than 36 mo). Second, the whole-mount section of the pathological examination was not used in these two studies, making the conclusion less convincing. Studies using whole-mount sections have shown that approximately 90% of patients receiving nCRT have a distal intramural extension of tumor budding within 5 mm, and 8% within 6-10 mm and less than 2% over 10 mm[32,36,37] (Table 1). Correspondingly, it has been suggested that the required length of the DRM should be shortened from 20 to 10 mm due to tumor remission induced by nCRT[36]. A DRM less than 10 mm is not yet justified for cases receiving nCRT based on current evidence. Therefore, following nCRT, the existence of budding cells is discontinuous and a supposed "negative" DRM less than 10 mm may not be a real negative margin for low-lying cancers.

MMF

Relationship with LR

Unlike tumor budding which is intramural, MMF, another risk factor for LR, is mesorectal. MMF is primarily defined as extranodal cancer deposits discontinuous with the primary tumor[38] in the mesorectum. The incidence of MMF is reported to be directly associated with the infiltrating depth of the primary tumor[38].

Ratto *et al*[39] specifically classified MMF into four major subtypes: endovascular (cancer deposits in blood vessels), endolymphatic (cancer deposits in lymphatic vessels but not in lymph nodes), perineural (cancer cell aggregates between the fasciculus and perineurium) and isolated (cancer deposits within the mesorectum, not a continuous extension from the main tumor mass). Clinically, MMF can be identified by careful pathological examination. Studies[39-41] have shown that MMF are detected in 13.8%-44.2% of cases after surgery despite downstaging induced by nCRT. Prabhudesai *et al*[38] reported that LR occurred in 17.2% (5/29) of patients with MMF and in 3.8% (1/26) of those without MMF, although the difference was not statistically significant.

Clinical significance: decide the status of circumferential resection margin and distal mesorectal margin

Similar to tumor budding, MMF may decide the status of the circumferential resection margin (CRM) and distal mesorectal margin (DMM) (Figure 2). However, no data are available regarding the appropriate CRM and DMM after nCRT. Should CRM and DMM be correspondingly shortened? Further pathological studies are required.

LYMPH NODES

Relationship with LR

Cancer cells harbored within LNs surrounding the rectum may serves as the seeds for LR. Although the nCRT-induced tumor regression does not necessarily parallel the sterilization of LNs metastasis, better tumor response may predict less LNs metastasis. Recent studies have proven that tumors at stage ypT0-1 correlate with a very low incidence of positive LN involvement[31,42-52] (Table 2). With regard to stage ypT2, LN involvement is present in about 20%-30% of cases[44,48].

Clinical significance: indication for local excision

With the belief that favorable tumor response may be equal to the disappearance of LNs metastasis, we propose that a proportion of pretreated T3 or T4 tumors might meet the requirements for local excision (LE). Several studies have shown that LR is not observed in ypT0 cases followed by LE, and the LR rate is around 3%-6% in ypT1 cases[53-60]. Moreover, the LR cases can be efficiently salvaged by subsequent radical dissection if early detection is achieved[54,61]. Therefore, LE is recommended by some authors for ypT0 or ypT1 cases due to its efficacy in local control which is equivalent to radical surgery[49,52,53,59,61-64]. Although these results are encouraging, the majority of the above-mentioned studies are retrospective and include small sample sizes. Thus, further

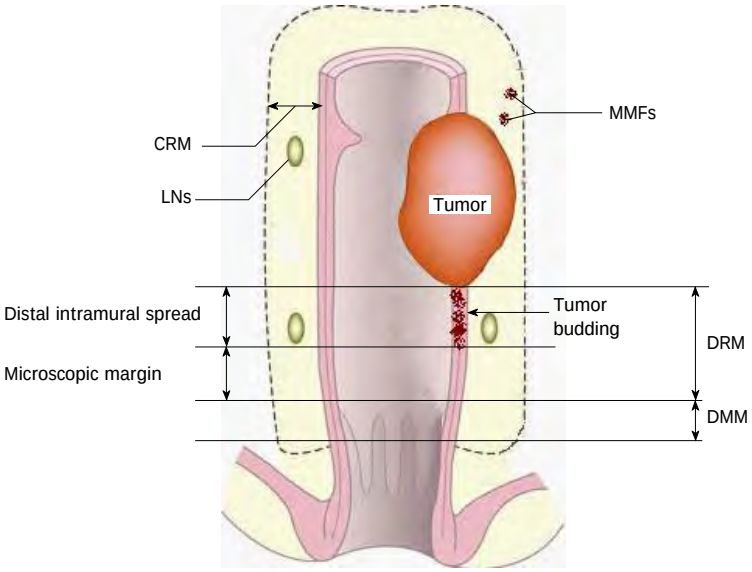

Figure 2 A diagram of resection margins of rectal cancer and their relationships with mesorectal microfoci and tumor budding. CRM: Circumferential resection margin; DRM: Distal resection margin. DMM: Distal mesorectal margin; LNs: Lymph nodes; MMF: Mesorectal microfoci.

Table 2 Association between ypT stage and ypN status *n* (%)

Ref.	No. of patients	Neoadjuvant therapy regimen		Time interval[1] (wk)	No. of patients with ypT0/T1	
		Radiotherapy (Gy)	Chemotherapy		ypN+/ypT0-1	ypN+/ypT2-4
Zmora *et al*[42]	109	45-50.4	5-Fu	6	4/33 (12.1)	30/61 (49.2)
Read *et al*[43]	644	20-45	5-Fu	NS	3/87 (3.4)	217/557 (39.0)
Bujko *et al*[44]	147	5 × 5		1	0/4 (0.0)	69/138 (50.0)
Bujko *et al*[44]	138	50.4	5-Fu	4-6	2/33 (6.1)	41/101 (40.6)
Pucciarelli *et al*[45]	235	45-50.4	5-Fu	6-8	3/69 (4.3)	45/166 (27.1)
Tulchinsky *et al*[46]	101	45	5-Fu	5-7	1/22 (4.5)	29/75 (38.7)
Habr-Gama *et al*[47]	401	50.4	5-Fu	8	3/25 (10.7)	75/224 (33.5)
Stipa *et al*[48]	187	50.4	5-Fu	NS	3/44 (6.8)	48/143 (33.6)
Kundel *et al*[49]	320	45	5-Fu	4-8	3/69 (4.3)	49/222 (22.1)
Gavioli *et al*[31]	139	50	5-Fu	4	2/34 (5.9)	38/105 (36.2)
Kim *et al*[50]	282	45	5-Fu	4-8	2/58 (3.4)	85/224 (37.9)
Lindebjerg *et al*[51]	135	60	5-Fu	8	8/47 (17.0)	32/88 (36.4)
Coco *et al*[52]	271	NS	NS	NS	3/71 (4.2)	70/200 (35.0)
Total	3109				37/596 (6.2)	828/2304 (35.9)

[1]Time interval refers to the time from the end of neoadjuvant therapy to subsequent operation. NS: Not specified; 5-Fu: 5-fluorouracil.

prospective, population-based and multi-center investigations are required to confirm these results.

With regard to ypT2 stage, 63% (53/88) of patients with ypT2 are reported to have at least one unfavorable pathological feature in addition to LNs metastases (vascular or perineural invasion, mucinous type and tumor size > 3 cm) for LE[65]. Perez *et al*[66] reported that the LR rate in patients with ypT2 who underwent LE was 9% (8/88) after nCRT. In cases with ypT3N0 or ypT4N0, the rate was up to 25% (14/25), including 14.7% (n = 8) systemic and 10.3% (n = 6) local relapse despite the absence of LNs micro-metastasis[66]. These findings indicate that ypT2-4 may have more residual cancer cells than detected and these tumor stages are not suitable for LE under the current nCRT regimen.

LATERAL LYMPH NODES

Relationship with LR

LLNs are a particular type of lymph nodes and dissection of LLNs is not included during regular TME. The incidence of LLN involvement varies from 7.7% to 20% in low and middle rectal cancer[67-69]. There is evidence to suggest that TME even with nCRT cannot completely remove remnant cancer cells in LLNs (Figure 1), especially in advanced tumors[45,70,71]. Kim *et al*[72] reported that 9 (7.9%) of 366 patients developed LR after nCRT and TME during a mean follow-up of 5 years, and lateral pelvic recurrence accounted for most (n = 24, 82.7%) of these cases. Patients with positive LLNs had a higher risk of lateral pelvic recurrence, compared with those with negative LLNs (LR rate: 26.6% *vs* 2.3%). Kusters *et al*[73] demonstrated that bilateral lateral lymph node dissection (LLND) generally resulted in better local control than unilateral LLND (LR rate: 15.4% *vs* 8.3%) in patients with advanced cancers after nCRT. When positive LLNs were detected preoperatively, the difference between unilateral and bilateral LLND was still significant (LR rate: 32.8% *vs* 14.2%). Furthermore, LR was detected on the contralateral side in a proportion of patients who

Table 3 Relationship between tumor response and local recurrence rate *n* (%)

Ref.	No. of patients	Neoadjuvant therapy regimen		No. of LR	
		Radiotherapy (Gy)	Chemotherapy	pCR LR/total	Non-pCR LR/total
Gavioli *et al*[31]	139	50	5-Fu	0/25 (0.0)	8/114 (7.0)
Stipa *et al*[57]	200	50	5-Fu	0/60 (0.0)	6/140 (4.3)
Hughes *et al*[71]	130	45	5-Fu	0/23 (0.0)	23/107 (17.7)
Kim *et al*[82]	114	50.4	5-Fu	0/10 (0.0)	17/104 (16.3)
Kuo *et al*[83]	248	50	5-Fu	2/36 (5.6)	66/212 (31.1)
Chan *et al*[84]	128	50	5-Fu	0/32 (0.0)	24/96 (18.4)
García-Aguilar *et al*[86]	168	40-65	5-Fu	0/21 (0.0)	7/147 (5)
Wheeler *et al*[87]	63	45-50	5-Fu	1/29 (3.4)	8/34 (23.5)
Theodoropoulos *et al*[88]	88	45	5-Fu	0/16 (0.0)	3/72 (4.2)
Total	1278			3/252 (1.2)	162/1026 (15.8)

LR: Local recurrence; pCR: Pathologic complete remission; 5-Fu: 5-fluorouracil.

underwent unilateral lymph node dissection. These data indicate that positive LLNs are a vital risk factor causing pelvic recurrence even after nCRT.

Clinical significance: application of LLND

There is controversy between Western and Japanese researchers concerning the application of LLND. Western researchers believe that nCRT plus TME may have a comparable outcome to that of LLND[74]. Moreover, resection of LLNs may result in injury to pelvic nerves. Thus, they recommend nCRT plus TME, not LLND. However, Japanese researchers indicate that LLND has a comparable outcome to that of nCRT plus standard TME regarding local control and the incidence of complications[75]. Thus, they recommend LLND. In our opinion, LLNs status is reflective of overall mesenteric LNs status and LLNs positivity may represent the poor response of rectal cancer to nCRT. LLND should be undertaken in selected patients, *e.g.*, those with tumor below the peritoneal reflection and poor tumor response. In addition, laparoscopic technology has unique advantages over laparotomy in terms of decreasing morbidity following LLND due to its high-definition close view in nerve-sparing.

TUMOR RESPONSES

Relationship with LR

A better tumor response may predict a more favorable prognosis for patients with advanced rectal cancer[76]. The response to neoadjuvant therapy includes remission in both primary tumor volume and lymphatic or vascular metastasis. Pathologic complete response (pCR) is defined as both ypT0 and ypN0, and the pCR rates range from around 10% to 30% in patients who underwent nCRT[77-80]. The final pathologic stage after nCRT and radical surgery is considered a vital factor in predicting LR. According to Mandard's Tumor Regression Grade (TRG) criteria[81], patients achieving a significant tumor remission (TRG1-3) displayed a relatively lower LR rate[71,82-87] compared with the non-downstaging group (TRG4-5). This figure decreased to 0%[31,71,82,86,88] (Table 3) in the pCR group. The reason for this may be that a pCR suggests a more favorable biological behavior and increases the chances of R0 resection. Moreover, complete regression of the primary cancer is paralleled with the disappearance of remnant cancer cells either in the mesorectum or lymph nodes[39].

Clinical significance: non-operative management

It has been shown that in patients with pCR, no residual cancer is found in resected specimens. This raises the question as to whether immediate radical surgery following nCRT is necessary, or, whether "watch and wait" is an appropriate strategy for these selected patients. Since pathological response can be judged only after tumor resection, a substitute parameter, clinical complete response (cCR), has been used to preoperatively screen potentially suitable patients[9,89]. A single-center study revealed that in patients treated with chemotherapy without surgery, only 5% of cCR cases (5 of 99) developed LR[9], whereas another study found that 8 of 10 patients had LR[90]. How do we explain such a big discrepancy? Actually, the critical premise for the "watch and wait" approach is to correctly identify the "real" suitable responders. A long-term persistent cCR may be a better representative of pCR. Only patients with sustained cCR for at least 12 mo were submitted to non-operative management in the study by Habr-Gama *et al*[9]. In contrast, the majority (75%) of patients with a short-term cCR (6-12 wk) were reported to have microscopic remnant cancers[70], at high risk of LR if subjected to "watch and wait". In addition, accuracy of staging in cases pretreated with nCRT is controversial. The absence of palpable tumors is not reliable evidence, nor is an invisible tumor on imaging methods, including transrectal ultrasonography, CT and MRI. Therefore, the overall attitude toward non-operative management remains critical and cautious, although the results from Habr-Gama *et al*[9,91] are promising. In our opinion, only selected cCR patients may undergo close observation without immediate radical surgery.

A CONTEMPORARY LOOK AT SURGERY-ASSOCIATED FACTORS

With the adoption of TME, LR and survival have im-

proved significantly in patients with rectal cancer, especially in those receiving anterior resection (AR)[92]. In comparison, abdominoperineal resection (APR) is reported to be related to a higher LR rate and poorer prognosis[93,94]. A possible explanation for the inferior outcome after APR is that surgeons often encounter more difficulties when resecting lower-lying tumors within a narrow pelvis[93]. Moreover, for those receiving nCRT, the appropriate surgical plane may be difficult to recognize due to tissue edema and fibrosis. These factors together may lead to inadequate excision of the mesorectum or of the tumor itself. In addition, the incidence of inadvertent intra-operative rectal perforation and post-operative anastomotic leak may increase, resulting in a higher LR rate[95-97].

With regard to AR, there is a legitimate concern about implanting exfoliated tumors cells when using circular staples. Despite the feasibility of low colorectal anastomosis, staples may also lead to implantation of viable tumor cells lying freely in the bowel lumen during staple firing[98,99]. That may also explain the mechanism of anastomotic recurrence in patients receiving nCRT (see Patterns of LR Following nCRT), who were expecting that tumor regression may translate to final sphincter-sparing surgery. Some authors[100,101] recommend intra-operative washout to eliminate exfoliated cancer cells because it is relatively risk-free and adds little to the operative trauma. However, it is difficult for surgeons to accomplish rectal washout in laparoscopic AR, as frequent laparoscopic manipulation probably increases tumor exfoliation, making wash-out even more crucial. Therefore, specific equipment or tools need to be designed to overcome the technical problems of laparoscopic rectal wash-out.

CONCLUSION

nCRT can downsize rectal cancer and facilitate subsequent radical resection. However, the impact of nCRT on downstaging of rectal cancer may also result in an altered pattern of LR and several distinctive risk factors for LR. These distinctive risk factors and altered patterns of LR are of clinical importance because they are decisive in treatment selection and follow-up. In future studies, we should not only identify but also improve our multidisciplinary approaches to minimize these factors.

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MINIREVIEWS

DNA methylation in inflammatory bowel disease and beyond

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Abstract

Inflammatory bowel disease (IBD) is a consequence of the complex, dysregulated interplay between genetic predisposition, environmental factors, and microbial composition in the intestine. Despite a great advancement in identifying host-susceptibility genes using genome-wide association studies (GWAS), the majority of IBD cases are still underrepresented. The immediate challenge in post-GWAS era is to identify other causative genetic factors of IBD. DNA methylation has received increasing attention for its mechanistical role in IBD pathogenesis. This stable, yet dynamic DNA modification, can directly affect gene expression that have important implications in IBD development. The alterations in DNA methylation associated with IBD are likely to outset as early as embryogenesis all the way until old-age. In this review, we will discuss the recent advancement in understanding how DNA methylation alterations can contribute to the development of IBD.

Key words: Intestinal inflammation; Crohn's disease; Colitis; DNA methyltransferase; Epi-therapy

Core tip: This review discuss the recent research advancement in the area of DNA methylation during the pathogenesis of inflammatory bowel disease (IBD) and IBD-associated cancer, with a focus on highlighting major players mediating DNA methylation alterations during IBD development. Temporal and spatial differential DNA methylation status that contributes to the disease, as well as epi-therapy treatment options for IBD patients, are also discussed. This emerging information will have important clinical significance, especially so in this post-genome-wide association studies era of IBD research.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic intestinal inflammatory condition that affects the intestine of millions of individuals throughout their lifetime[1]. IBD is classified into two major forms, Crohn's disease (CD) and ulcerative colitis (UC), which both exhibit etiologically and clinically distinct features. Patients with IBD have a 2-3 fold greater life time risk of developing IBD-associated colorectal cancer (IBD-CRC)[2]. Although numerous clinical and experimental reports have given large amount of insights on the pathogenesis of IBD, the complexity of the initiation of IBD renders an incom-

plete understanding. Recently, there has been significant progress in identifying risk loci that are associated with IBD patients through genome-wide association studies (GWAS). These robust analyses have identified 163 IBD susceptible gene loci[3-6]. Genome-wide meta-analysis has confirmed that 71 of these loci are associated with CD, but only accounts for 25% of disease heritability[4]. The immediate challenge of the post-GWAS era is to unravel other parameters that may be less obvious from a genetic point of view. One of such emerging fields is epigenetics, in particularly DNA methylation. In this review, we will discuss the recent progress in DNA methylation analysis in IBD and how it can be used as a potential therapeutic target.

DNA METHYLATION ENSEMBLE IN IBD

By definition, epigenetics refers to a heritable change in gene expression phenotype that does not involve alterations in DNA sequence. DNA methylation, histone modifications and non-coding RNA are the three major components involved in epigenetic mechanism. In DNA methylation, the addition of a methyl group at the 5th position of cytosine (5mC) is common on CpG dinucleotides in eukaryotic genomes[7]. Methylation of CpG rich regions (CpG islands) are relative lower and are usually associated with transcription silencing when the methylated CpG islands occur at gene promoters[8]. DNA methylation is catalyzed by enzymes known as DNA methyltransferases and the reaction is reversible. Methyl groups can be edited and removed *via* actions of DNA demethylases during specific time points such as gametogenesis and disease onset including IBD. In this section, we discuss the roles of players mediating DNA methylation in the context of IBD development.

DNA methylation authors

Highly heritable and bona fide DNA methylation is attributed towards the actions of DNA methyltransferase. In the pathogenesis of IBD and IBD-CRC, three major DNA methyltransferases (DNMT) have been proposed to be involved, including DNMT1, DNMT3a and DNMT3b (Figure 1).

DNMT1 is a key maintenance methyltransferase that primarily methylates hemimethylated DNA in the genome during DNA replication. During IBD and IBD-CRC development, DNMT1 activity is significantly upregulated[9-11]. DNMT1 is highly expressed in actively inflamed colonic mucosa in UC patients as compared to normal or quiescent UC colonic mucosa[11]. In IBD-CRC, Foran *et al*[9] compared the methylation profiles of 36 IBD-CRC *vs* 44 sporadic CRC tumour specimens and demonstrated increased nuclear localization of DNMT1 in IBD-CRC than in sporadic-CRC, evidence linking inflammation-mediated DNMT1 activity. In addition, overexpression of DNMT1 is proposed to correlate with an abundance of CD68 positive macrophages, suggesting direct involvement of DNA methylation in a pro-inflammatory response[9]. Stimulation of HCT116 human colon cancer cells with interleukin (IL)-6 increases and stabilizes DNMT1 expression, leading to increase levels of global methylcytosine, especially at gene promoter regions[9]. This effect by IL-6 is mediated through AKT (Protein Kinase B), but not signal transducer and activator of transcription 3 (STAT3) or c-Jun N-terminal kinase (Jnk), pathway in Hela human cervical cancer cells[9]. Alternatively, another group showed that STAT3 binds directly onto the *DNMT1* promoter in malignant T cell lymphoma that is responsible for inducing DNMT1 expression[12]. All these suggest that specific cell type, temporal, or even inflammatory *vs* non-inflammatory mechanisms, affect DNMT1 expression and activity.

DNMT1 binds to non-intronic upstream enhancer of *Foxp3* (forkhead box P3), a locus required to induce the development of regulatory T cells (Treg) capable of suppressing broad ranges of inflammatory responses such as colitis[13]. Stimulation with IL-6 has been proposed to increase methylation in upstream enhancer regions of *Foxp3* in Treg cells, resulting in down-regulation of both mRNA and protein expression. This effect was not observed in STAT3-deficient Treg, providing additional evidence on the involvement of the STAT3-signalling pathway in the methylation process[13]. In a separate study, Li *et al*[14] reported that IL-6-associated STAT3 signalling is highly dependent on DNMT1 enzymatic activity. They showed that IL-6-induced DNMT1 expression results in hypermethylation on the promoter of suppressor of cytokine-signaling-3 (*SOCS3*), a negative regulator of IL-6 signalling. The decreased SOCS3 expression may then promote full pro-oncogenic effects of STAT3.

DNMT3 (includes three known members: DMNT3A, DMNT3B and DMNT3L) is another family of DNA methyltransferase. Although DNMT3 acts primarily for *de novo* methylation during gametogenesis and development, many reports have shown that DNMT3 can serve cooperatively with DNMT1 to regulate bona fide DNA methylation maintenance. Active UC colonic mucosa showed higher DNMT3B expression as compared to normal colonic samples or quiescent UC colon patient samples, but relatively lower than that of DNMT1[11]. Similarly, IBD-associated neoplasm lesions showed upregulation of DNMT3B expression as compared to colonic epithelium without any neoplastic changes[15]. Conversely, human colorectal cancer cell lines (HCT15, DLD1, Col15, HT29, SW480 and RKO) are hypermethylated on the distal *DNMT3B* promoter as compared to healthy colon tissues, correlating it to the low expression level that results in hypomethylation of many of its target gene promoters[16]. These different observations may suggest that the etiology of IBD-CRC and sporadic-CRC are mechanistically distinct.

DNMT3A has been shown to play an important role in both innate and adaptive immune responses. For example, DNMT3A affects T cell polarization through *IL-4* and interferon gamma (*IFN*γ) promoter methylation upon ligation of T cell receptors[17]. In UC patient's

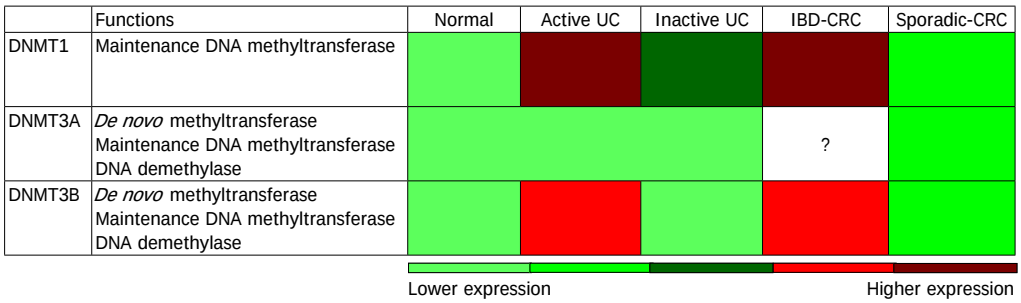

Figure 1 Potential relative expression levels of DNA methyltransferase in active-ulcerative colitis, inactive-ulcerative colitis, inflammatory bowel disease-associated colorectal cancer and sporadic-colorectal cancer patient specimens consolidate from several studies. DNA methyltransferase (DMNTs) is primarily responsible for DNA methylation maintenance, whereas DNMT3A/B have additional roles in *de novo* DNA methylation and demethylation functions. The relative DNMTs expressions were built on consolidated reports that were normalized to healthy controls to display potential relative expression in different inflammatory bowel disease associated diseases. UC: Ulcerative colitis; IBD-CRC: Inflammatory bowel disease-associated colorectal cancer.

peripheral T cells, levels of methylation within *IFNγ* promoter regions have been reported to correlate to the immune response against microbial antigens[18]. In addition, DNMT3A hypermethylates the CpG islands within the tumour necrosis factor alpha (*TNFα*) promoter region in the context of LPS stimulation[19]. However, another study has proposed no alteration of DNMT3A expression levels in colonic mucosa of UC patients[11]. It is possible that a modification of DNA methylation status during UC pathogenesis is mediated primarily *via* DNMT1 and DNMT3B. In contrast, meta-analysis of GWAS data has suggested *DNMT3A* as an important risk loci associated with CD[4]. Therefore, it is likely that methylation status in CD *vs* UC is controlled by different mechanisms.

In a clinical setting, the differential expression, involvement, and activities of DNMTs can provide additional options as a diagnosis marker tool to monitor IBD and IBD-CRC progression in patients.

DNA methylation editors

Editing and removal of methyl groups from 5mC can be actively or passively achieved through actions of DNA demethylases. Passive DNA demethylation blocks additional methylation during DNA replication by methylation dilution, or by inactivating DNMTs. Over the years, the search for active demethylases has been hindered by the fact that demethylation process is controlled by indirect multi-step mechanisms. DNA demethylation processes appear to be executed through DNA repair and base excision mechanisms, rather than direct removal of the methyl group from the 5mC moiety[20]. Recently, three proteins have been reported to potentially possess demethylase activity, including ten-eleven translocation (TET) methylcytosine dioxygenase, thymine DNA glycosylase (TDG), and activation-induced cytidine deaminase (AID).

TET converts 5mC to 5-hydroxymethylcytosine (5hmC) that is predicted to lift the repression of gene expression imposed by 5mC in both humans and mice[21]. Recently, Neves-Costa *et al*[22] demonstrated that TET1 negatively regulates the expression and secretion of a pro-inflammatory cytokine IL-1β in a THP-1 monocytic leukemia cell line. In addition, TET co-operates with TDG in the process of active DNA demethylation. TDG excises the mismatch bases at the deaminated 5mC or its derivatives caused by TET[23]. However to date, neither TET nor TDG has been implicated in the pathogenesis of IBD.

AID is another candidate involved in DNA demethylation[24,25]. AID belongs to the family of apolipoprotein B mRNA-editing catalytic polypeptide (APOBECs), which were extensively studied due to its master regulatory function in antibody diversification in B cells[26]. A process for this antibody diversification includes immunoglobulin class switch recombination (CSR), immunoglobulin somatic hypermutation (SHM), and gene conversion (GC)[27]. AID was originally demonstrated as an enzyme to convert cytosine (C) to uracil (U) for induction of SHM[28]. Subsequently, Morgan *et al*[24] unveiled an additional and unexpected ability of AID to convert 5mC to thymidine *in vitro* (5mC→T), suggesting the involvement of AID in DNA demethylation. This conversion of 5mC to T creates a T:G mismatch, which will be excised by T:G mismatch-specific glycosylases (*i.e.*, TDG). The T position will then be replaced with unmethylated C through base excision repair process, thereby concluding a 5mC to unmethylated C transition[29]. Recently, AID has been implicated in the pathogenesis of IBD and IBD-CRC[30,31]. Endo and colleagues showed that colonic AID expression is up-regulated under Th2-mediated colonic inflammatory conditions seen in T cell receptor (*TCR*)-α knockout mice[30]. In addition, ectopic expression of AID in colonic epithelial cells (CECs) was elicited in UC (54%) and IBD-CRC (80%) patients[30]. In contrast, AID expression was seen in only 40% of sporadic colon cancer, indicating the differential pathogenesis between IBD-CRC and sporadic-CRC with respect to AID functions. AID expression may be induced *via* IKK (IκB kinase)-dependent NF-κB signalling and further enhanced by Th2 cytokines such as IL-4 and IL-13[30]. Functionally, overexpression of AID in CECs has been reported to tremendously increase mutations within some, but not all, oncogenes including p53. Importantly, such mutations were significantly reduced in AID deficient mice[30,31].

However, it still remains largely unknown whether AID plays any specific roles in IBD and/or IBD-CRC through its epigenetic (demethylation) modification ability rather than its classical functions (SHM, CSR, and/or GC). The co-relationship between aberrant AID expression and IBD/IBD-CRC progression suggests that further studies on the role of AID-mediated epithelial homeostasis can potentially be translated into a therapeutic strategy for IBD patients by targeting AID.

In 2008, Kangaspeska *et al*[32] and Métivier *et al*[33] reported that DNMT3A and DNMT3B are recruited to gene promoters during transcription and they directly mediate cyclical demethylation and also remethylation processes. The identification of deaminase activity in DNMT3A and DNMT3B has received tremendous attention in the epigenetic field. Since it is clear now that DNMT3A and DNMT3B have dual functions for demethylation and methylation, the idea of dynamic methylation patterns during transcription will be further discussed in the following section.

DYNAMICS OF DNA METHYLATION FROM AN IBD PERSPECTIVE

Covalent modification of DNA through the addition of methyl moieties on CpG dinucleotides is highly stable and conserved. These epigenetic marks, however, do undergo dynamic changes at specific time points, including embryonic development and during perturbed cellular homeostasis such as increased cellular stress and disease onset. Thus, these temporal changes will have important implications that are relevant to the development of IBD.

During germ cell specification and post-fertilization, 5mC undergo *de novo* erasure and subsequent reprogramming[34]. The consequences of such wholescale DNA methylation reprogramming include formation of parental specific gene expression, including X-linked effects and genomic imprinting, of which gene expression are predominately contributed by specific parental allele. Several lines of evidence have demonstrated the parent-of-origin effects in IBD. As one of the earliest reports, Akolkar *et al*[35] demonstrated a familial association of IBD. In this study, clinical data analysis of 135 families showed that offspring of IBD affected mothers had higher risk for CD than offspring of fathers with IBD ($P = 0.00001$). Indeed, sex of parent seemed to play a role in IBD susceptibility and genetic imprinting process, at least in part, by DNA methylation. Fransen *et al*[36] recently present limited evidence for genomic imprinting effects of IBD susceptibility genes. They analysed 28 IBD susceptibility gene locus and found that *IL12B*, PR domain containing 1 (*PRDM1*) and nucleotide-binding oligomerization domain containing 2 (*NOD2*; L1007fs variant) have genomic imprinting effect. Recently, Schaible *et al*[37] showed that the offspring from female mice fed with methyl donor supplements (folic acid, betaine and vitamin B12) had a striking susceptibility towards dextran sulfate sodium (DSS)-induced colitis as compared to control mice fed with regular diet. These effects were also reflected with colonic mucosal DNA methylation profile alterations and prolonged gene expression changes, as well as difference in bacteria microflora when compared to mice with control diet. Therefore, better characterization of the effects and mechanisms of imprinting and parent-of origin can be utilized as a clinical risk predictor of IBD for offsprings of IBD susceptible parents in the future.

Another incidence where DNA methylation dynamics is activated is when colonic cellular homeostasis is perturbed such as during oxidative stress, which results in global loss or gain in DNA methylation. Oxidative stress and damage are common phenomenon in IBD and IBD-CRC that are mainly contributed by the reactive oxygen species produced by inflammatory cells[38,39]. Oxidative damage in cells induces recruitment of DNMT1 to the affected chromatin and forms a complex consisting of DNMT3B and members of the polycomb repressive complex 4, including Sirtuin-1 (SIRT1), Enhancer of zeste homolog 2 (EZH2) and embryonic ectoderm development (EED), to re-establish DNA methylation pattern after the DNA is repaired[40]. These key components re-localize from non-GC-rich regions to GC-rich regions[40]. The observation was validated in an *in vivo* model of colitis where infection with human commensal enterotoxigenic *Bacteroides fragilis* (ETBF) into a mouse model of adenomatous polyposis coli in Multiple intestinal neoplasia (Min) mice induced inflammation and tumorigenesis[40]. This model may provide a good putative explanation on the mechanism of how certain specific genes are hypermethylated, whereby other loci are hypomethylated, within the same cell, during disease onset.

Despite well-established consensus that DNA methylation is a highly stable modification on the DNA under steady state, two recent reports have changed this conventional perspective. In the first report, Kangaspeska *et al*[32] showed that estrogen receptor α (ERα) induces waves of transcription of its target promoter that involves series of active and cyclical demethylation and remethylation during the course of transcriptional activation. DNA methylation status were quantified using glutathione S-transferase tagged methyl binding domain (GST-MBD) pull-down assay, which showed a periodicity of 100 min at the ERα target *pS2* gene promoter. The second report by Métivier *et al*[33] showed similar cyclical demethylation-methylation effects at the *pS2* promoter, and further provided evidence of DNMTs are present at the promoter during transcription activation and is involved in both demethylation and remethylation processes. Specifically, methylated CpG were deaminated by DNMT3A and DNMT3B, resulting in a base-pair mismatch that is subsequently repaired by base-excision machinery. These two reports pioneered a previously unreported cyclical methylation-demethylation association with transcription and that this process is mediated by DNMTs, proteins previously tightly linked to only methylation but not demethylation. The authors have validated this observation in other promoters including *ER*α, trefoil

factor 3 (*TFF3*), and potassium inwardly-rectifying channel, subfamily J, member 8 (*KCNJ8*). Interestingly, these selected validated genes have previously been implicated in different studies of IBD and IBD-CRC in patients or animal models[41-44]. TFF3 is secreted by intestinal goblet cells that forms part of the enteric mucus layer and has a role in epithelial repair and restitution[45]. *TFF3*$^{-/-}$ mice are less reactive towards mounting repair response during colonic injury induced by chemical, hypoxia and radiation stress[46-48]. Another of the validated candidates is KCNJ8 (also known as KIR6.1), which forms the pore-forming sub-unit of the ATP-sensitive potassium channel (K_{ATP}). hydrogen sulphide (H_2S), produced by colonic smooth muscles, neurons and other enteric cell types, activates and opens K_{ATP} channels in a 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced murine colitis model[44]. Similarly in TNBS-induced colitis in rats, the production and effects of H_2S is associated with the resolution of colitis[49]. Nevertheless, whether cyclical demethylation-remethylation process plays any roles in the pathogenesis of IBD remains elusive, and further extensive studies will be required.

IMPACT OF ENTERIC MICROBES IN IBD HOST DNA METHYLATION

It has become increasingly apparent that dysregulated host microbial interactions contribute to the induction, exacerbation and perpetuation of IBD. Importantly, commensal microbes have an ability to alter DNA methylation status. Mice that were housed in germ free (GF) conditions exhibited hypermethylation of the chemokine ligand *CXCL16* [chemokine (C-X-C motif) ligand 16] in the colon, as compared to mice kept under specific pathogen-free (SPF) environment[50]. CXCL16 expressed on the surface of antigen-presenting cells, including subsets of CD19+ B cells and CD14+ monocytes/macrophages, mediates the adhesion and phagocytosis of gram-negative and positive bacteria[51,52]. Soluble CXCL16 can also act as a strong chemo-attractant for CXCR6+ [chemokine (C-X-C motif) receptor 6] T cells[53,54]. Up-regulation of CXCL16 mRNA and protein has been reported in CD patients[55]. Hypermethylation of *CXCL16* gene in GF mice leads to the gene activation and accumulation of invariant natural killer T (iNKT) cells, in the colonic lamina propria. iNKT cells are highly conserved subset of T cells expressing a semi-invariant T cell receptor, which is restricted to CD1d and specific for the glycosphingolipid antigen α-galactosylceramide. Furthermore, the activated CXCL16 pathway made GF mice more susceptible against oxazolone-induced Th2-type of acute colitis as compared to SPF mice[50]. Importantly, colonization of neonatal GF mice with a conventional microbiota reduced hypermethylation of *CXCL16* to SPF level[50]. However, this phenomenon was not observed when adult GF mice were colonized with the same conventional microbiota, indicating that early-life microbial exposure has a significant impact on host epigenetic status[50]. In addition, recent studies showed that oral inoculation of lipoteichoic acid (LTA)-deficient *Lactobacillus acidophilus* bacteria (NCK2025), protect mice from colitis-associated cancer presumably by restoring aberrant DNA methylation pattern of cancer-specific genes[56,57]. LTA is a major immunostimulatory component of cell wall of Gram-positive bacteria, which can specifically bind to CD14 and toll-like receptors (TLRs) such as TLR2 on host cells. It is well known that host-microbial recognition is attributed to TLRs. Of note, TLR2 deficient (*Tlr2*$^{-/-}$) mice were characterized by low abundance of intestinal *Firmicutes* and high proportion of *Proteobacteria*, *Bacteroidetes* and *Actinonbacteria*, as compared to wild-type mice[58]. This specific change in microbial composition was associated with epigenomic alterations. For instance, 1.4% of the interrogated genome in *Tlr2*$^{-/-}$ mice was differentially methylated[58]. Female wild-type C57BL/6J mice that were given methyl-donor supplemented diet produce offspring that exhibit different microbiome profile at postnatal day 30, as compared to control diet offspring[59]. All these data cumulatively suggest that the commensal microbiota can directly influence the status of host DNA methylation and therefore may have important implications in IBD development.

In addition to how bacteria affect the host DNA methylome, the status of DNA methylation on exogenous sources of DNA, in this case bacterial DNA and host self-DNA, also plays a role in the pathogenesis of autoimmune diseases such as IBD. Bacterial DNA has high CpG frequencies but is predominately unmethylated and has immunostimulatory effect[60]. It was originally shown that the introduction of bacterial CpG motifs oligodeoxynucleotides exacerbates existing intestinal inflammation in DSS-treated mice[61]. Recent studies showed that the unmethylation status of bacterial DNA is the predominate factor to induce human plasmacytoid dendritic cells to produce high levels of interferon-alpha (IFN-α), since methylation of the bacterial DNA abolished this induction[62]. These unmethylated CpG DNAs are recognised by the host toll-like receptor 9 (TLR9)[63]. Specific CpG motifs (purine-purine-CpG-pyrimidine-pyrimidine) common in microbial DNA, but which are rare in mammalian DNA, have the strongest activation potential of TLR9[64]. In contrast to bacterial DNA, mammalian DNA has lower CpG frequencies and is predominately methylated, with an exception of CpG islands. There are now increasing evidence that these mammalian self-DNA, presumably released from necrotic cells, can also be an effective TLR9 ligand[64]. Under normal circumstances, the host immune system is protected against self-DNA because of the intracellular location of TLR9. However, during IBD progression, natural antimicrobial peptide LL37 is expressed on the mucosa surfaces and form an immuno-complex with self-DNA, which may lead to the activation of TLR9[65]. Yasuda *et al*[64] showed that CpG-rich DNA from mammalian DNA, commonly found on CpG islands, are optimal sequence to activate TLR9 and suggested a possible contribution towards

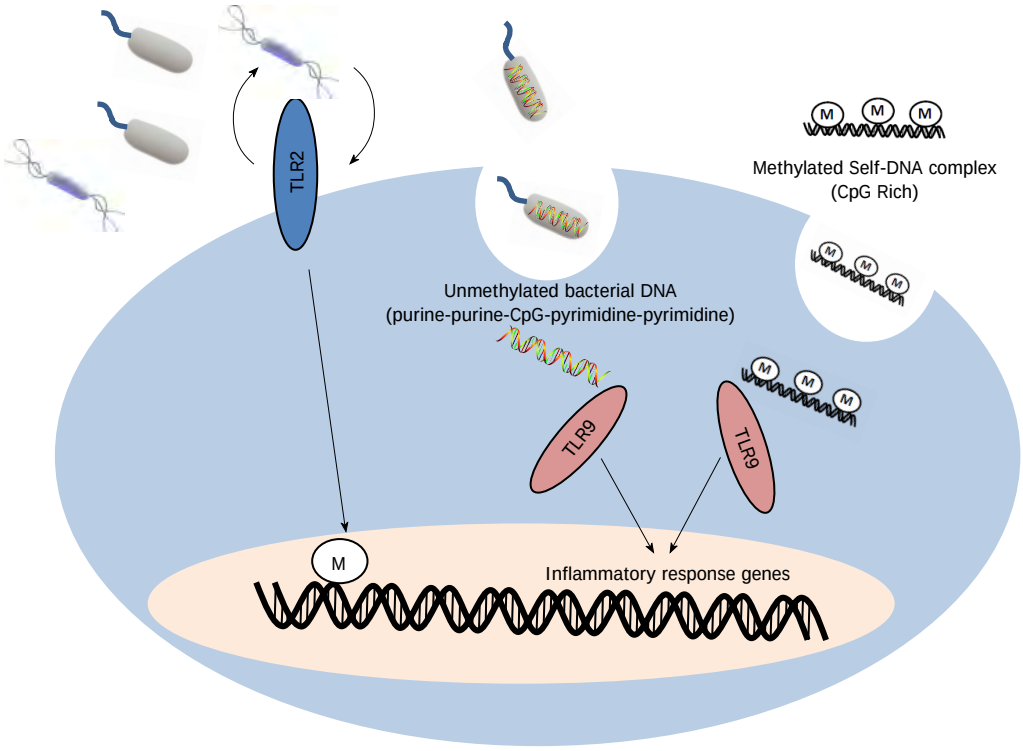

Figure 2 Host genetics and epigenetics alterations by commensal bacterial and self-DNA. Alterations in intestinal microflora or host pathogen recognition functions, such as toll-like receptor (TLR)2, directly affect host DNA methylation. Endocytosis of bacterial and release of unmethylated bacterial DNA into host cell triggers inflammatory response *via* TLR9. Strong activation requires a purine-purine-CpG-pyrimidine-pyrimidine bacterial DNA motif. Endocytosis of CpG rich methylated self-DNA also activates TLR9 to induce similar inflammatory response *via* TLR9, but with on a less magnitude compared to stimulation *via* bacterial DNA.

autoimmune diseases pathogenesis. However, this TLR9 activation by methylated self-DNA is still comparatively lower than those of unmethylated bacterial DNA[62]. As such, it was proposed that the initiation of autoimmune disease, such as IBD, is initiated by unmethylated microbial DNA whereas subsequent autoimmunity is mediated by methylated (or unmethylated) self-DNA[62]. Therefore, appropriately targeting self-DNA mediated immune responses may be another attractive option to reduce the perpetuation of inflammation in IBD.

In summary, bacterial genetics have a direct impact on host epigenetics. Similarly, bacterial and host (self-DNA) epigenetics can also directly affect host genetics to trigger inflammatory responses (Figure 2).

GENOME-WIDE DNA METHYLOME PROFILES IN IBD

Recent advances on genomic/epigenetic technologies targeting the "omics" level have contributed to a plethora of reports on genome-wide DNA methylome analysis to study the pathogenesis of IBD. The information derived from the analyses in IBD will provide significant rationale to open up a new avenue to develop novel diagnostic and therapeutic strategies. Indeed, genome-wide altered methylation patterns have been shown to be enriched around GWAS identified loci[66,67]. In addition, methylome profiling may also resolve the differences in etiology and pathogenesis of UC *vs* CD.

Nimmo *et al*[67] recently profiled the methylome of whole blood genomic DNA from 21 ileal CD patients and 19 healthy controls. They identified 1117 CpG sites that are differentially methylated. Within the list, 35 genes overlapped with previous GWAS identified CD loci, including *NOD2*, *TNF*α and caspase recruitment domain family, member 9 (*CARD9*). Comparative analysis of these gene hits showed that differentially methylated CpG sites are located within 25-100 kb of the 71 previously identified GWAS CD loci. Importantly, sex, environmental and individual lifestyle (*e.g.*, non-smoking and immunomodulatory therapy status) factors were taken into consideration for the selection of cohort in this study because these factors are influential in determining IBD, as well as epigenetic changes. This is especially apparent as seen from the high discordance rate of CD (68%) and UC (85%) in monozygotic twins, who had identical genomes[68]. The immediate question is how these identical genomes in monozygotic twins divert into different phenotypes outcome. A recent report studied 20 monozygotic twins discordant for UC and investigated the genomic profile based on three-layers of genome-wide scans, including transcriptome profiling, genome-wide methylation variable positions (MVPs) and genome-wide differentially methylation regions (DMRs)[69]. In this study, they identified 61 disease loci defined by differential gene expression profile and at least one MVP or DMR position within 50 kb from

Table 1 High throughput DNA methylome profiling in inflammatory bowel disease

Ref.	Disease	Tissue/cell	Array platform	Significant differential methylation	GWAS overlap
	Human patients				
Lin *et al*[73]	UC and CD	Intestinal	Illumina goldengate	7 CpG sites	Not reported
Cooke *et al*[66]	UC and CD	Rectal	Illumina infinium human methylation 27	3604 (UC) and 472 (CD) loci	Yes
Lin *et al*[72]	UC and CD	B cell	Illumina goldengate	24 (UC) and 14 (CD) CpG sites	Not reported
Häsler *et al*[69]	UC	Intestinal	Illumina human methylation 27 and nimblegen custom 385K	61 loci	No
Nimmo *et al*[67]	CD	Whole blood	Illumina human methylation 27	1117 CpG sites	Yes
	Mouse				
Kellermayer *et al*[59]	DSS colitis (postnatal day 30 *vs* day 90)	Colon	Custom array (Agilent)	271 intervals	Not reported
Kellermayer *et al*[58]	*Tlr2-/-*	Colon	Custom array (Agilent)	387 intervals	Not reported

UC: Ulcerative colitis; CD: Crohn's disease; GWAS: Genome-wide association studies; DSS: Dextran sulfate sodium.

the transcription start site. Promoter regions of these hits showed prominent hypomethylation, whereas gene-intronic regions were more frequently hypermethylated. However, none of these 61 loci overlapped with the previously reported 47 UC GWAS risk loci[5]. Nevertheless, environmental factors and lifestyle surely contribute to the pathogenesis of IBD and provide the most direct clues to understand how identical genomes from monozygotic twins can have distinct susceptibility to IBD. IBD usually occurs during young adulthood and the peak age of onset is around 15-30 years old[70,71]. Thus, identification of the changes in methylome during crucial developmental time point can provide great insights on IBD risk. Studies showed that postnatal day 90 mice had increase susceptibility to DSS-induced colitis as compared to postnatal day 30 mice[59]. Methylation specific amplification microarray (MSAM) revealed 271 differential methylation genomic intervals between the above two mice groups[59]. These results suggest that age-dependent methylation dynamics is another important aspect to consider in the risk of IBD.

In addition to prying into the individual genomic status in UC or CD as compared to normal individuals, epigenome-wide profiling can also dissect the differences in disease-associated loci between UC and CD. Cooke *et al*[66] recently characterized the genome-wide methylation changes in the rectal samples obtained from patients with inflamed UC/CD and non-inflamed UC/CD. Consistent with other reports, many identified loci in this study overlapped with GWAS-identified risk loci, including *CARD9*, intercellular adhesion molecule 3 (*ICAM3*) and cadherin 1 (*CDH1*). Inflamed UC and CD, as well as non-inflamed UC formed individual methylome signatures when compared to normal control individuals. Interestingly, there was no difference in the methylation profile between inflamed UC and inflamed CD. In contrast, 13 differentially methylated loci were identified between non-inflamed UC and non-inflamed CD. These multiple comparison suggests that the different sub-types of IBD, as well as disease severity, may be distinguished by their methylome status. In addition, Lin *et al*[72,73] also reported the methylome profiles of UC and CD patients derived B cells and intestinal tissues. Therefore, the methylome may be one of the useful clinical diagnostic biomarkers in IBD (Table 1). However, much more careful attention would be necessary in this regard because different cell types exhibit different methylomes in IBD.

EPI-THERAPY TARGETING DNA METHYLATION IN IBD

Several compounds targeting DNA methylation status has been demonstrated to have potential therapeutic effects on animal models of IBD and/or human IBD patients. One of these compounds is folate, a methyl donor that exerts an effect to increase global methylation. Chronic UC patients that were given dietary folinic acid, a vitamer of folic acid, supplementation (15 mg/d) had a lower risk of colon cancer[74]. Kominsky *et al*[75] recently also showed that intraperitoneal injection of folate (50 mg/kg) into DSS-treated mice results in less severe colitis. In addition, dietary folate deficiency led to aggravation of DSS-induced colitis in rats[76]. These results are consistent with clinical reports showing that folate deficiencies are common in IBD patients[77-79]. However, oral dietary supplementation of folate did not seem to have an effect on the suppression of IBD-CRC in an azoxymethane/DSS-associated cancer model[80]. In this model, diet supplementation with folic acid (8 mg/kg) did not show any alterations in intestinal microflora or difference in tumor initiation, growth and progression as compared to the control mice without receiving folic acid supplement. One possible reason for this failure is that the chronic inflammation that has transited into tumorigenic stage would have acquired more stable genetic changes including chromosomal instability and translocation, as well as genetic mutations, as compared to acute intestinal inflammation. These alterations in DNA sequence may occur at critical DNA methylated CpG sites and hence global methylation effects of folate can no longer re-establish methylation at these mutated CpG target sites.

Development of small compounds that can directly or indirectly affect DNA methylated mediated gene ex-

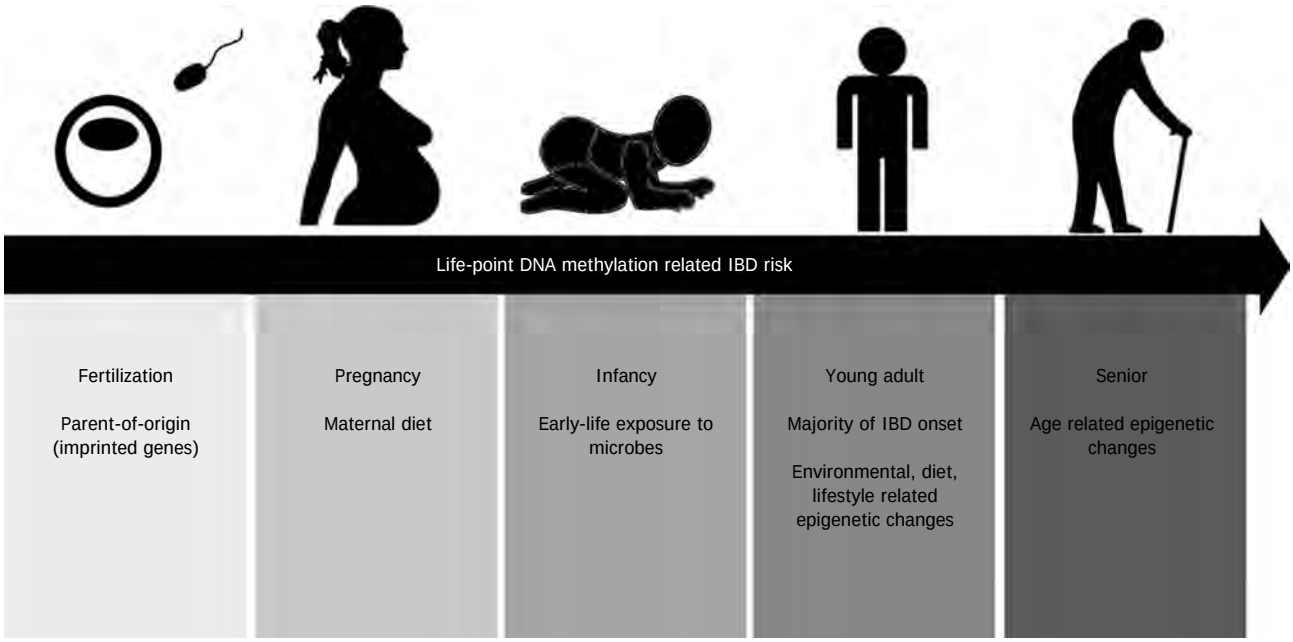

Figure 3 Life-stages with an impact on epigenetic changes that increase inflammatory bowel disease risk. Alterations in DNA methylome in inflammatory bowel disease (IBD) pathogenesis begin right from the fertilized egg. The risk alleles are inherited, and its expression is dependent on the parent-of-origin (imprinting). Maternal diet during pregnancy may also potentially alter the fetal IBD-associated-methylome. Exposures to certain microbes during infancy can also have lasting effects on DNA methylation alteration towards IBD susceptibility. Environment-, lifestyle- and diet- associated DNA methylation changes are important aspects during young adulthood where the majority of IBD onset occurs.

pression may also be useful targets of IBD treatment. Meng *et al*[81] demonstrate that using a combination of a novel tylophorine analog W-8, together with TGF-β (transforming growth factor), demethylates *Foxp3* promoter. Tylophorine analogs, including W-8, are phenanthroindolizidine alkaloids that have anti-cancer and anti-inflammatory effects. The effect of W-8 is mediated through ERK (extracellular signal-regulated kinase) pathway inhibition that results in the down-regulation of DNMT1 expression. Therefore, W-8 appears to up-regulate *Foxp3* expression by demethylating the promoter in the presence of TGF-β and promotes differentiation of naïve CD4+ T cells into Foxp3+ Treg cells with immunosuppression capabilities.

The challenge in developing innovative therapies for IBD has been on-going. Currently, oral and topical aminosalicylates are usually the first-line medication to treat IBD. Other immunosuppressive agents including azathioprine, methotrexate and cyclosporine are also in used. However, the beneficial effects of these drugs are accompanied with detrimental side effects, such as allergy. In addition, not all patients respond to these treatments. Recently, the use of anti-TNFα antibody has also been deployed to control IBD in patients. However, on top of the adverse side effects of anti-TNFα antibody, the administration of the treatment requires invasive intravenous infusion or subcutaneous injection and the high cost of this form of medication, which range from US$3000 to US$8000 per infusion, is a major disadvantage. Therefore epi-therapy drug design is an attractive alternative method to develop an effective, low-cost and non-invasive therapy for IBD patients.

CONCLUSION

DNA methylation has great heuristic potential in improving our understanding of the IBD pathogenesis in the post-GWAS era. Individuals who inherited a normal set of DNA may still be susceptible to IBD depending on epigenetic changes during their course of life. As described in this review, epigenetics changes that may account for IBD risk begin right from the fertilized egg to entire life period (Figure 3). Further advancements in this promising field would allow the discovery of new mediators to control DNA methylation/demethylation, aiming to improve the lives of patients with IBD and IBD-CRC.

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ORIGINAL ARTICLE

Human platelets inhibit liver fibrosis in severe combined immunodeficiency mice

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Abstract

AIM: To investigate the role of human platelets in liver fibrosis.

METHODS: Severe combined immunodeficiency (SCID) mice were administered CCl_4 and either phosphate-buffered saline (PBS group) or human platelet transfusions (hPLT group). Concentrations of hepatocyte growth factor (HGF), matrix metallopeptidases (MMP)-9, and transforming growth factor-β (TGF-β) in the liver tissue were compared between the PBS and the hPLT groups by enzyme-linked immunosorbent assay (ELISA) and Western blotting. The effects of a human platelet transfusion on liver fibrosis included the fibrotic area, hydroxyproline content, and α-smooth muscle actin (α-SMA) expression, which were evaluated by picrosirius red staining, ELISA, and immunohistochemical staining using an anti-mouse α-SMA antibody, respectively. Phosphorylations of mesenchymal-epithelial transition factor (Met) and SMAD3, downstream signals of HGF and TGF-β, were compared between the two groups by Western blotting and were quantified using densitometry. Hepatocyte apoptosis was evaluated by terminal deoxynucleotidyl transferase dUTP nick end labeling. Furthermore, the accumulation of human platelets in the liver 2 h after platelet transfusion was compared between normal and fibrotic livers by immunohistochemical staining using an anti-human CD41 antibody.

RESULTS: The fibrotic area and hydroxyproline content in the liver were both significantly lower in the hPLT group when compared to the PBS group (fibrotic area, 1.7% ± 0.6% *vs* 2.5% ± 0.6%, $P = 0.03$; hydroxyproline content, 121 ± 26 ng/g liver *vs* 156 ± 47 ng/g liver, $P = 0.04$). There was less α-smooth muscle actin staining in the hPLT group than in the PBS group (0.5% ± 0.1% *vs* 0.8% ± 0.3%, $P = 0.02$). Hepatic expression levels of mouse HGF and MMP-9 were significantly higher in the hPLT group than in the PBS group (HGF, 109 ± 13 ng/g liver *vs* 88 ± 22 ng/g liver, $P = 0.03$; MMP-9, 113% ± 7%/GAPDH *vs* 92% ± 11%/GAPDH, $P = 0.04$). In contrast, the concentration of mouse TGF-β in the liver tissue was significantly lower in the hPLT group than in the PBS group (22 ± 5 ng/g liver *vs* 39 ± 6 ng/g liver, $P = 0.02$). Phosphorylation of Met was more prevalent in the hPLT group than in the PBS group (37% ± 4%/GAPDH *vs* 20% ± 8%/GAPDH, $P = 0.03$). Phosphorylation of SMAD3 was weaker in the hPLT group than in the PBS group (60% ± 12%/GAPDH *vs* 84% ± 12%/GAPDH, $P = 0.1$), although this difference was not significant. Furthermore, a lower rate of hepatocyte apoptosis was observed in the hPLT group than in the PBS group (5.9% ± 1.7% *vs* 2.9% ± 2.1%, $P = 0.02$). Significant human platelet accumulation was observed in the fibrotic liver tissues, whereas few platelets accumulated in the normal liver.

CONCLUSION: Human platelets inhibit liver fibrosis in SCID mice. Increased concentration of HGF in the liver suppresses hepatic stellate cell activation, induces MMPs, and inhibits hepatocyte apoptosis.

Key words: Human platelet; Liver fibrosis; Hepatocyte apoptosis; Hepatocyte growth factor; Transforming growth factor-β; Matrix metallopeptidases

Core tip: We assessed the effects of human platelet transfusion on liver fibrosis. Severe combined immunodeficiency (SCID) mice were administered CCl_4 and either phosphate-buffered saline or human platelets. The effects of a human platelet transfusion on liver fibrosis and hepatocyte apoptosis were compared. The fibrotic area, hydroxyproline content, and α-smooth muscle actin expression were decreased in mice that received human platelet transfusions. Transfusion increased mouse hepatocyte growth factor (HGF) and matrix metallopeptidases (MMP)-9 levels in the liver and decreased mouse transforming growth factor-β. Furthermore, transfusion suppressed hepatocyte apoptosis. Human platelets inhibited liver fibrosis in SCID mice. Increased concentration of HGF in the liver suppresses hepatic stellate cell activation, induces MMPs, and inhibits hepatocyte apoptosis.

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INTRODUCTION

Chronic liver disease and liver cirrhosis are major causes of morbidity and mortality worldwide. In chronic liver disease, normal repair of hepatocyte damage and tissue remodeling is lost, resulting in fibrosis and ultimately cirrhosis, which leads to portal hypertension, hepatocellular carcinoma, and lethal hepatic failure[1]. The most common etiological factors in chronic liver disease are chronic hepatitis C virus infection, excessive alcohol consumption, non-alcoholic fatty liver disease, and non-alcoholic steatohepatitis. Liver transplantation is the only curative approach, and specific treatments that stop progressive fibrosis are currently unavailable[1].

Liver fibrosis is characterized by the excessive production and deposition of the extracellular matrix (ECM) proteins, such as collagen, proteoglycans, fibronectins, and hyaluronic acids[2]. Accumulation of the ECM results in remodeling of the hepatic structure. Among the deposited ECM proteins, collagen type I is a major constituent, which is mainly produced by hepatic stellate cells (HSCs). Matrix metallopeptidases (MMPs) are the key enzymes responsible for the degradation of all protein components of the ECM[3]. Recently, it has been reported that hepatocyte apoptosis in cirrhotic liver induces HSC activation, which promotes liver fibrosis[4].

Liver cirrhosis has traditionally been viewed as an irreversible state in which the normal hepatocellular structures and organization are destroyed and fibrosis is firmly established. However, several reports have opposed this conventional concept. Lang *et al*[5] reported that blocking transforming growth factor-β (TGF-β) with small interference RNA suppressed HSC activation and decreased liver fibrosis in mice. Iimuro *et al*[6] showed that the delivery of MMP-1 attenuated established liver fibrosis in rats. In recent years, platelets have been shown to exert both anti-fibrotic and fibrolytic effects on the liver[7-10].

In this study, we transfused human platelets into severe combined immunodeficiency (SCID) mice to examine the effects of human platelet transfusion on liver fibrosis. This model was used for the following two reasons: first, there is no direct evidence that human platelets inhibit liver fibrosis. Second, because *in vivo* human studies are difficult, xenotransfusion of human platelets into SCID mice has been used to examine the functions of human platelets[11,12]. Using this model, we evaluated the effects of human platelet transfusion on liver fibrosis and hepatocyte apoptosis.

MATERIALS AND METHODS

Animals

Experiments were performed using 8-12-wk-old male C.B-17/lcr-scid/scid Jcl mice weighing 20-26 g (CLEA, Tokyo, Japan). Mice were maintained in a temperature-controlled room on a 12-h light-dark cycle with free access to water and standard chow. After an acclimation period of at least 7 d, mice were divided into two groups: CCl_4 plus phosphate-buffered saline (PBS) administration (PBS group), and CCl_4 plus human platelet transfusion (hPLT group). All experiments complied with the Guidelines for the Care and Use of Laboratory Animals (University of Tsukuba).

Models for liver cirrhosis

To induce liver fibrosis, each mouse received an intraperitoneal injection of CCl_4 (200 μL/kg body weight) in a 1:3 ratio with corn oil twice a week for 8 wk. PBS or concentrated human platelets was transfused once a week from weeks 5 to 8. A 500-μL aliquot of PBS or concentrated human platelets was injected into the retro-orbital vein one day after the administration of CCl_4. Mice were sacrificed 96 h after the final administration of PBS or human platelet transfusion, and livers were removed and divided into two samples; One liver section was fixed in 10% buffered formalin for subsequent immunohistochemical analysis, and the other section was snap-frozen in liquid nitrogen and kept at -80 ℃ until use.

Transfusion preparations

Human whole blood was obtained from healthy volunteers. Platelet-rich plasma was obtained by centrifuging anticoagulated blood containing acid-citrate-dextrose at a 1:4 volume ratio at 120 *g* for 10 min. Samples were then

centrifuged at 1000 *g* for 15 min, and resuspended in citrate buffer (120 mmol/L NaCl, 4.26 mmol/L $NaHPO_4$, 5.5 mmol/L glucose, 4.77 mmol/L sodium citrate, and 2.35 mmol/L citric acid at pH 6.5). Platelets were then suspended in PBS and counted using a hematology analyzer (MICROS abc LC-152; Horiba Ltd., Kyoto, Japan).

Transfusion conditions and flow cytometric analysis of transfused platelets

To determine the number of cells for transfusion, 2.5×10^8, 5.0×10^8, or 10.0×10^8 of human platelets were transfused into naive SCID mice, and the post-transfusion percentage of transfused platelets was measured after 6 h ($n = 3$). We examined at 6 h because a 10% increase in peripheral platelet count 6 h after platelet transfusion improved liver function of the patients with liver cirrhosis in our clinical study. Because it required approximately 15 mL of human whole blood to prepare 10.0×10^8 of human platelets, 10×10^8/body weight was determined to be the upper limit.

Peripheral blood was collected from the lateral tail vein. Blood samples were incubated for 30 min with a biotin-conjugated rat anti-mouse CD41 antibody (AbD Serotec, Oxford, United Kingdom) that specifically detected murine platelets. Samples were then washed in platelet HEPES buffer (137 mmol/L NaCl, 2 mmol/L KCl, 0.4 mmol/L NaH_2PO_4, 1 mmol/L $MgCl_2$, 5.6 mmol/L glucose at pH 7.4) containing 10% acid-citrate-dextrose, and centrifuged at 500 *g* for 5 min. Supernatants were removed and the cells were resuspended in platelet HEPES buffer containing 10% acid-citrate-dextrose. Samples were incubated with a FITC-conjugated mouse anti-human CD41 antibody (Dako, Glostrup, Denmark) that specifically detected human platelets and streptavidin-phycoerythrin (PE)/Cy5 (Biolegend, San Diego, CA, United States) for 30 min and then analyzed using a flow cytometer (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ, United States). The post-transfusion percentage of human platelets was defined as human platelets/(human platelets + murine platelets).

After 6 h, the post-transfusion percentages of human platelets in naive mice that received 2.5×10^8, 5.0×10^8, and 10.0×10^8 of human platelets were 0.6% ± 0.3%, 2.0% ± 1.6%, and 10.3% ± 1.4%, respectively (Figure 1). We used 10.0×10^8 of human platelets for each mouse in this study.

Platelet count and chemical parameters

Blood samples were collected at the time of sacrifice. Platelet count was measured, and serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (T-Bil), albumin (Alb), and total cholesterol (T-CHO) were measured and compared between the PBS group and the hPLT group (Fuji Drichem; Fuji Film Inc, Tokyo, Japan) ($n = 8$).

Histological examination

Liver samples were fixed in 10% buffered formalin, and stained with picrosirius red solution, and the liver fibrotic area was quantified using the winROOF visual system (Mitani Co., Tokyo, Japan) ($n = 8$). In addition, specimens were immunostained with an anti-α-smooth muscle actin (SMA) antibody (Dako) and counterstained with hematoxylin. α-SMA expression was also quantified using the winROOF visual system (Japan) ($n = 6$). To assess the hepatocellular mitotic index, liver sections were stained with hematoxylin and eosin, and the number of hepatocytes undergoing mitosis was calculated. In addition, proliferating cell nuclear antigen (PCNA) staining was conducted using a PCNA staining kit (Invitrogen Co., Carlsbad, CA, United States). PCNA-positive hepatocytes and hepatocytes undergoing mitosis were counted in four randomly selected high-power fields (× 200). Liver sections were also incubated with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) antibody (Promega KK, Tokyo, Japan). TUNEL-positive hepatocytes were counted in four randomly selected high-power fields (× 200) on each slide, and calculated as TUNEL-positive hepatocytes/total hepatocytes ($n = 6$).

Hepatocyte growth factor and TGF-β levels in the liver tissue

An enzyme-linked immunosorbent assay (ELISA) kit was used to measure mouse hepatocyte growth factor (HGF) (Institute of Immunology Co., LTD, Tokyo, Japan) and mouse TGF-β (R and D Systems, Minneapolis, MN, United States). ELISAs were used to measure levels of these proteins in 10% liver tissues lysates ($n = 8$).

Detection of liver hydroxyproline content

Hydroxyproline content was determined as described previously[13]. Briefly, 50 mg liver samples were hydrolyzed in 6 mol/L HCl at 120 ℃ for 16 h. After centrifugation, the supernatant was removed and neutralized with 6 mol/L NaOH. The solution was oxidized with Chloramine T (Sigma-Aldrich Corp., St Louis, MO, United States) in acetate/citrate buffer, followed by the addition of Ehrlich's solution (p-dimethylamino-benzaldehyde in 60% HCl4 with isopropanol). The final mixture was incubated at 60 ℃ for 30 min and then at room temperature for 10 min. Absorbance was determined at 560 nm. The value of the hepatic hydroxyproline concentration was expressed as μg/g wet tissue.

α-SMA and MMP-9 expression levels, and signal transduction cascades

For Western blotting analysis, protein was obtained from liver tissues lysates, separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes (Millipore, Bedford, MA, United States). We used primary antibodies specific for α-SMA (Dako), MMP-9 (AB1916) (Chemicon International, Temecula, CA, United States), phosphoserine mesenchymal-epithelial transition factor (Met) (3127), Met (3135S), phosphotyrosine SMAD3 (9529S), SMAD3 (9513), caspase-3 (9662), cleaved caspase-3 (9962), Bcl-2 (2876), glyceraldehyde-3-phosphate dehydrogenase

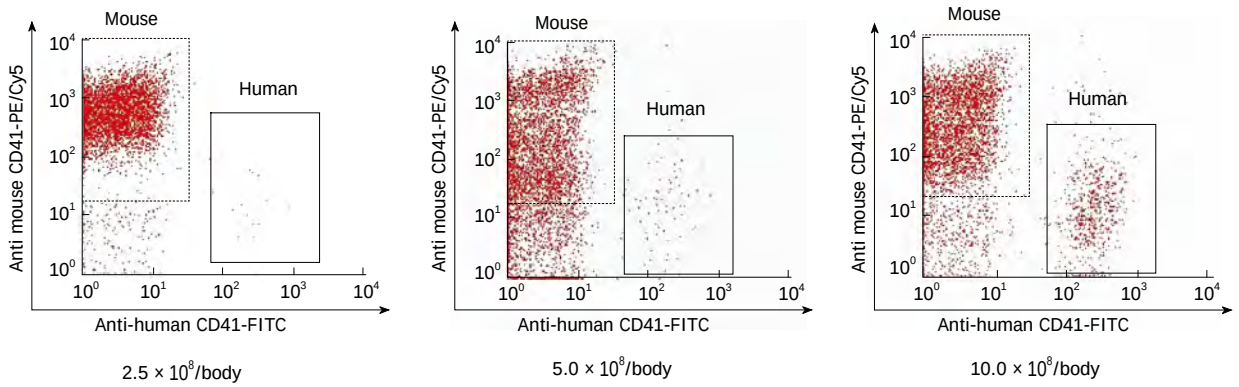

Figure 1 Transfusion conditions. The post-transfusion percentages of human platelets in naïve mice receiving 2.5 × 10^8, 5.0 × 10^8, and 10.0 × 10^8 human platelets. The post-transfusion percentage of human platelets was defined as human platelets/(human platelets + murine platelets). The post-transfusion percentages of human platelets in mice receiving 2.5 × 10^8, 5.0 × 10^8, and 10.0 × 10^8 of human platelets were 0.6% ± 0.3%, 2.0% ± 1.6%, and 10.3% ± 1.4%, respectively. *n* = 3 per group. Data are expressed as the mean ± SD. CD41-FITC: Cluster of differentiation 41-fluorescein isothiocyanate.

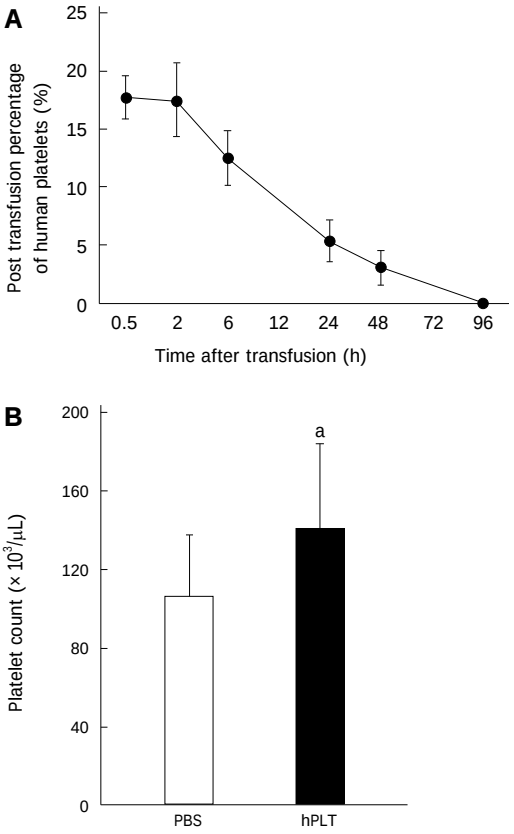

Figure 2 Post-transfusion percentages of human platelets and peripheral platelet counts. A: The post-transfusion percentages of human platelets. Human platelets disappeared from the circulation at 96 h post-transfusion. *n* = 3 per group. Data are expressed as the mean ± SD; B: Peripheral platelet counts. The peripheral platelet count was significantly higher in the human platelet transfusions group than in the phosphate-buffered saline group. *n* = 8 per group. Data are expressed as the mean ± SD. [a]*P* < 0.05 using an unpaired *t*-test. PBS: Phosphate-buffered saline; hPLT: Human platelet transfusions.

(GAPDH) (2118), and β-actin (4970) (Cell Signaling Technology, Beverly, MA, United States) and secondary mouse or rabbit antibodies conjugated with horseradish peroxidase (Invitrogen Co.). Immunoblots were analyzed using an enhanced chemiluminescence system. Protein band densities were quantified using densitometry. Band intensities were normalized to those of GAPDH, caspase-3, Met, or SMAD3 (n = 3).

Immunohistochemistry for human platelets

Human platelets were transfused to SCID mice with normal or fibrotic livers, and accumulation of the transfused human platelets in the liver 2 h after transfusion was measured and compared between the two groups.

Immunofluorescence staining was performed on 5 μm thick sections of tissue that had been fixed in 4% paraformaldehyde, immersed in OCT compound, and incubated with FITC-conjugated anti-human CD41 antibody (Dako). Stained sections were examined under a confocal laser-scanning microscope (BZ-9000, Keyence Co., Tokyo, Japan).

Statistical analysis

All data are expressed as means ± SD. Unpaired *t*-tests were used to compare two groups. P values < 0.05 were considered significant.

RESULTS

The post-transfusion ratio of human platelets and peripheral platelet counts

Human platelets disappeared from the peripheral blood 96 h after transfusion (Figure 2A). The peripheral platelet counts at the time of sacrifice, *i.e.*, 96 h after transfusion, were significantly higher in the hPLT group than in the PBS group (P < 0.05) (Figure 2B).

Liver/body weight ratio, PCNA labeling index, mitotic index, and spleen/body weight ratio

There were no significant differences in the liver/body weight ratio, PCNA index, mitotic index, and spleen/body weight ratio between the hPLT and PBS groups (Table 1).

Serum AST, ALT, T-bil, Alb, and T-CHO concentrations

There were no significant differences in the serum AST,

Table 1 Liver regeneration indices and spleen/body weight ratios, serum asparatate aminotransferase, alanine aminotransferase, total bilirubin, albumin, and total cholesterol concentrations

	Liver/body weight ratio	PCNA labeling index (/HPF)	Mitotic index (/HPF)	Spleen/body weight ratio	AST (U/mL)	ALT (U/mL)	T-bil (mg/mL)	Alb (g/mL)	T-CHO (g/mL)
PBS	6.2% ± 0.5%	2.1 ± 0.9	0.6 ± 0.3	0.23% ± 0.04%	50 ± 21	122 ± 56	1.0 ± 0.2	3.0 ± 1.0	77.8 ± 5.4
hPLT	6.7% ± 0.5%	2.4 ± 0.9	0.6 ± 0.5	0.24% ± 0.05%	50 ± 15	104 ± 64	0.8 ± 0.2	3.0 ± 1.5	82.7 ± 4.4[a]

n = 8 per group. [a]P < 0.05 for the human platelet transfusions (hPLT) group *vs* the phosphate-buffered saline (PBS) group. PCNA: Proliferating cell antigen; HPF: High-power field; AST: Asparatate aminotransferase; ALT: Alanine aminotransferase; T-bil: Total bilirubin; Alb: Albumin; T-CHO: Total cholesterol.

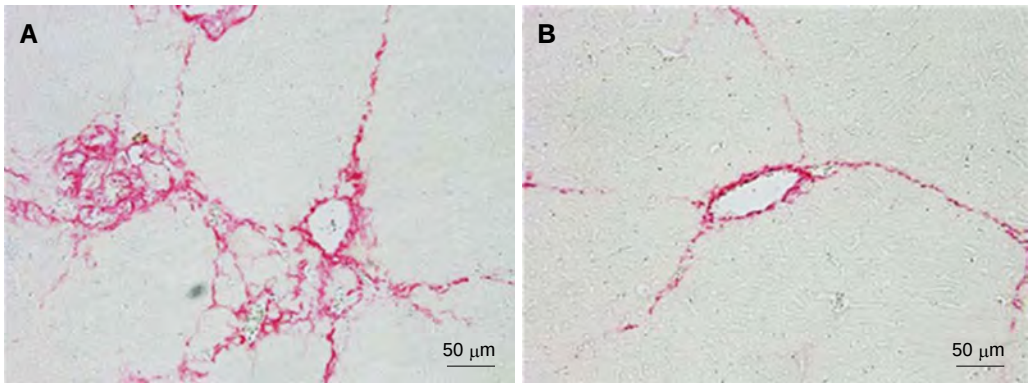

Figure 3 Fibrotic index and hydroxyproline contents. A: The fibrotic index, which was calculated based on the area stained by picrosirius red solution, was significantly lower in the human platelet transfusions (hPLT group) group than in the phosphate-buffered saline (PBS) group; B: The hydroxyproline content in the liver tissue was significantly lower in the hPLT group than in the PBS group. n = 8 per group.

T-bil, and Alb levels between the PBS and hPLT groups. Despite the lack of statistically significant differences, there was a tendency for the serum ALT level to be lower in the hPLT group than in the PBS group (P = 0.3). The serum T-CHO level was significantly higher in the hPLT group than in the PBS group (P < 0.05) (Table 1).

Fibrotic index and liver hydroxyproline content

The fibrotic index, which was calculated based on the area stained with picrosirius red solution, was significantly lower in the hPLT group than in the PBS group (P < 0.05) (Figure 3A). In addition, the liver hydroxyproline content was significantly lower in the hPLT group than in the PBS group (P < 0.05) (Figure 3B).

α-SMA and TUNEL stainings and MMP-9, Bcl-2, caspase-3, and cleaved caspase-3 expression levels

There was less α-SMA staining in the hPLT group compared to the PBS group (Figure 4A and B). TUNEL staining revealed only a few apoptotic cells in the hPLT group, whereas several apoptotic hepatocytes were observed in the PBS group (Figure 4C and D). α-SMA expression calculated based on the area stained by anti-α-SMA antibody and TUNEL positive hepatocytes/total hepatocytes were significantly lower in the hPLT group than in the PBS group (both P < 0.05) (Figure 4E).

MMP-9 expression was significantly higher in the hPLT group than in the PBS group (P < 0.05) (Figure 4F and G). Cleaved caspase-3 expression was significantly lower in the hPLT group than in the PBS group (P < 0.05), whereas Bcl-2 was more robustly expressed in the hPLT group as compared to the PBS group (P < 0.01) (Figure 4F and G).

Mouse HGF and TGF-β levels in the liver tissues and cellular signal transduction

Expression of mouse HGF in the liver tissue was significantly higher in the hPLT group than in the PBS group (P < 0.05) (Figure 5A). The concentration of mouse TGF-β was significantly lower in the liver tissues of the hPLT group than in the PBS group (P < 0.05) (Figure 5B).

There was increased Met phosphorylation in the hPLT group compared to the PBS group (P < 0.05) (Figure 5C and D). Although the difference was not statistically significant, SMAD3 phosphorylation was lower in the hPLT group than in the PBS group (P = 0.1) (Figure 5C and D).

Accumulation of human platelets in the liver

Significant human platelet accumulation in the liver was observed in the fibrotic liver tissues, whereas fewer platelets accumulated in the normal liver (Figure 6).

DISCUSSION

We demonstrated that human platelets suppressed liver fibrosis in SCID mice. It was suspected that these anti-fibrotic effects were due to an increased concentration of HGF in the liver, resulting in decreased TGF-β concentrations and increased MMP-9 levels. Furthermore, inhibition of hepatocyte apoptosis by HGF may have suppressed

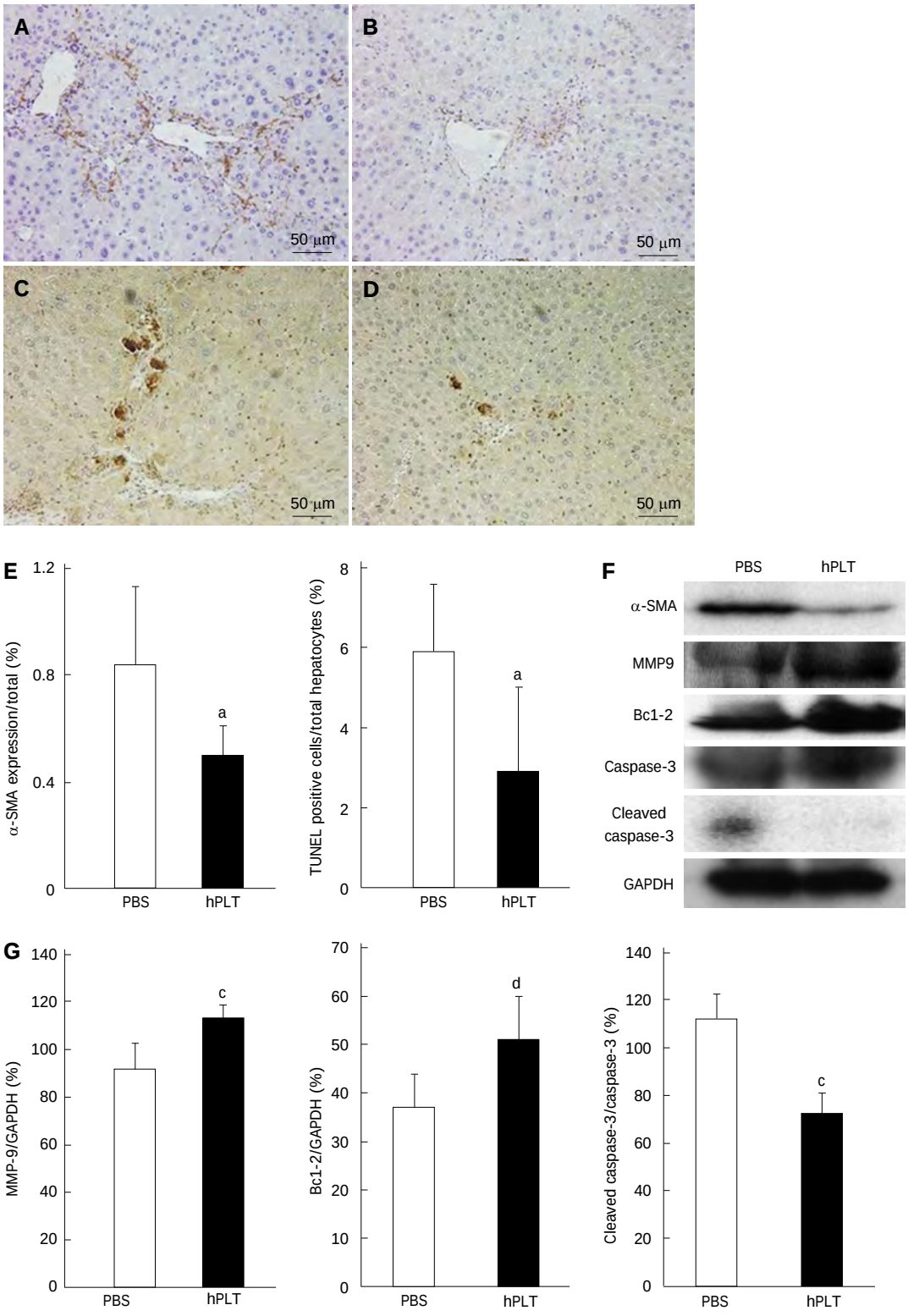

Figure 4 α-smooth muscle actin and TUNEL staining. Matrix metalloproteinase-9 (MMP-9), Bcl-2, caspase-3, cleaved caspase-3 expression levels. A, B: Immunostaining of α-smooth muscle actin (α-SMA) in the phosphate-buffered saline (PBS group) or human platelet transfusions (hPLT group). The α-SMA staining was less robust in the hPLT group than in the PBS group; C, D: Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining in the PBS and hPLT groups. Few apoptotic cells were observed in the hPLT group, whereas several apoptotic hepatocytes were observed in the PBS group; E: α-SMA expression calculated based on the area stained by anti-α-SMA antibody and TUNEL-positive hepatocytes/total hepatocytes in the PBS and hPLT groups. *n* = 6 per group. Data are expressed as the mean ± SD. $^{a}P < 0.05$ using an unpaired *t*-test. α-SMA expression and the number of apoptotic hepatocytes in the hPLT group were lower than those in the PBS group; F: α-SMA, MMP-9, Bcl-2, caspase-3, and cleaved caspase-3 expression levels assessed with Western blotting. α-SMA and cleaved caspase-3 expression levels were less intense in the hPLT group than in the PBS group, whereas MMP-9 and Bcl-2 expression levels were stronger in the hPLT group than in the PBS group; G: MMP-9, Bcl-2, and cleaved caspase-3 expression levels were quantified using densitometry. *n* = 3 per group. Data are expressed as the means ± SD. $^{c}P < 0.05$ and $^{d}P < 0.01$ using an unpaired *t*-test. MMP-9 and Bcl-2 expression levels were significantly higher in the hPLT group than in the PBS group, whereas cleaved caspase-3 expression was significantly lower in the hPLT group than in the PBS group. Bcl-2: B-cell lymphoma-2.

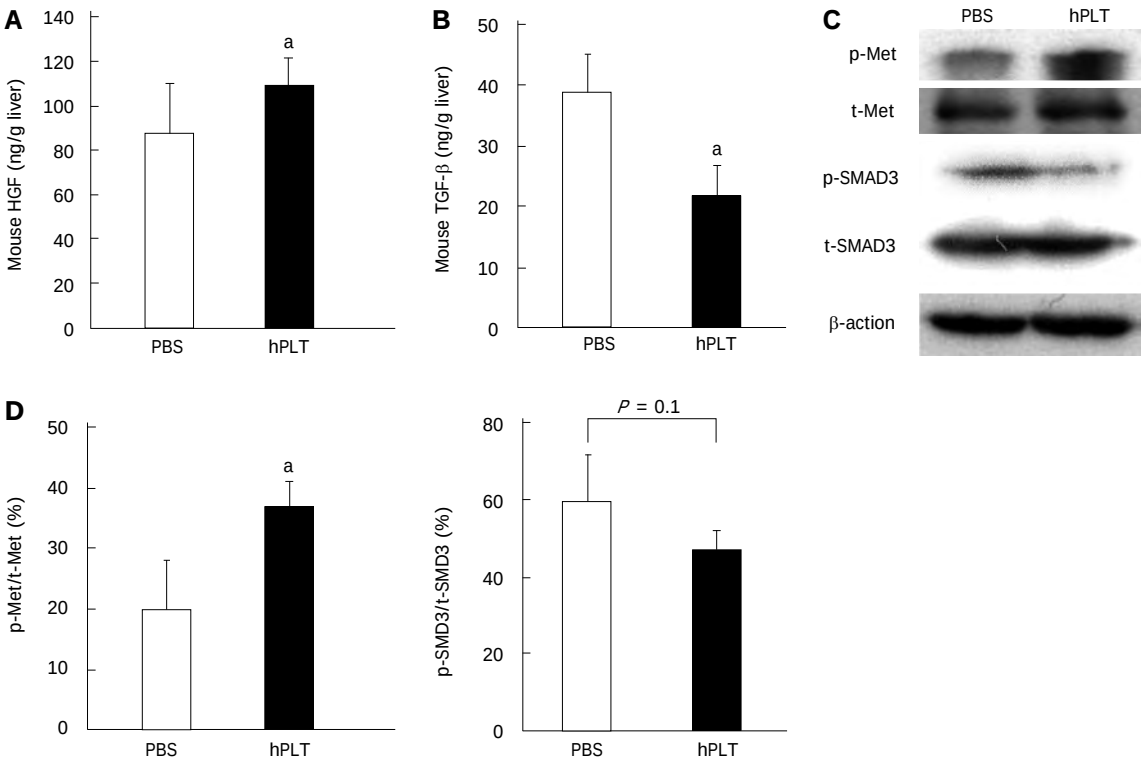

Figure 5 Mouse hepatocyte growth factor and transforming growth factor-β in liver tissue and cellular signal transductions. A: Mouse hepatocyte growth factor (HGF) concentrations in liver tissue. $n = 8$ per group. Data are expressed as the means ± SD. $^{a}P < 0.05$ for the human platelet transfusions (hPLT) group *vs* the phosphate-buffered saline (PBS) group using an unpaired *t*-test. Mouse HGF expression was significantly higher in the hPLT group than in the PBS group; B: Mouse transforming growth factor-β (TGF-β) concentrations in liver tissue. $n = 8$ per group. Data are expressed as the mean ± SD. $^{a}P < 0.05$ using an unpaired *t*-test. Mouse TGF-β expression was significantly lower in the hPLT group than in the PBS group; C: Phosphorylation of mesenchymal-epithelial transition factor (Met) and SMAD3 in the PBS and hPLT groups. Met was more highly phosphorylated in the hPLT group than in the PBS group, whereas phosphorylation of SMAD3 was weaker in the hPLT group than in the PBS group; D: Met and SMAD3 phosphorylation levels were quantified using densitometry. $n = 3$ per group. Data are expressed as the mean ± SD. $^{a}P < 0.05$ using an unpaired *t*-test. Phosphorylation of Met was significantly higher in the hPLT group than in the PBS group. Although the difference was not statistically significant, phosphorylation of SMAD3 tended to be lower in the hPLT group than in the PBS group.

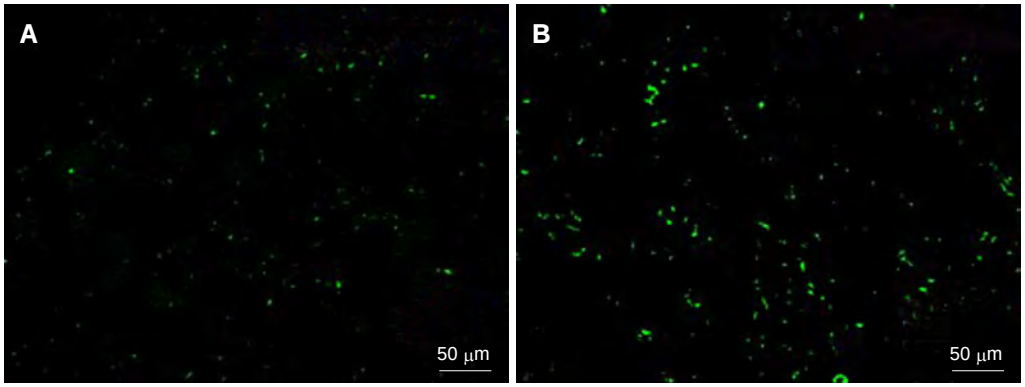

Figure 6 Accumulation of transfused human platelets in the liver. A: Normal liver; B: Fibrotic liver. Immunostaining images obtained using anti-human CD41 antibody 2 h after transfusion. Significant human platelet accumulation in the liver was observed in the fibrotic liver, whereas few platelets accumulated in the normal liver.

HSC activation, resulting in decreased fibrotic changes. These results, together with recent reports showing that platelets contribute to liver regeneration[12,14-21], suggest that platelet increment therapy, such as thrombopoietin administration and platelet transfusions, may provide new clinical approaches for the treatment of liver diseases.

Platelets contain three types of secretory granules, notably α-granules, dense-granules, and lysosomal granules[22]. Each granule contains growth factors, such as platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), HGF, vascular endothelial growth factor, serotonin, ATP, and epidermal growth factor, among others[22]. The granule constituents of platelets exhibit species differences, *i.e.*, although rodent platelets contain a large amount of HGF[16,23], human platelets

do not[24]. Platelets accumulate in the liver in response to various conditions, such as ischemia and reperfusion[25], cirrhosis[26], cholestasis[27], and viral hepatitis[28]. Although most studies have evaluated platelets as promoters of inflammatory responses and liver injury[25,26,28], recent scientific[12,14-19] and clinical data[20,21] have revealed additional and different roles for platelets in the liver. We previously showed that platelets accelerate liver regeneration through three different mechanisms: a direct effect on hepatocytes[14,16], a cooperative effect with liver sinusoidal endothelial cells[18], and a collaborative effect with Kupffer cells[12]. Furthermore, platelets are reported to have anti-fibrotic and fibrolytic effects on the liver[7-10]. We have indicated that thrombopoietin-induced thrombocytosis attenuated fibrotic changes in rodents[7,8]. Kodama *et al*[9] reported that platelets exert an anti-fibrotic role by suppressing collagen type I expression *via* the HGF/Met signaling pathway. Ikeda *et al*[10] demonstrated that human platelet-derived ATP suppressed the activation of HSCs through the adenosine-cyclic 5'-adenosine monophosphate signaling pathway. In addition, Maruyama *et al*[29] reported that platelet transfusion once a week for 12 wk decreased serum hyaluronic acid concentrations, a fibrotic marker, in chronic hepatitis patients with Child-Pugh class A or B. In the present study, human platelet transfusion inhibited liver fibrosis in SCID mice. The elevated peripheral platelet counts and the higher serum T-CHO concentrations after transfusion were consequences of reduced liver cirrhosis. Furthermore, the increased number of platelets that accumulated in the fibrotic liver implied that transfused platelets accumulation was induced in the fibrotic liver and released biologically-active substances, such as ATP, which directly suppresses HSC activation and decreases fibrosis[10].

HSCs undergo a complex transformation and activation process during which the cells morphologically change from quiescent oval-shaped cells to activated spindle-shaped cells. The activation of HSCs correlates with α-SMA expression[30]. TGF-β is produced by HSCs and Kupffer cells and is recognized as the main pro-fibrogenic mediator that triggers HSC activation. Hepatic TGF-β concentrations have been shown to be increased among patients with liver cirrhosis[31]. The effects of TGF-β are mediated by intracellular signaling via SMAD proteins, which modulate the transcription of target genes[32]. Following ligand binding to the TGF-β type Ⅱ receptors, the TGF-β type Ⅰ receptor becomes activated. SMAD3 proteins associate with the activated receptor and become phosphorylated, allowing the formation of oligomeric complexes with SMAD4. This heterotrimeric complex translocates into the nucleus and binds to specific nucleotide motifs to regulate transcription of target genes such as *COL1A2*, which encodes the collagen α-2 (1) chain in HSCs[32]. In the present study, although there were no significant differences in the liver/body weight ratio, spleen/body ratio, and liver regeneration indexes, fibrogenic markers such as the fibrotic index, hydroxyproline content, and expression of α-SMA were decreased upon human platelet transfusion. In addition, TGF-β concentration decreased with subsequent suppression of SMAD3 phosphorylation after platelet transfusion. These results indicated that human platelet transfusion might have suppressed liver fibrosis by reducing the TGF-β concentration in the liver.

HGF is predominantly produced by Kupffer cells[33]. HGF is known for its major roles in liver development and regeneration by exerting mitogenic and morphogenic effects on hepatocytes. After HGF binds to Met, Met is phosphorylated and intracellular adapter proteins activate distinct intracellular signals, such as the PI3K, Ras, and ERK pathways, and execute pro-mitogenic and anti-apoptotic functions[34]. HGF contributes to the resolution of fibrosis by regulating TGF-β and MMP levels[35]. Giebeler *et al*[36] reported that hepatocyte-specific Met knockout mice exhibited increased expression of TGF-β, α-SMA, and collagen-1α messenger RNA, and enhanced collagen fiber staining. Kanemura *et al*[37] reported that up-regulated HGF expression after human *HGF* gene delivery induced higher MMP activities. In the present study, the mouse HGF concentration in the liver tissue was elevated after human platelet transfusion. Because human platelets do not contain significant amounts of HGF[24], it was suspected that the expression of HGF in the liver might be elevated because of enhanced release from Kupffer cells or an increased amount of mouse platelet accumulation in the liver, leading to a reduction in the TGF-β concentration and attenuated HSC activation. Furthermore, HGF might have enhanced the production of MMP-9, which promotes fibrinolysis in the liver.

In recent years, liver fibrosis has been considered to be associated with hepatocyte apoptosis[4]. Hepatic fibrosis was shown to be significantly reduced when Fas-mediated apoptosis was impaired or when caspases were inhibited[38]. Moreover, persistent hepatocyte apoptosis has been shown to lead to liver fibrosis due to hepatocyte disruption of Bcl-xL[39]. Engulfment of apoptotic bodies by Kupffer cells has been demonstrated to promote TGF-β production, and phagocytosis of apoptotic bodies by HSCs leads to their activation and increased production of TGF-β and collagen type Ⅰ. Hisakura *et al*[40] reported that platelets protect against hepatocyte apoptosis and induce immediate activation of the Akt pathway, followed by an increase in Bcl-xL and a decrease in cleaved caspase-3 in hepatocytes. In the present study, hepatocyte apoptosis and expression of cleaved caspase-3 were suppressed and Bcl-2, an inhibitor of caspase-3, was increased by human platelet transfusion. It was hypothesized that inhibition of apoptosis by human platelet transfusion might help suppress liver fibrosis. Specifically, because HGF has an anti-apoptotic effect[34], elevated HGF levels may contribute to the inhabitation of hepatocyte apoptosis.

However, several questions remain. First, there are several types of growth factors in platelets that exert pro-fibrotic or anti-fibrotic effects. For example, platelet-derived chemokine ligand 4[26] and PDGF[41] induce HSC activation, whereas ATP[10] and IGF-1[42] suppress

HSC activation. It is difficult to explain the pro-fibrotic or anti-fibrotic effects by one or two substances within platelets. In addition, there are many cell types in the liver, such as hepatocytes, Kupffer cells, HSCs, and liver sinusoidal endothelial cells, that are involved in liver fibrogenesis. Therefore, it is important to view these results from a comprehensive perspective. Second, in this study, there were no differences in liver regeneration between the PBS and hPLT groups, which differed from our previous study[7]. It has been reported that a higher dose of CCl_4 is necessary to induce liver fibrosis in SCID mice compared to wild-type mice[43]. In this study, the degree of liver fibrosis was reduced compared to the previous study. The reduced fibrosis in the current model may have contributed to the low PCNA labeling index and hepatocyte mitosis in the hPLT group. Furthermore, in our previous study, we induced thrombocytosis using thrombopoietin, which resulted in higher peripheral platelet counts than those observed in this study. These differences in the degree of fibrosis and peripheral platelet counts may underlie the discrepancies in the results related to the requirement for the hepatocyte cell cycle and mitosis. Third, HGF and TGF-β are both produced by Kupffer cells, and the discrepancy in the dynamics of these growth factors was not clear. Because TGF-β is also produced by HSCs, it is possible that the increased HGF levels resulting from human platelet transfusion mainly suppressed HSC activity and down-regulated TGF-β expression in the liver. Fourth, although there was a significant difference in hepatocyte apoptosis as evaluated by TUNEL staining, serum AST and ALT concentrations were not significantly different. In our fibrosis model using CCl_4 with this duration and dose, it was difficult to induce strong fibrosis and apoptosis of hepatocytes in SCID mice. Despite statistically significant differences in the number of apoptotic hepatocytes between the PBS and hPLT groups, the difference was small considering the damage to the entire liver. Therefore, the damage did not reflect the serum AST and ALT concentrations.

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COMMENTS

Background

Liver cirrhosis is the ultimate stage of liver fibrosis, and there are currently no specific treatments that inhibit progressive fibrosis. Hepatocyte growth factor (HGF) helps resolve fibrosis by regulating transforming growth factor-β (TGF-β), matrix metallopeptidases (MMPs), and hepatocyte apoptosis.

Research frontiers

Platelets have been conventionally regarded as an exacerbating factor to inflammatory response and injury in the liver. However, recent studies have demonstrated the role of platelets in promoting liver regeneration, improving liver fibrosis, and attenuating hepatitis. In this study, authors assessed the effects of human platelet transfusion on liver fibrosis.

Innovations and breakthroughs

Platelets contain three types of secretory granules: α-granules, dense-granules, and lysosomal granules. Each granule contains growth factors. The granule constituents of platelets exhibit species differences, *i.e.*, human platelets do not contain significant amounts of HGF. This is the first study to show that human platelets have a role in suppressing liver fibrosis.

Applications

By demonstrating that human platelets suppress liver fibrosis, this study represents a potential future strategy for platelet therapy in the treatment of patients with liver cirrhosis.

Terminology

HGF is known for its major roles in liver development and regeneration. After HGF binds to mesenchymal-epithelial transition factor (Met), Met is phosphorylated, and intracellular adapter proteins activate distinct intracellular signals, and execute pro-mitogenic and anti-apoptotic functions. HGF is known to contribute to the resolution of fibrosis by regulating TGF-β and MMP levels.

Peer review

The authors examined the role of human platelets on liver fibrosis. It was revealed that increased concentrations of HGF in the liver suppressed hepatic stellate cell activation, induced MMPs, and inhibited hepatocyte apoptosis. The results are interesting and may provide new clinical approaches for the treatment of liver cirrhosis.

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ORIGINAL ARTICLE

Human development index is associated with mortality-to-incidence ratios of gastrointestinal cancers

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Author contributions: Hu QD and Liang TB conceived the study; Hu QD and Zhang Q, Liang TB designed the study; Hu QD collected data; Hu QD, Zhang Q, Chen W and Bai XL analyzed the data; Hu QD, Zhang Q and Chen W drafted the manuscript; Liang TB finalized the manuscript, and took responsibility for the integrity of the data and the accuracy of the data analysis; all authors have read and approved the final manuscript.

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Abstract

AIM: To identify the role of human development in the incidence and mortality rates of gastrointestinal cancers worldwide.

METHODS: The age-standardized incidence and mortality rates for gastrointestinal cancers, including cancers of the esophagus, stomach, pancreas, liver, gallbladder, and colorectum, were obtained from the GLOBOCAN 2008 database and United States Cancer Statistics (USCS) report. The human development index (HDI) data were calculated according to the 2011 Human Development Report. We estimated the mortality-to-incidence ratios (MIRs) at the regional and national levels, and explored the association of the MIR with development levels as measured by the HDI using a modified "drug dose to inhibition response" model. Furthermore, countries were divided into four groups according to the HDI distribution, and the MIRs of the four HDI groups were compared by one-way ANOVA followed by the Tukey-Kramer *post-hoc* test. State-specific MIRs in the United States were predicted from the estimated HDI using the fitted non-linear model, and were compared with the actual MIRs calculated from data in the USCS report.

RESULTS: The worldwide incidence and mortality rates of gastrointestinal cancers were as high as 39.4 and 54.9 cases per 100000 individuals, respectively. Linear and non-linear regression analyses revealed an inverse correlation between the MIR of gastrointestinal cancers and the HDI at the regional and national levels ($\beta < 0$; $P = 0.0028$ for regional level and < 0.0001 for national level, ANOVA). The MIR differed significantly among the four HDI areas (very high HDI, 0.620 ± 0.033; high HDI, 0.807 ± 0.018; medium HDI, 0.857 ± 0.021; low HDI, 0.953 ± 0.011; $P < 0.001$, one-way ANOVA). Prediction of the MIRs for individual United States states using best-fitted non-linear models showed little deviation from the actual MIRs in the United States. Except for 28 data points (9.93% of 282), the actual MIRs of all gastrointestinal cancers were mostly located in the prediction intervals *via* the best-fit non-linear regression models.

CONCLUSION: The inverse correlation between HDI and MIR demonstrates that more developed areas have a relatively efficacious healthcare system, resulting in low MIRs, and HDI can be used to estimate the MIR.

Key words: Gastrointestinal neoplasms; Mortality-to-incidence ratio; Human development index; Healthcare disparities; Socioeconomic factors

Core tip: This study is the first to explore the exact re-

lationship between the epidemiology of gastrointestinal cancers and area-specific development disparities. We showed the association between the mortality-to-incidence ratios (MIRs) and the human development index at the regional and national levels using a modified "drug dose to inhibition response" model. Further prediction of the MIRs for individual United States states on the basis of best-fitted non-linear models showed little deviation from the actual MIRs in the United States.

Hu QD, Zhang Q, Chen W, Bai XL, Liang TB. Human development index is associated with mortality-to-incidence ratios of gastrointestinal cancers. *World J Gastroenterol* 2013; 19(32): 5261-5270 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5261.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5261

INTRODUCTION

The digestive system includes multiple organs within or alongside the alimentary tract and is of vital importance in the proper functioning of the body. Currently, gastrointestinal cancer is a leading cause of cancer-related deaths in many developed countries, and it has been predicted to have the highest incidence and mortality rates worldwide, irrespective of the level of a country's resources[1-3]. Gastrointestinal cancers are known to notably affect the pathophysiological condition and functioning of the digestive system. Both cancer incidence and mortality in highly developed countries such as the United States peaked in the early 1990s and have since declined because of enhanced awareness, preventive measures, earlier detection and the availability of new and more effective treatment regimens, although very little progress has been made in the treatment of some cancers such as pancreatic cancer[4]. In contrast, limited or inaccessible healthcare resources in developing areas remain barriers to the effective control of future changes in incidence and mortality rates[5,6]. The expected cancer burden will continue to be a serious public health problem in the coming decade, particularly in developing countries[3,7,8].

Disparities in healthcare have received considerable attention from international organizations and national governments[9,10]. The socioeconomic determinants of the inequality reflect regional imbalances in human development. A previous study found that 35% of the cancer deaths may be attributable to nine modifiable risk factors: alcohol, smoking, low fruit and vegetable intake, overweight and obesity, physical inactivity, urban air pollution, unsafe sex, contaminated injections in health care settings, and indoor smoke from household activities such as cooking or indoor heating[11]. Most of these risk factors vary widely among populations in areas with different levels of development[12,13]. However, there is little knowledge about the healthcare disparities in the individuals suffering from gastrointestinal cancers. This study is the first to explore the exact relationship between the epidemiology of gastrointestinal cancers and area-specific development disparities. We aimed to identify the role of human development in the incidence and mortality rates of gastrointestinal cancers worldwide.

MATERIALS AND METHODS

Incidence and mortality data

The global incidence and mortality estimates for gastrointestinal cancers in 184 countries were obtained from the GLOBOCAN 2008 database (http://globocan.iarc.fr/) maintained by the WHO International Agency for Research on Cancer[14,15]. GLOBOCAN also provided regional estimates for each continent.

United States Cancer Statistics (USCS) reported the incidence and mortality rates associated with cancers in United States states[16]. State-specific incidence data were collected from the National Program of Cancer Registries and the Surveillance, Epidemiology, and End Results Program. Mortality information was collected by the National Vital Statistics System, National Center for Health Statistics and United States Centers for Disease Control and Prevention (United States-CDC).

We obtained the data of the incidence and mortality rates of gastrointestinal cancers in six major sites, namely, the esophagus, stomach, pancreas, liver, gallbladder, and colorectum. The USCS did not provide gallbladder cancer data. The overall rates of gastrointestinal cancers were estimated by addition of the rates of the six cancers. The rates were age-standardized using the world standard population and a previously proposed method[17], and presented as age-standardized rates (ASR). ASR is a summary measure of the rate that a population distribution would have if it had a standard age structure. Since age has a powerful influence on the risk of cancer, standardization is necessary when comparing several populations that differ with respect to age[14].

Mortality-to-incidence ratio

The mortality-to-incidence ratios (MIRs) were calculated from the obtained incidence and mortality rates provided by the GLOBOCAN database[14] and USCS report[16]. Extreme MIRs (0, 1, or > 1) were considered abnormal because of (1) illogical data (zero mortality or mortality more than incidence); and (2) zero incidence, and these results were excluded from the regression fit and further analysis. Respectively, 25, 13, 62, 82, 46, and 0 extreme MIR results were excluded in the analysis for cancers of the esophagus, stomach, pancreas, liver, gallbladder, and colorectum.

Estimated human development index

The human development index (HDI) data of Union Nation members in 1980-2011 were available in the United Nations Development Programme (UNDP) database (http://hdr.undp.org/en/statistics). The HDI was calculated according to the 2011 Human Development Report (HDR 2011)[18]. The HDI of Taiwan was

obtained from the National Statistics (Taiwan) website (http://www.stat.gov.tw), and subsequently verified.

We further estimated the state-specific HDI in the United States on the basis of data provided by various data agencies. Information on life expectancy at birth provided by the CDC was adapted by the American HDI Project[19]. The gross domestic product (GDP) per capita was acquired from the Bureau of Economic Analysis at the United States Department of Commerce, and compiled by the Bureau of Business and Economic Research, University of New Mexico[20]. The GDP values were converted to international dollars using purchasing power parity rates. The mean duration of education was estimated from the 2009 American Community Survey data provided by the United States Census Bureau[21], according to the method of Barro and Lee[22]. The expected duration of education in the United States was defined as 12 years; this value was adapted from the United Nations Educational, Scientific and Cultural Organization Institute for Statistics[23].

Statistical analysis

Only the countries with both epidemiologic data from the GLOBOCAN database and HDI from the UNDP program were included in the analysis. Taiwan was not excluded because its HDI value was available at the National Statistics (Taiwan) website. The number of countries included in our research was 165. Patterns in the MIR of gastrointestinal cancers with respect to the levels of socioeconomic development were investigated by correlating the MIRs to the corresponding HDIs *via* linear or non-linear regression. Linear regression fit was conducted to determine the existence of correlations. Derivation of the slope parameter β from 0 was defined by ANOVA. Correlation existence referred to the significantly non-zero β value. Non-linear regression fit was based on a modified "drug dose to inhibition response" model using the formula:

$$\mathrm{MIR} = \frac{1}{1+10^{(\mathrm{HDI}_{50}-\mathrm{HDI})\times\mathrm{Slope}}},$$

where "HDI_{50}" was the HDI value at half maximal MIR and "slope" was a parameter that indicated the steepness of the slope. The MIRs of the four HDI groups were compared by one-way ANOVA followed by the Tukey-Kramer *post-hoc* test. A P value of less than 0.05 was considered statistically significant. Statistical analysis and plotting were performed using Prism 5 (GraphPad, San Diego, CA, United States). The geographical map showing MIR was created using the open source software TileMill (a GitHub project maintained by MapBox, Washington, WI, United States), with map data sources from the Natural Earth database rendered by the Mapnik Library.

RESULTS

Global incidence and mortality of gastrointestinal cancers

In 2008, gastrointestinal cancers were estimated to have affected a total of 3878986 individuals and caused 2824985 deaths worldwide. The global mortality and incidence rates were as high as 39.4 and 54.9 cases per 100000 individuals, respectively. Colorectal cancer was the third most common cancer with 1235108 incidences among the 27 cancers included in the GLOBOCAN database, and it was the most common cancer among the six gastrointestinal cancers included in the current study. Other prevalent cancers according to the incidences reported in the database included stomach cancer (ranked 4th, with 988602 incidences), liver cancer (6th, 749744 incidences), esophageal cancer (8th, 481645 incidences), pancreatic cancer (13th, 278684 incidences), and gallbladder cancer (21st, 145203 incidences). However, stomach cancer had the highest mortality rate (26.1%, 737419 deaths) among all gastrointestinal cancers. In terms of the mortality rate, liver cancer (ranked 3rd with 695726 deaths), colorectal cancer (4th, 609051 deaths), esophageal cancer (6th, 406533 deaths), pancreatic cancer (8th, 266669 deaths) and gallbladder cancer (17th, 109587 deaths) contributed to 24.6%, 21.6%, 14.4%, 9.4% and 3.9% of all deaths caused by gastrointestinal cancers, respectively.

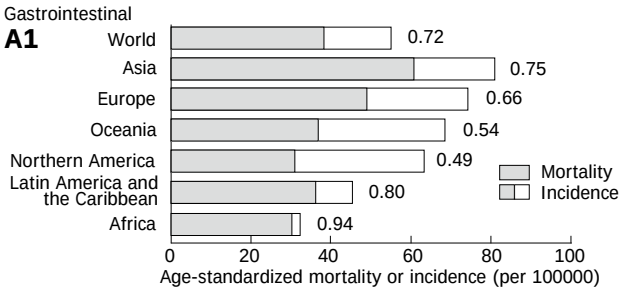
Differences in the regional incidence and mortality

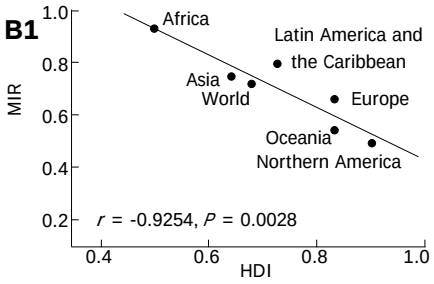
The regional incidence and mortality rates varied among different continents and regions (Figure 1A). Asia had the highest incidence rates of esophageal, stomach and liver cancers, as well as gastrointestinal cancers overall. Interestingly, the MIRs for gastrointestinal cancers, except for pancreatic cancer, were higher in Africa compared with other continents. Linear regression analysis revealed a significant inverse correlation between the regional HDI and MIR of stomach, gallbladder and colorectal cancers and gastrointestinal cancer overall ($P < 0.05$, ANOVA) (Figure 1B).

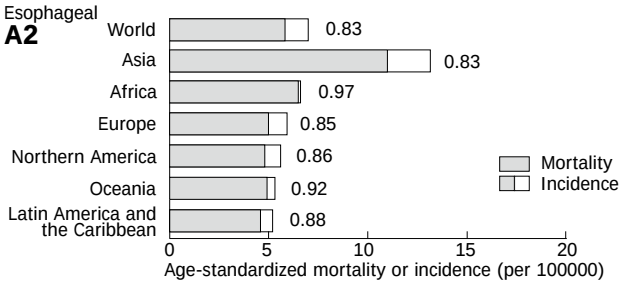
Association between national HDI and MIR

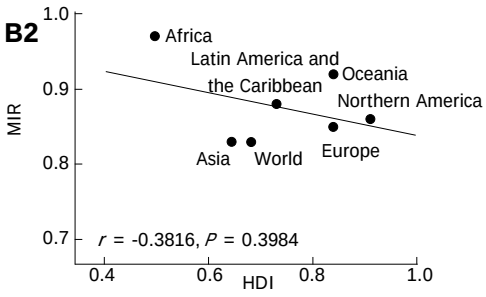
The national MIR varied across different countries with different levels of development, as measured by HDI (Figure 2). Countries with high HDI tended to have relatively low MIR. Cross-national analysis demonstrated that the MIRs of gastrointestinal cancers consistently showed an inverse correlation with the national HDI values *via* linear regression ($\beta < 0$; $P < 0.05$, ANOVA; Table 1, Figure 3A). Furthermore, non-linear regression based on the "drug dose to inhibition response" model was used to analyze this correlation, and a more satisfactory fitting result with larger R square values was achieved for all gastrointestinal cancers (Table 1, Figure 3B). The HDI values at half-maximal MIR (HDI_{50}) in gastrointestinal cancers overall and colorectal cancer were 0.946 and 0.825, respectively. Five other cancers had an HDI_{50} of more than 1.

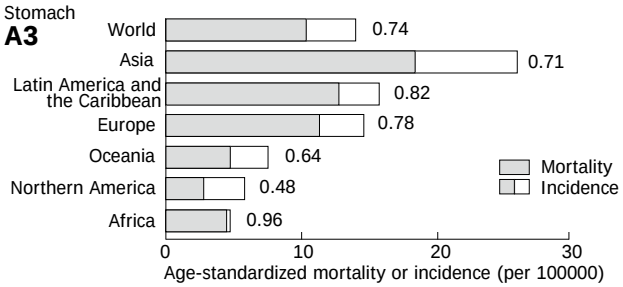
Countries were divided into four groups according to the HDI distribution reported in HDR 2011[18]. The MIR of gastrointestinal cancers differed significantly among these four groups ($P < 0.001$, one-way ANOVA). The mean MIR of countries with very high HDI was

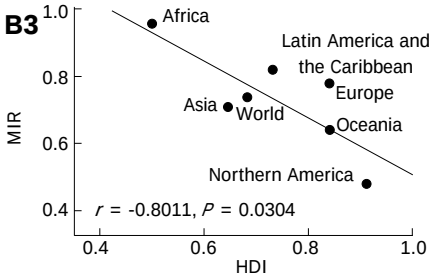

Gastrointestinal
A1
World
Asia
Europe
Oceania
Northern America
Latin America and the Caribbean
Africa
0.72
0.75
0.66
0.54
0.49
0.80
0.94
Mortality
Incidence
0
20
40
60
80
100
Age-standardized mortality or incidence (per 100000)

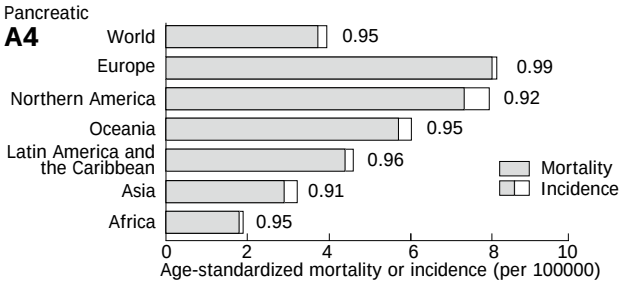

B1
MIR
HDI
Africa
Latin America and the Caribbean
Asia
World
Europe
Oceania
Northern America
r = -0.9254, P = 0.0028

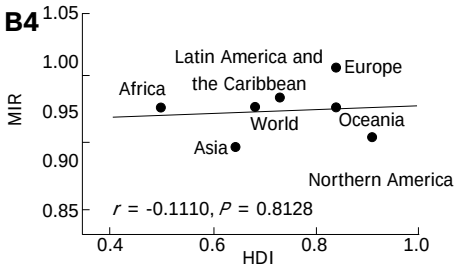

Esophageal
A2
World
Asia
Africa
Europe
Northern America
Oceania
Latin America and the Caribbean
0.83
0.83
0.97
0.85
0.86
0.92
0.88
Mortality
Incidence
0
5
10
15
20
Age-standardized mortality or incidence (per 100000)

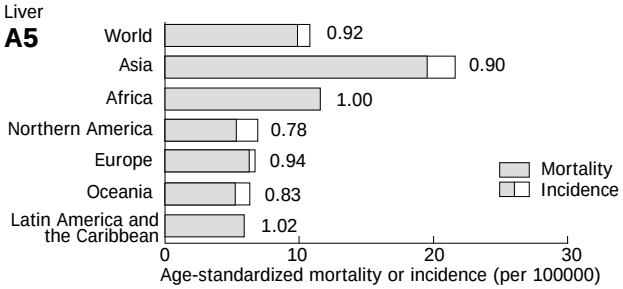

B2
MIR
HDI
Africa
Latin America and the Caribbean
Oceania
Northern America
Asia
World
Europe
r = -0.3816, P = 0.3984

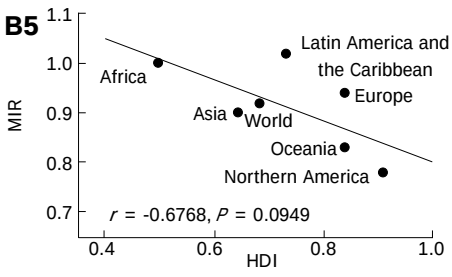

Stomach
A3
World
Asia
Latin America and the Caribbean
Europe
Oceania
Northern America
Africa
0.74
0.71
0.82
0.78
0.64
0.48
0.96
Mortality
Incidence
0
10
20
30
Age-standardized mortality or incidence (per 100000)

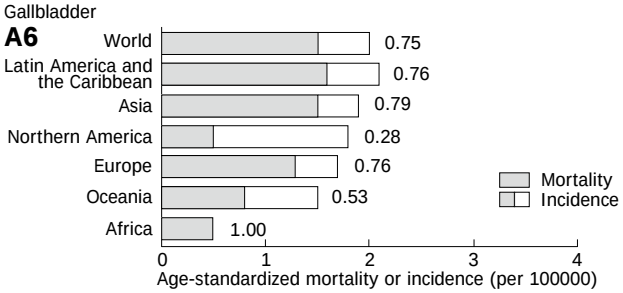

B3
MIR
HDI
Africa
Latin America and the Caribbean
Europe
Asia
World
Oceania
Northern America
r = -0.8011, P = 0.0304

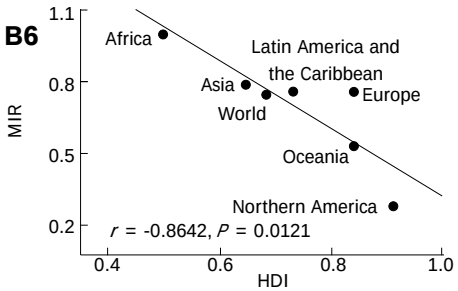

Pancreatic
A4
World
Europe
Northern America
Oceania
Latin America and the Caribbean
Asia
Africa
0.95
0.99
0.92
0.95
0.96
0.91
0.95
Mortality
Incidence
0
2
4
6
8
10
Age-standardized mortality or incidence (per 100000)


B4
MIR
HDI
Latin America and the Caribbean
Europe
Africa
World
Oceania
Asia
Northern America
r = -0.1110, P = 0.8128


Liver
A5
World
Asia
Africa
Northern America
Europe
Oceania
Latin America and the Caribbean
0.92
0.90
1.00
0.78
0.94
0.83
1.02
Mortality
Incidence
0
10
20
30
Age-standardized mortality or incidence (per 100000)


B5
MIR
HDI
Latin America and the Caribbean
Africa
Europe
Asia
World
Oceania
Northern America
r = -0.6768, P = 0.0949


Gallbladder
A6
World
Latin America and the Caribbean
Asia
Northern America
Europe
Oceania
Africa
0.75
0.76
0.79
0.28
0.76
0.53
1.00
Mortality
Incidence
0
1
2
3
4
Age-standardized mortality or incidence (per 100000)


B6
MIR
HDI
Africa
Latin America and the Caribbean
Asia
Europe
World
Oceania
Northern America
r = -0.8642, P = 0.0121

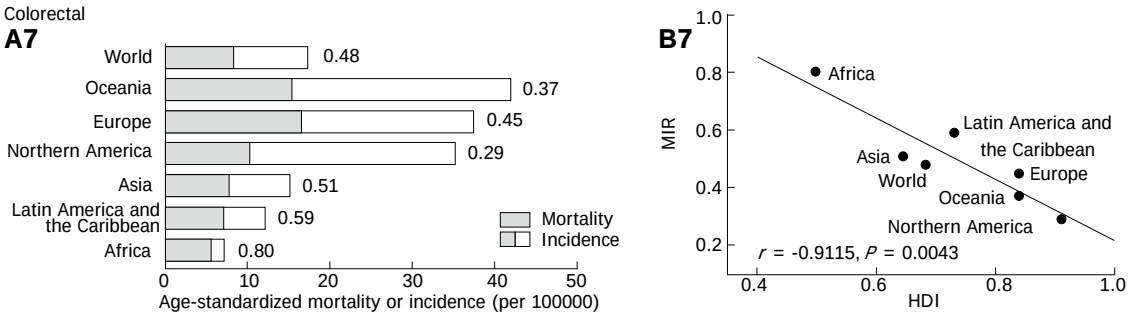


Figure 1 Association between the mortality-to-incidence ratio and human development at the regional level. A: Regional age-standardized mortality (grey) and incidence (white and grey) rates per 100000 individuals for gastrointestinal cancers. The mortality-to-incidence ratios (MIRs) are denoted; B: The regional MIRs of gastrointestinal cancers overall and stomach, liver and colorectal cancers correlate with the human development index (HDI). Best-fit lines by linear regression (solid) are indicated.

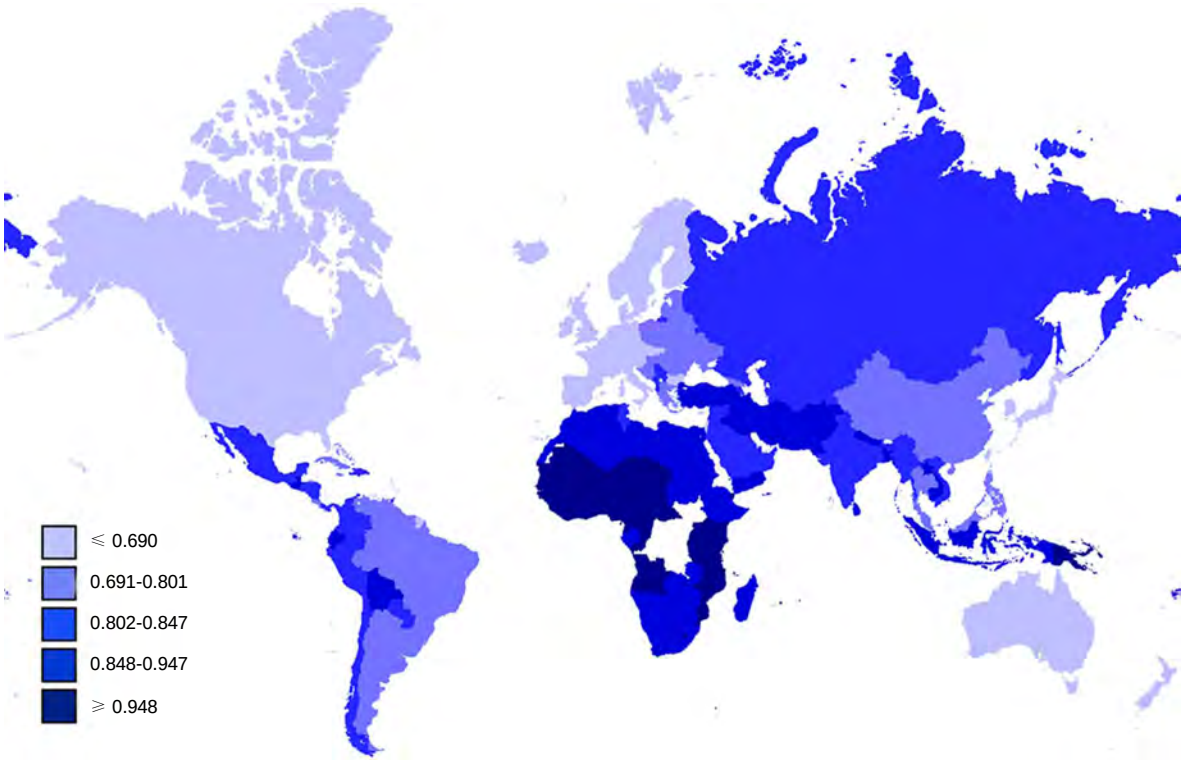


Figure 2 Global mortality-to-incidence ratios of gastrointestinal cancers. Mortality-to-incidence ratios (MIRs) varied across different countries.

0.620 ± 0.033 (95%CI), which was significantly lower than the corresponding values of countries with high, medium, and low HDIs (0.807 ± 0.018, 0.857 ± 0.021, and 0.953 ± 0.011, respectively; $P < 0.05$, Tukey-Kramer *post-hoc* test; Figure 4). Furthermore, there was a significant difference among the four groups in each specific cancer ($P < 0.001$, one-way ANOVA).

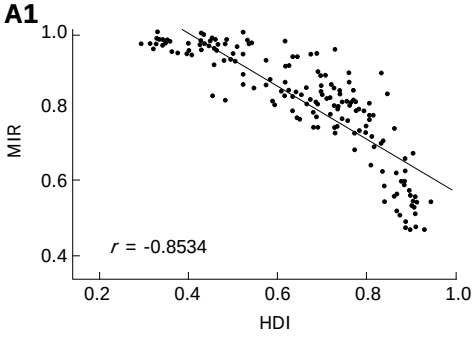
Prediction of MIR in individual United States states

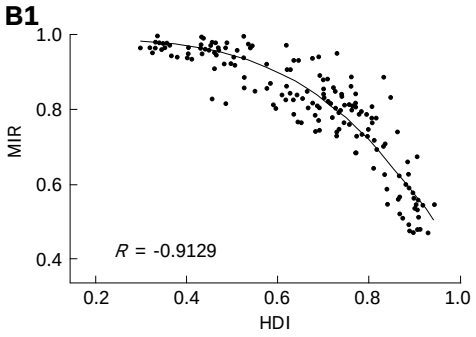
The individual HDIs of 51 United States states were calculated as previously described in HDR 2011. The HDI values in each state ranged from 0.847 to 0.962. To verify the effectiveness of the fitted models, the MIRs of gastrointestinal cancers in each of the United States states were predicted using respective best-fit equations. Except for 28 data points (9.93% of 282), the actual MIRs of all gastrointestinal cancers were mostly located in the prediction intervals *via* the best-fit non-linear regression models. In California, for example, the estimated HDI was 0.907 and the predicted MIR of gastrointestinal cancers was 0.560 ± 0.118 (95% prediction interval, 95%PI). The actual MIR calculated from the reported incidence and mortality was 0.533, and the difference between the actual and predicted MIRs (Δ_{MIR}) was -0.027 (23.1% of 95%PI). The actual MIRs of the six cancers were also in the 95%PI predicted by the respective regression fitting equations (Table 2).

DISCUSSION

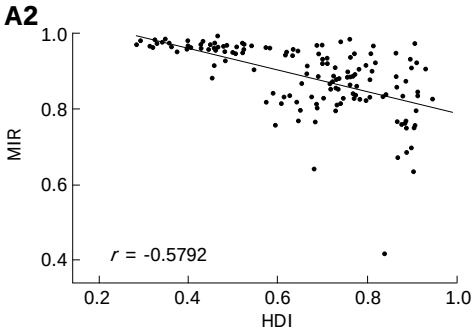
Gastrointestinal cancers have high incidence and mor-

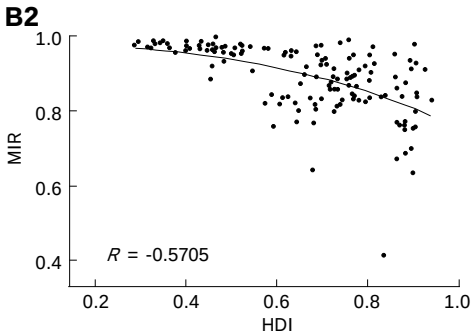
Gastrointestinal



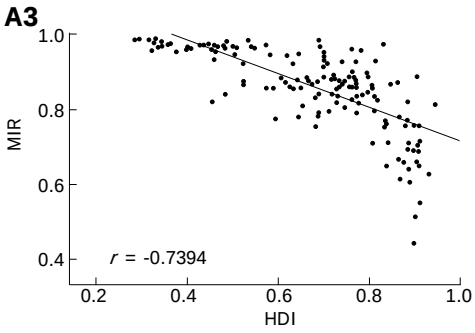


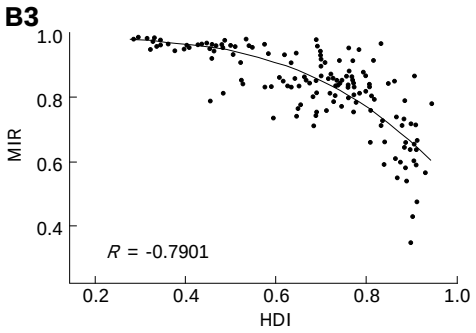
Esophageal



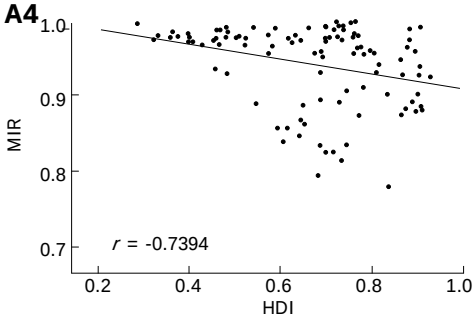


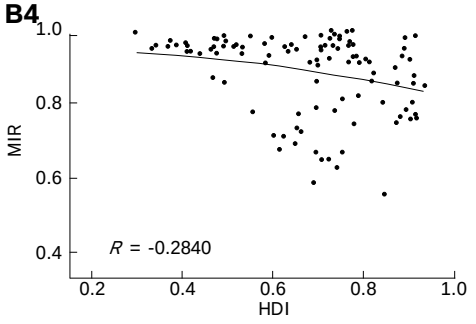
Stomach



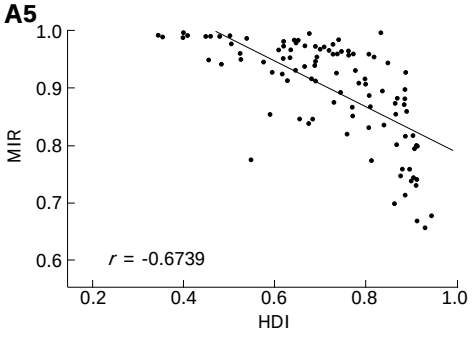


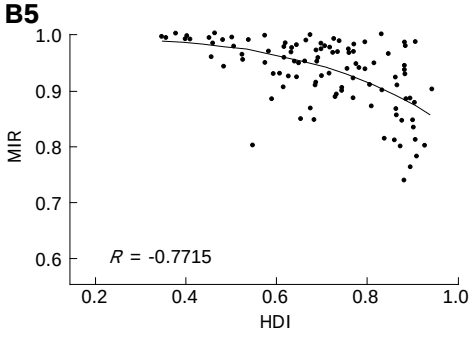
Pancreatic





Liver, gallbladder and pancreatic





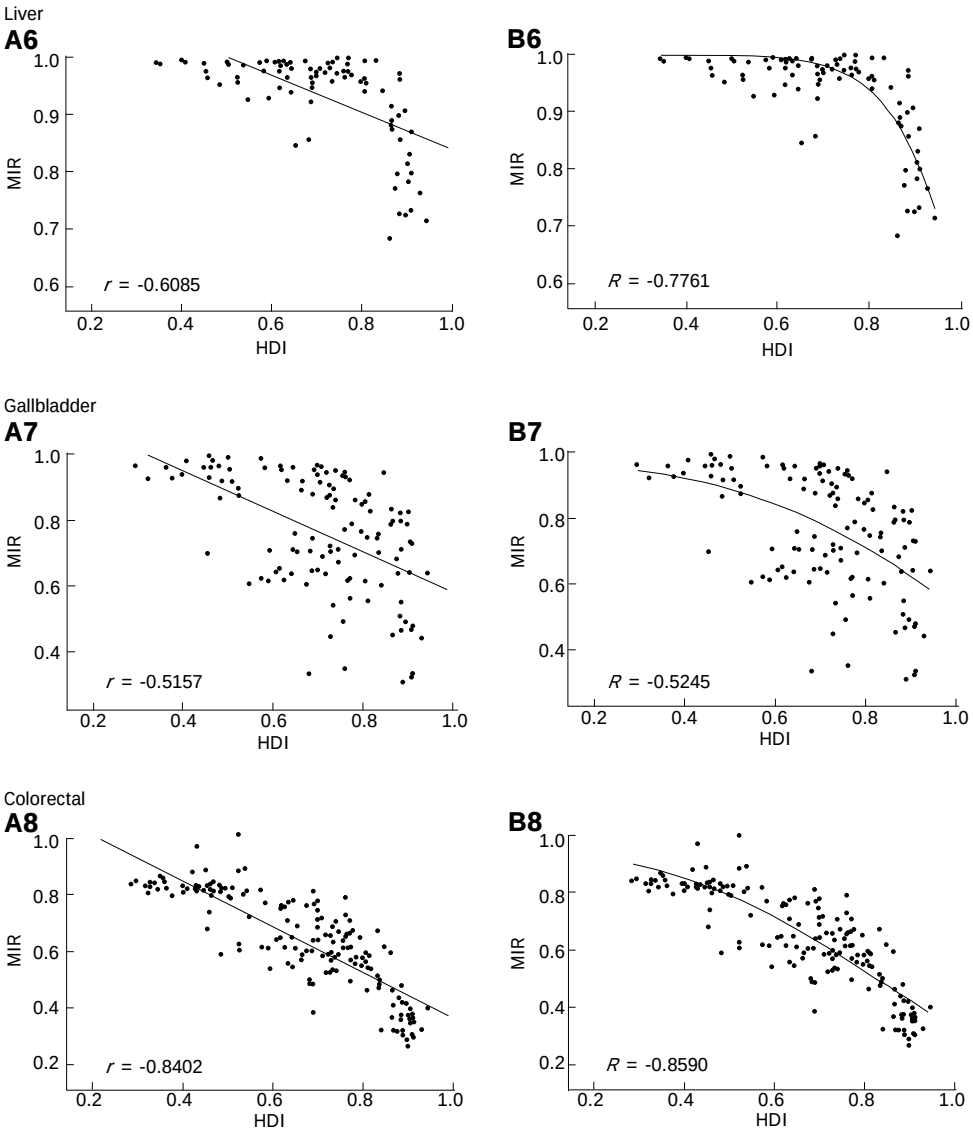


Figure 3 Correlation between national human development index and mortality-to-incidence ratio of gastrointestinal cancers *via* (A) linear or (B) non-linear regression. Best-fit line by regression (solid) is indicated, with *r* or *R* values denoted.

tality rates worldwide[1,24]. We found that both the incidence and mortality rates differed greatly from region to region. Interestingly, the ratio of the mortality rate to the incidence rate, *i.e.*, the MIR, appeared to be higher in less developed regions such as Africa. The development level was quantified by HDI, which is a composite measure of human development. Estimation of national HDI is based on the following parameters: a long and healthy life, access to knowledge, and a decent standard of living[18]. As an indicator of the socioeconomic factor of health, the HDI may serve as the gold standard for international comparisons of development.

MIR is derived as a surrogate indicator of the effectiveness of the health system. It has been proposed as an indirect measure of true biological differences in disease phenotypes or health system-related attributes such as screening, diagnostic modalities, treatment and follow-up[25,26]. An MIR-associated derivative form was identified as a good approximation of the 5-year relative survival for most, but not all, cancers[27]. The MIR is computed from age-standardized rates, and it also reflects a population-based approximation of survival[25]. Accordingly, it could be used to assess the diagnosis proficiency and treatment effectiveness in gastrointestinal cancers.

Africa, which had a relatively low HDI, showed a high MIR for most gastrointestinal cancers, whereas Northern America, which had a higher HDI, showed a low MIR. Furthermore, we found a significant inverse correlation between the regional MIR and corresponding HDIs in some, but not all, gastrointestinal cancers. However, only seven data points were included in the region-specific linear regression analysis. Insufficient sample size for regression analysis might cause fitting inaccuracy[28]. To avoid such inaccuracies, country-specific regression was performed. Linear regression analysis in this study revealed a correlation between the national HDI and MIR in all gastrointestinal cancers. The impact of hu-

Table 1 Results of regression in a cross-national analysis of human development index and mortality-to-incidence ratio

Cancer	Linear regression			Non-linear regression[1]		
	β	*P*	*r*	HDI_{50}	Slope	*R*
All gastrointestinal	-0.703	< 0.001	-0.853	0.946	2.746	-0.9129
Esophageal	-0.295	< 0.001	-0.579	1.362	1.344	-0.5705
Stomach	-0.536	< 0.001	-0.739	1.023	2.372	-0.7901
Pancreatic	-0.097	0.0019	-0.301	2.391	0.706	-0.2840
Liver	-0.322	< 0.001	-0.609	1.026	5.247	-0.7761
Gallbladder	-0.611	< 0.001	-0.516	1.027	1.697	-0.5245
Liver, gallbladder and pancreas	-0.216	< 0.001	-0.543	1.386	1.726	-0.5704
Colorectal	-0.808	< 0.001	-0.840	0.825	1.785	-0.8590

[1]Non-linear regression based on the "drug dose to inhibitory response" model, and human development index $(HDI)_{50}$ and slope were the two parameters used. $P < 0.01$ was defined as significantly non-zero β; ANOVA.

Table 2 Actual and predicted mortality-to-incidence ratio values of gastrointestinal cancers in California

Cancer	Incidence (ASR, per 100000)	Mortality (ASR, per 100000)	Actual MIR	Predicted MIR (95%PI)[1]	Δ_{MIR}
All gastrointestinal	74.3	39.6	0.533	0.560 ± 0.118	-0.027
Esophageal	3.8	3.4	0.895	0.803 ± 0.148	0.091
Stomach	7.4	4.3	0.581	0.653 ± 0.158	-0.072
Pancreatic	11.3	10.3	0.912	0.918 ± 0.104	-0.006
Liver	8.4	6.8	0.810	0.807 ± 0.101	0.002
Colorectal	43.4	14.8	0.341	0.416 ± 0.173	-0.075

[1]MIR values were predicted using California's human development index (HDI) (0.907) and the best-fit regression models. Δ_{MIR} = actual MIR-predicted MIR. ASR: Age-standardized rate; MIR: Age-standardized mortality-to-incidence ratio; PI: Prediction interval.

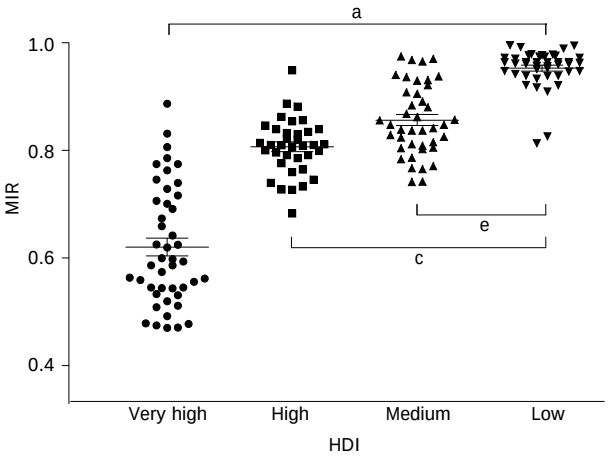


Figure 4 Overall mortality-to-incidence ratio of gastrointestinal cancers in four human development index groups. The mortality-to-incidence ratio (MIR) differs significantly among areas having very high, high, medium and low human development index (HDI); [a]$P < 0.05$ *vs* very high HDI areas; [c]$P < 0.05$ *vs* high HDI areas; and [e]$P < 0.05$ *vs* medium HDI areas; one-way ANOVA followed by the Tukey-Kramer *post-hoc* test.

man development on the effectiveness of healthcare for gastrointestinal cancers, as reflected by the relationship between HDI and MIR, was assumed to bear a similarity to the dose-dependent inhibitory response by anticancer drugs. HDI-to-MIR and dose-to-response patterns both have several characteristics in common, such as (1) MIR or response approaches 1 as HDI or dose approaches 0; (2) MIR or response decreases as HDI or dose increases; and (3) MIR or response approaches 0 as HDI or dose approaches infinity. Non-linear regression using the modified "drug dose to inhibition response" model confirmed the assumption and provided the HDI_{50} value, which was found to be a potential estimate of healthcare effectiveness on gastrointestinal cancers. The progress in screening, diagnostic and therapeutic techniques for colorectal cancer in recent decades[1,29] has resulted in an HDI_{50} of 0.825, which is the lowest value among all the gastrointestinal cancers investigated in this study.

Inequality in healthcare has been regarded as a major cause of variation in the effectiveness of cancer care[30], reflected by the inverse correlation between MIR and HDI. Although eliminating such disparities in healthcare has become the focus of an initiative of healthcare reform in many countries, quality improvement in medical care is not yet obvious[9]. Region- or country-specific disparities in cancer care still exist, even in highly developed countries[7]. Apart from healthcare inequality, the inverse correlation between HDI and MIR is also influenced by the factors such as socioeconomic conditions, lifestyle (particularly diet and tobacco use), and genetic variances among individuals or races[7,9]. Infection with *Helicobacter pylori*, hepatitis virus or other cancer-inducing micro-organisms is another risk factor for gastrointestinal cancers[31-33]. A very recent study analyzed world cancer burden by HDI groups and suggested disparities in cancer distributions[3]. We further demonstrated that HDI influenced cancer MIRs on a country level, which resembled the effect of drug dose on inhibitory response. Therefore, relatively high MIRs indicate the premature mortality from cancer in lower HDI areas. Healthcare disparities emphasize the need for efforts in cancer control in low human development settings.

Cancer health disparities occur not only between countries, but also within a single country[7,34,35]. The health outcomes in the United States were related to socioeconomic factors and racial diversity[9,36]. Health inequality between different socioeconomic levels also contributed to the health disparities observed in the United States. Therefore, we supposed that the observed association between HDI and MIR could be applied to United States states. Prediction based on the modified "drug dose to inhibition response" model turned out to be relatively satisfactory.

The methods used to estimate cancer-specific incidence and mortality rates at the national level in the GLOBOCAN database depend on the availability and accuracy of local data sources[3]. Despite the various provisos concerning data quality and methods of estimation, the estimates in GLOBOCAN are the most accurate that can be made at present, and may be used in the setting of priorities for cancer control actions in different regions and countries of the world[14]. Countries

without high quality data are usually those countries with lower development levels. Limiting analysis to high quality data could eliminate biases due to data inaccuracy, but would lead to excessive absence of epidemiological data in the less developed countries. Since our study aimed to show the disparities of cancer MIRs between low and high HDI countries, the data with relatively low quality were essential to this study and therefore remained in our analysis.

In conclusion, the results of this study obtained by collating excellent data resources revealed an inverse correlation between HDI and MIR at the regional and national levels. This association illustrated that more developed areas tend to have relatively more effective healthcare systems, resulting in low MIRs. Further prediction of the state-specific MIR of gastrointestinal cancers obtained using a fitted non-linear regression model revealed the potential application of HDI for estimation of the MIR.

COMMENTS

Background

Gastrointestinal cancer is a common, highly fatal disease. The expected cancer burden will be a serious public health problem in the coming decade, particularly in developing countries. However, little is known about healthcare disparities in individuals suffering from gastrointestinal cancers.

Research frontiers

There is little knowledge about the healthcare disparities in individuals suffering from gastrointestinal cancers. Inequality in healthcare has been regarded as a major cause of variation in the effectiveness of cancer care. Region- or country-specific disparities in cancer care still exist, even in highly developed countries.

Innovations and breakthroughs

According to the authors of this study, this study is the first to explore the exact relationship between the epidemiology of gastrointestinal cancers and area-specific development disparities. The authors showed the association between the mortality-to-incidence ratios (MIRs) and the human development index (HDI) at the regional and national levels using a modified "drug dose to inhibition response" model. Further prediction of the MIRs for individual United States states on the basis of best-fitted non-linear models showed little deviation from the actual MIRs in the United States.

Applications

Based on the modified "drug dose to inhibition response" model, more developed areas have relatively more effective healthcare systems, resulting in low MIRs. Prediction of the state-specific MIR of gastrointestinal cancers obtained using a fitted non-linear regression model revealed the potential application of HDI for estimation of the MIR.

Terminology

MIR is derived as a surrogate indicator of the effectiveness of the health system, and is proposed as an indirect measure of true biological differences in disease phenotypes or health system-related attributes such as screening, diagnostic modalities, treatment and follow-up. HDI is a composite measure of human development based on the following parameters: a long and healthy life, access to knowledge, and a decent standard of living.

Peer review

The authors, using the GLOBOCAN 2008 database, obtained age-standardized incidence and mortality rates for gastrointestinal cancers. They estimated the MIRs at the regional and national levels, and explored the association between the MIR and development levels as measured by the HDI. Furthermore, they have predicted state-specific MIRs in the United States from the estimated HDI using the fitted non-linear model. Finally, the authors have managed to show an inverse correlation between HDI and MIR at the regional and national levels and that more developed areas tend to have relatively more effective healthcare systems, resulting in low MIRs. Overall, the manuscript is very well written and well organized. The language is satisfactory and the tables along with the figures are well structured.

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BRIEF ARTICLE

Sessile serrated adenomas in the proximal colon are likely to be flat, large and occur in smokers

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Abstract

AIM: To examine the epidemiology and the morphology of the proximal sessile serrated adenomas (SSAs).

METHODS: We conducted a retrospective study to identify patients with SSAs using a university-based hospital pathology database query from January 2007 to April 2011. Data collected included: age, gender, ethnicity, body mass index, diabetes, smoking, family history of colorectal cancer, aspirin, and statin use. We collected data on morphology of SSAs including site (proximal or distal), size, and endoscopic appearance (flat or protuberant). We also compared proximal SSAs to proximal tubular adenomas detected during same time period.

RESULTS: One hundred and twenty patients with SSAs were identified: 61% were distal and 39% were proximal SSAs. Proximal SSAs were more likely to be flat than distal (100% *vs* 78% respectively; $P = 0.0001$). Proximal SSAs were more likely to occur in smokers (OR = 2.63; 95%CI: 1.17-5.90; $P = 0.02$) and in patients with family history of colorectal cancer (OR = 4.72; 95%CI: 1.43-15.55; $P = 0.01$) compared to distal. Proximal SSAs were statistically more likely to be $\geqslant$ 6 mm in size (OR = 2.94; $P = 0.008$), and also more likely to be large ($\geqslant$ 1 cm) (OR = 4.55; $P = 0.0005$) compared to the distal lesions. Smokers were more likely to have proximal ($P = 0.02$), flat ($P = 0.01$) and large ($P = 0.007$) SSAs compared to non-smokers. Compared to proximal tubular adenomas, proximal SSAs were more likely to be large and occur in smokers.

CONCLUSION: Proximal SSAs which accounted for two-fifths of all SSAs were more likely to present as flat lesions, larger SSAs, and were more likely to occur in smokers and in patients with family history of colorectal cancer. Our data has implications for colorectal cancer screening.

Key words: Proximal; Sessile; Serrated; Adenoma; Colonoscopy; Colorectal cancer; Smoking

Core tip: Sessile serrated adenomas (SSAs) have been implicated in the alternative pathway for colorectal carcinoma. Proximal SSAs might account for higher incidence of interval colorectal cancers (CRC) on the right side given the fact that these are often flat and difficult to detect. Our study is first to compare the morphology and epidemiology of proximal SSAs with distal SSAs.

We found proximal SSAs are more likely to present as flat lesions, larger SSAs, and were more likely to occur in smokers and in patients with family history of CRC. These findings have implications for CRC screening.

Rustagi T, Rangasamy P, Myers M, Sanders M, Vaziri H, Wu GY, Birk JW, Protiva P, Anderson JC. Sessile serrated adenomas in the proximal colon are likely to be flat, large and occur in smokers. *World J Gastroenterol* 2013; 19(32): 5271-5277 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5271.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5271

INTRODUCTION

Colorectal cancer is the fourth most common form of cancer and the second most frequent cause of cancer deaths in the United States[1]. The majority of colorectal cancers arise from the adenoma-carcinoma sequence where mutations in the *APC* gene play an early role. However, an alternative pathway exists in which there is an increased frequency of CpG island methylation of gene promoter. These abnormalities are associated with *BRAF* mutations which have been observed in sessile serrated adenomas (SSAs)[2,3] as well as serrated aberrant crypt foci[4]. Large serrated polyps (≥ 1 cm) have been shown to have a strong association with synchronous advanced colorectal neoplasia[5,6]. SSAs are often flat and proximally located. Interval cancers have been shown to be associated with the methylation pathway[7]. In addition to the fact that they may be difficult to detect, SSAs may provide an explanation for the reason why rates of right sided colorectal neoplasia remain high while the left sided lesions have decreased in patients who have had a colonoscopy in the recent past[8,9].

Very few studies have examined the epidemiology of the various types of serrated polyps. A recent study has shown smoking to be strongly associated with SSAs of all sizes, including the clinically important large (≥ 1 cm) lesions[10]. Multivariate logistic regression found that age, smoking and obesity were statistically significant predictive factors for any SSA as compared to controls[10]. Most of the preceding studies have focused on the relatively common left-sided serrated polyps and little is known about the proximal SSAs. Our goal was to examine the epidemiology and the morphology of the proximal SSA in comparison to the distal lesions.

MATERIALS AND METHODS

Patient selection and data collection

The retrospective study was approved by the Institutional Review Board of the University of Connecticut Health Center. We defined cases as patients diagnosed to have SSAs from January 2007 to April 2011, identified by a pathology database query. We identified all lesions diagnosed by our pathologists as SSA. We excluded the traditional serrated adenomas or the subgroup of serrated polyps that not only share serrated crypt architecture with hyperplastic polyps, but also have cytologic dysplasia. SSAs were those serrated polyps with abnormal proliferation and/or abnormal architecture, but without the cytological dysplasia seen in adenomatous polyps. All of the SSAs were confirmed or had a clinical description that alerted the pathologist that the endoscopist was suspecting a SSA. We defined a large SSA as any SSA of size greater than or equal to 1 cm in diameter.

We collected the following data from the patient's charts: age, gender, ethnicity, height, weight, clinically diagnosed type Ⅱ diabetes mellitus, smoking exposure, a family history of colorectal cancer, lipid profile, use of aspirin, calcium, hormone replacement therapy and statin use. From an electronic database at our University Hospital, we were able to use several different primary care and sub specialty notes to collect and confirm the data. Thus, most of our information had at least one source.

With regard to smoking, we calculated the exposure in the form of pack-years (*i.e.*, number of packs smoked per day multiplied by the number of years smoked). We defined a smoker as someone who smoked at least 20 pack-years or more regardless of whether they quit smoking. Family history of colorectal cancer was defined as having at least one first degree relative or two second degree relatives with the disease. Obesity was defined as a body mass index ≥ 30 kg/m^2. We also randomly selected patients with adenomas who had colonoscopies during the same time period as the patients with serrated lesions.

High-definition (1080i signal) wide-angle (170° field of view) Olympus 180-series colonoscopes (Olympus America, Center Valley, PA, United States) were used to perform all of the colonoscopies. All polyps were photo documented next to a snare catheter for *in vivo* measurement and retrieved for histology, and morphology was classified according to the Japanese Research Society for Cancer of Colon and Rectum guidelines[11,12]. We used a standard method to visualize the polyp for morphologic classification. Specifically, the colon was insufflated so that the polyp was visualized and photo documented in this setting. Any lesion that was determined to be Ip, Is, or Ips was considered to be polypoid or protuberant, and those that were Ⅱa, Ⅱb, or Ⅱc were considered to be flat or non-polypoid. Adenoma size was confirmed by the pathology report[13]. One experienced endoscopist (Anderson JC) confirmed the morphology from the photodocumentation for a representative group of adenomas that were randomly selected from our analyzed sample. The colon was divided into proximal and distal by the splenic flexure which was considered proximal. A colonoscopy was considered complete if the following criteria were fulfilled: transillumination of the right lower quadrant, visualization of the ileocecal valve, or appendiceal orifice.

Statistical analysis

Our main outcomes were detection of SSAs, and proximal SSAs. SPSS version 20.0 (Chicago, IL, United States)

Table 1 Comparison of patient characteristics among proximal and distal sessile serrated adenoma group *n* (%)

	Proximal SSA (*n* = 47)	Distal SSA (*n* = 73)	Univariate OR (95%CI)	*P* value	Multivariate OR (95%CI)
Race (CC)	37 (78.7)	54 (74.0)	1.30 (0.54-3.12)	0.66	-
Gender (male)	21 (44.7)	27 (37.0)	1.38 (0.65-2.90)	0.45	-
Age (yr) (≥ median)	28 (59.6)	40 (54.8)	1.22 (0.58-2.56)	0.71	-
Obesity	22 (46.8)	36 (49.3)	0.90 (0.43-1.88)	0.85	-
Family history	11 (23.4)	5 (6.8)	4.16 (1.34-12.89)	0.01	4.72 (1.43-15.55) *P* = 0.01
Diabetes mellitus	14 (29.8)	23 (31.5)	0.92 (0.42-2.05)	1.00	-
Smoking	30 (63.8)	30 (41.0)	2.53 (1.19-5.39)	0.02	2.63 (1.17-5.90) *P* = 0.02
Triglyceride (mean ± SD, mg/dL)	124.9 ± 63.9	129.7 ± 67.9	-4.80 (-29.38-19.78)	0.69	-
Cholesterol (mean ± SD, mg/dL)	179.1 ± 45.8	180.9 ± 43.8	-1.80 (-18.31-14.71)	0.83	-

SSA: Sessile serrated adenoma.

was used for all statistical analysis. Univariate analyses were performed using Fisher's test or χ^2 for dichotomous variables and unpaired *t*-test for non-parametric continuous variables. After univariate analyses, all variables with a *P* value of 0.10 or less were entered into the equation and only those variables with $P < 0.10$ were used in the final multivariate logistic regression equation to estimate Odds ratios and 95% confidence intervals for proximal SSAs. We considered results to be significant if the *P* value was < 0.05.

RESULTS

From January 2007 to April 2011, 120 patients (mean age: 59.72 ± 10 years, males: 40%) with SSAs were identified. This included 90 patients searched through the same pathology database query that were part of the earlier study focused on identifying risk factors associated with any SSAs[10]. Thirty additional patients were added to this database between October 2010 and April 2011. Proximal SSAs constituted two-fifths (47/120) of all SSAs. Fifty-seven (78%) of the distal lesions were flat as compared to the 47 (100%) proximal lesions which were all flat ($P = 0.0001$). Proximal SSAs were more likely to occur in smokers compared to distal (30/47 *vs* 30/73; $P = 0.02$) as shown in Table 1. Similarly, smokers were more likely to have proximal SSAs compared to non-smokers (30/60 *vs* 17/60; $P = 0.02$). Compared to non-smokers, smokers were also more likely to have flat SSAs (57/60 *vs* 47/60; $P = 0.01$). Proximal SSAs were more likely to be found in subjects with a family history of colorectal cancer compared to distal SSAs (11/47 *vs* 5/73; $P = 0.01$) as shown in Table 1.

We also examined the site of the SSA in relation to the adenoma size and morphology. Proximal SSAs were more likely to be ≥ 6 mm in size and also more likely to be large (≥ 1 cm) compared to the distal lesions, as shown in Table 2. Smokers were significantly more like to have large SSAs (23/60 *vs* 9/60; $P = 0.007$; multivariate OR = 3.93; 95%CI: 1.52-10.17) compared to non-smokers.

We compared SSA group to a control group consisting of 122 patients with conventional tubular adenomas identified from the same time period. Proximal tubular adenoma constituted 64% of all tubular adenomas compared to proximal SSA which constituted 39% of all SSAs. Proximal SSAs were significantly more likely to be flat, large (≥ 1 cm), and occur in smokers compared to the proximal tubular adenomas, as shown in Table 3.

DISCUSSION

Our data suggest that proximal SSAs are more likely to occur in smokers and in patients with family history of colorectal cancer. Proximal SSAs are also more likely to present as large lesions, including the significant (≥ 6 mm) adenomas and clinically important large (> 1 cm) adenomas. In addition, we found proximal SSAs to be more likely to present as flat lesions. To our knowledge, this is the first study examining the morphology of SSAs specifically, and their association with smoking with respect to anatomical location.

We found smokers to have proximal, flat and large SSAs. Smoking has been associated with key mutations in cancer-related genes such as *hMLH1*, CPG island methylation phenotype (CIMP) and *BRAF* mutation, with multiple studies establishing a definitive link between smoking and microsatellite instability-high (MSI-H) colorectal cancers[14-19]. Molecular studies have shown serrated polyps including SSAs to be associated with a higher frequency of CIMP and *BRAF* mutations[3,20-22]. Several large studies have reported the association of serrated adenoma- carcinoma pathway *via* the microsatellite instability[23-28]. With respect to the link between smoking and serrated lesions, multiple studies have shown that cigarette smoking has a stronger association with serrated polyps than it does with adenomatous polyps[29-34]. Wallace *et al*[35] identified smoking as one of the major risks for serrated polyps. Current smokers were found more likely to have proximal nondysplastic serrated polyps in a study by Schreiner *et al*[6]. A recent study by Anderson *et al*[10] demonstrated smoking to be a major risk factor for the presence of SSAs. Our current study further links smoking strongly with proximal SSAs compared to distal lesions. Thus, smoking is not only a major risk factor for all SSAs, but is a much stronger predictor of proximal SSAs. Our study demonstrates smoking to be strongly

Table 2 Comparison of adenoma characteristics in the proximal and distal sessile serrated adenoma *n* (%)

	Proximal SSA (*n* = 47)	Distal SSA (*n* = 73)	Univariate OR (95%CI)	*P* value
Flat SSA	47 (100.0)	57 (78.0)	-	0.0001
⩾ 6 mm SSA	31 (66.0)	29 (39.7)	2.94 (1.37-6.31)	0.0080
⩾ 1 cm SSA	21 (44.7)	11 (15.0)	4.55 (1.92-10.77)	0.0005

SSA: Sessile serrated adenoma.

Table 3 Comparison of patient and adenoma characteristics among proximal sessile serrated adenoma and proximal tubular adenoma groups *n* (%)

	Proximal SSA (*n* = 47)	Proximal TA (*n* = 78)	Univariate OR (95%CI)	*P* value
Family history	11 (23.4)	14 (18.0)	1.40 (0.57-3.40)	0.4900
Smoking	30 (63.8)	26 (33.3)	3.53 (1.65-7.54)	0.0010
Adenoma size ⩾ 1 cm	21 (44.7)	11 (14.1)	4.92 (2.08-11.61)	0.0002
Flat morphology	47 (100.0)	46 (59.0)	-	< 0.00001

SSA: Sessile serrated adenoma; TA: Tubular adenoma.

linked with flat and proximal SSAs, which were more likely to present as large lesions having higher neoplastic potential.

Several studies have explored the association between smoking and anatomical site-specific lesions. Colorectal cancers arising from the serrated pathway that are *BRAF*-mutated, CIMP-high and MSI-H, and are specifically associated with smoking[17,18] occur most often in the proximal colon[36,37]. Limsui *et al*[16] also reported an association between proximal colon cancer and cigarette smoking in a large cohort study of over 37000 women. However, few studies, including a meta-analysis of the association between colorectal cancer and smoking, suggest a specific association with distal/rectal neoplasia[15,38,39]. A recent case-control study by Burnett-Hartman *et al*[29] also reported a stronger association between distal/rectal colorectal polyps and smoking. Botteri *et al*[40] showed a strong association between smoking and cancers in the rectum and proximal colon. They postulated that this could be due to the differential anatomical location of serrated colorectal cancers. Although non-serrated polyps tend to have no site predilection[40-42], studies have reported that serrated neoplasia arise more frequently in the proximal colon and in the rectum[43-45]. Microsatellite instability has been associated with proximal lesions[46,47] and has been shown to develop late in serrated adenoma-carcinoma pathway[3]. This could possibly explain our observation of large and proximal SSAs in smokers. As with microsatellite instability, studies have shown that tumors involving *BRAF* mutations arise more frequently in the proximal colon than in the distal colon[7,48-50]. Our study shows proximal SSAs comprise two-fifths of all SSAs, but are clinically more important given the finding that they are larger and all have flat morphology compared to the distal lesions which were more common. Smoking was found to be a much stronger risk factor for proximal SSAs compared to proximal tubular adenomas, likely due to high frequency of CIMP and *BRAF* mutations which are involved in serrated lesions.

Another interesting observation was the link between family history of colorectal cancer and proximal SSAs on both univariate and multivariate analyses. Family history of colorectal cancer has been shown to be a predictor of proximal significant adenomas on previous studies[51]. Schreiner *et al*[6] also found patients with family history of colorectal cancer to be more likely to have proximal non-dysplastic serrated polyps. However, this study did not include an analysis that distinguished hyperplastic polyps and SSAs. Our study is the first to show similar association of family history of colorectal cancer with proximal SSAs. Anderson and colleagues did not find family history of colorectal cancer to be a risk factor for SSAs compared to controls[10]. This might be because of the relatively small sample size and the fact that distal lesions accounted for two-third of all SSAs. Our results show family history of colorectal cancer is associated with proximal and not distal SSAs. Patients with family history of colorectal cancer might have an alternative involvement of *BRAF*-serrated pathways predisposing them to proximal SSA, which might account for the increased risk of adenoma and colorectal neoplasia.

There are many implications for our findings with respect to colorectal screening. The majority of our SSAs were flat. Those located proximally were all flat as opposed to the distal lesions. These lesions may be difficult to detect and may be associated with synchronous advanced neoplasia[5,6]. Proximal SSAs would be theoretically much more difficult to detect given their location: incomplete colonoscopies, variation in cecal intubation rates, variation in detection of proximal serrated polyps[52]. Given the potential for malignancy of SSAs as well as their proclivity to a flat morphology, these lesions may explain the lack of protection of colonoscopy in the proximal colon. Studies have shown the limitations of colonoscopy in reduction of right sided colon cancers[8,9]. Interval colorectal cancers are three times as likely to occur in the right colon[53] and proximal SSAs might account for significant proportion of these interval colorectal cancers. Recent study by Arain *et al*[7] also found interval cancers to be more likely to arise in the proximal colon and found both CIMP and MSI to be independently associated with interval cancers. This might pose an important concern from a screening perspective. Proximal SSAs are more likely to occur in smokers which may require special screening techniques to identify these lesions in this high risk group. We further divided our SSAs into the larger lesions due to their malignant potential and those > 6 mm. We chose the latter measurement since lesions of this size are considered important clinically with regard to optical colonoscopy as well as computer tomographic colonography (CTC)[54,55]. We observed that most of these lesions were found proximal to the splenic flex-

ure. Therefore, if chromoendoscopy is found to be beneficial in detecting flat adenomas, the entire colon, with special attention to the right side, would be important in smokers and in patients with family history of colorectal cancer. Therefore, great attention to the proximal colon with a detailed evaluation for flat adenomas should be undertaken. Perhaps different techniques, such as special high-definition colonoscopes, narrow band imaging or chromoendoscopy may be required to detect these flat adenomas[56]. The role of CTC in screening smokers for colorectal cancer may also change as it may be more difficult to identify lesions with a flat morphology by this method of screening.

We acknowledge that the retrospective design of the study is a potential limitation for our results. Our retrospective data collection included data regarding known colorectal neoplasia risk factors such as smoking history, family history of colorectal cancer and obesity in addition to medication use, dietary intake, lipid profile and patient demographics. However, we acknowledge that there may have been factors that were missed. Another limitation of this study is the relatively small sample size and single center study.

In conclusion, our study is the first to suggest that proximal SSAs are more likely to present as flat and large adenomas, and also more likely to occur in smokers and in patients with family history of colorectal cancer compared to distal SSAs. Smokers are more likely to have proximal, flat, and large SSAs. Increased malignant potential from larger size and difficulty in detection given their flat morphology might contribute to higher risk of interval colorectal cancer in the proximal colon, particularly in smokers.

COMMENTS

Background

Sessile serrated adenomas (SSAs) have been implicated in the alternative pathway for colorectal carcinoma (CRC) and might account for significant proportion of interval CRC given the fact that these are often flat and difficult to detect. Lesions in this pathway and interval cancers share a common proximal location as well as molecular mutations. Many of the epidemiological studies have focused on the relatively common left-sided serrated polyps and little is known about proximal SSAs.

Research frontiers

Smoking, age, obesity, diabetes have been identified as risk factors for SSAs. Proximal serrated polyps have attracted more attention based on their premalignant potential and their association with synchronous and metachronous lesions.

Innovations and breakthroughs

Their results show differences in risk factors, epidemiology and morphology between proximal and distal SSAs. These novel data show that proximal SSAs are all flat and more likely to present as larger lesions. Proximal SSAs are more likely to occur in smokers and in patients with family history of CRC.

Applications

Smokers are more likely to have proximal SSAs which are flat and larger. This might have implications for CRC screening, recommending use of new or different techniques such as chromoendoscopy in smokers for detection of these lesions which account for significant proportion of interval cancers in the right colon. Future studies should focus on techniques and procedure-related factors to enhance the detection of these clinically important proximal SSAs.

Terminology

Sessile serrated adenoma are characterized by the presence of a disorganized and distorted crypt growth pattern that is usually easily identifiable upon low-power microscopic examination. Crypts, particularly at the basal portion of the polyp, may appear dilated and/or branched, particularly in the horizontal plane, which leads to the formation of "boot", "L", or "anchor"-shaped crypts. The terms "SSAs" and "sessile serrated polyp" are considered synonyms, and both are acceptable. Proximal colon location is defined as proximal to the splenic flexure (transverse colon, ascending colon, cecum, ileocecal valve).

Peer review

This is a nice and well written retrospective case-control study showing that SSAs in the proximal colon were more associated with smoking compared to distal SSAs and tubular adenoma in the proximal colon.

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BRIEF ARTICLE

Long-term leukocyte natural α-interferon and ribavirin treatment in hepatitis C virus recurrence after liver transplantation

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Abstract

AIM: To evaluate the effect of long-term treatment with leukocyte natural α-interferon (ln-α-IFN) plus ribavirin (RBV).

METHODS: Forty-six patients with hepatitis C virus (HCV) recurrence received 3 MU three times a week of ln-α-IFN plus RBV for 1 mo; then, patients with good tolerability (n = 30) were switched to daily IFN administration, while the remaining were treated with the same schedule. Patients have been treated for 12 mo after viral clearance while non-responders (NR) entered in the long-term treatment group. Liver biopsies were planned at baseline, 1 year after sustained virological response (SVR) and at 36 mo after start of therapy in NR. MedCalc software package was used for statistical analysis.

RESULTS: About 16.7% of genotype 1-4 and 70% of genotype 2-3 patients achieved SVR. Nine patients withdrew therapy because of non-tolerance or non-compliance. A significant improvement in serum biochemistry and histological activity was observed in all SVR patients and long-term treated; 100% of patients with SVR achieved a histological response (fibrosis stabilization or improvement) with a significant reduction in mean staging value (from 2.1 to 1.0; P = 0.0031); histological response was observed in 84% of long-term treated patients compared to 57% of drop-out. Six patients died during the entire study period (follow-up 40.6 ± 7.7 mo); of them, 5 presented with severe HCV recurrence on enrollment. Diabetes (OR = 0.38, 95%CI: 0.08-0.59, P = 0.01), leukopenia (OR = 0.54, 95%CI: 0.03-0.57, P = 0.03) and severe HCV recurrence (OR = 0.51, 95%CI: 0.25-0.69, P = 0.0003) were variables associated to survival. Long-term treatment was well tolerated; no patients developed rejection or autoimmune disease.

CONCLUSION: Long-term treatment improves histology in SVR patients and slows disease progression also in NR, leading to a reduction in liver decompensation, graft failure and liver-related death.

Key words: Hepatitis C virus; Hepatitis C recurrence; Interferon; Ribavirin; Liver transplantation

Core tip: Recurrent hepatitis C virus hepatitis is associated with a significant increase in morbidity and mortality of transplanted patients; biochemical and necro-inflammatory improves in transplanted patients who achieved a virological response after a course of antiviral treatment. Although the relative small sample size of our study, we demonstrated the efficacy of long-term antiviral treatment on disease progression despite the virological response, without significant side effects.

Tamè M, Buonfiglioli F, Del Gaudio M, Lisotti A, Cecinato P, Colecchia A, Azzaroli F, D'Errico A, Arena R, Calvanese C, Quarneti C, Ballardini G, Pinna AD, Mazzella G. Long-term leukocyte natural α-interferon and ribavirin treatment in hepatitis C virus recurrence after liver transplantation. *World J Gastroenterol* 2013; 19(32): 5278-5285 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5278.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5278

INTRODUCTION

Hepatitis C virus (HCV) -related end-stage liver disease is the main indication for liver transplantation (LT) in Western countries[1]. However, graft re-infection is almost universal, leading to accelerated, severe liver disease with a 30% rate of graft cirrhosis after 5 years[2,3]. Antiviral treatment is indicated for all patients with evidence of recurrent HCV hepatitis[4]; patients with signs of severe HCV recurrence, such as fibrosing cholestatic hepatitis (FCH), must be treated because of the aggressive disease course. The combination of interferon (IFN)-α (both standard and pegylated) plus ribavirin (RBV) is the treatment of choice; however, in the transplant setting, antiviral therapy is less effective. Indeed, IFN plus RBV combination therapy leads to a sustained virological response (SVR) rate of 17%-30%[5,6]. PEG-IFN plus RBV treatment has an SVR rate of approximately 30%[7-9], while in immunocompetent patients, the SVR rate ranges from 40%-82% according to the viral genotype[10]. The decreased efficacy of antiviral treatment in post-transplant patients may be explained by the low tolerability and the high rate of dose reduction and therapy discontinuation due to adverse events[5]. As previously reported, PEG-IFN-based treatment appears to be associated with more hematological and autoimmune adverse events than natural IFN-based therapy[11-13].

Previous studies[14,15] have reported that daily IFN administration leads to good virological and histological outcomes with an acceptable tolerability profile; we hypothesized that daily IFN administration could induce an higher, stable serum IFN concentration, similar to PEG-IFN therapy.

To the best of our knowledge, patients with a SVR to antiviral treatment have improved biochemical and necro-inflammatory activity, while the effect of antiviral treatment on disease progression in non-responders is still controversial[16]. Kornberg *et al*[17] first described the effect of long-term IFN and ribavirin treatment in transplant patients; the authors reported that antiviral maintenance treatment could prevent disease progression, leading to improved long-term survival. Walter *et al*[18] in their retrospective analysis, confirmed the previous results and reported that even in non-responders, long-term antiviral treatment significantly slowed the progression of fibrosis.

Our study aimed to evaluate the virological and histological effects of long-term leukocyte natural α IFN (ln-α-IFN) plus RBV treatment in patients with recurrent HCV hepatitis.

MATERIALS AND METHODS

Patients

From January 2003 to January 2008, 46 patients with recurrent HCV after liver transplantation were prospectively enrolled in our study. The diagnosis of recurrent hepatitis C was made using a combination of biochemical [increase in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) of at least 2x the ULN], virological (positive serum HCV-RNA) and histological findings. Patients with evidence of decompensated liver disease, histological evidence of rejection or drug-related injury, HBsAg positivity, HIV positivity, moderate to severe anemia (Hb < 10 g/dL), leukopenia (WBC < 1500/μL), thrombocytopenia (platelet count < 50000), impaired renal function (creatinine clearance < 50 mL/min), significant history of cardiovascular and psychiatric diseases or ongoing alcohol abuse were excluded.

Treatments

After the diagnosis of recurrent hepatitis C, all of the patients received a standard dose of leuckocyte natural α-IFN (Alfaferone, Alfawasserman, Bologna, Italy), 3 MU three times a week (*tiw*) and ribavirin. After one month of treatment, patients with good tolerance received an increased ln-α-IFN dose of 3 MU daily (Group A), while patients with poor tolerance to the antiviral treatment were maintained on *tiw* dosing (Group B). Tolerance to antiviral treatment was evaluated based on hematological side effects and patient compliance.

Patients who achieved undetectable HCV-RNA levels continued treatment for 12 mo after viral clearance. Non-responders and relapsers entered the long-term treatment group and were treated with ln-α-IFN plus RBV. The S. Orsola-Malpighi internal review board performed a case-by case evaluation for the use of off-label, daily IFN treatment and long-term antiviral therapy. The patients gave informed consent.

Standard immunosuppressive treatment was prescribed to all of the patients at the S. Orsola-Malpighi Hospital; 7 patients received a cyclosporine-based regi-

Table 1 Patients' baseline characteristics (mean ± SE)

Characteristic	Patients (n = 46)
Sex (M/F)	30/16
Age (yr)	57.9 ± 1.28
Time from OLT (mo)	26.3 ± 5.1
ALT (IU/L)	152.3 ± 17.8
AST (IU/L)	105.9 ± 11.5
Gamma-GT (IU/L)	188.7 ± 39.3
Alkaline phosphatases (IU/L)	364.7 ± 39.9
Bilirubin (mg/L)	1.7 ± 0.37
Viral load (log_{10})	6.25 ± 0.09
Genotypes 1/4 *vs* 2/3	36/10
F1/F2/F3/F4	13/16/9/8
Fibrosing cholestatic hepatitis	3
Cyclosporine A *vs* tacrolimus	7/39

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Gamma-GT: Gamma-glutamyl transpeptidase; M/F: Male/female.

men (CyA), while 39 received a tacrolimus-based one (FK).

Patients who presented with anemia or neutropenia received the scheduled IFN and RBV doses; then, erythropoietin was prescribed when the hemoglobin level fell below 10 g/dL, while granulocyte-colony stimulating factor was administered when the neutrophil count was < 700 mmc. When the anemia or neutropenia did not improve with growth factors, the IFN or RBV dose was reduced.

Biochemistry and virological assessment

Quantitative and qualitative HCV-RNA (Versant HCV-RNA 3.0 bDNA, and Versant TMA; Bayer Diagnostics) were measured before starting treatment, after 1 mo and every 3 mo for the first year; then, serum HCV-RNA was checked every 6 mo. Routine blood tests (blood cell counts and liver and renal function tests) were performed at baseline and weekly for the first 4 wk and then monthly.

Liver biopsy

A liver biopsy was performed for all of the patients before enrollment. For patients who achieved a virological response, a liver biopsy was repeated 1 year after the end of treatment; the non-responders and relapsers had a repeat liver biopsy after 30 mo of treatment. The histological staging and grading of chronic, recurrent HCV were evaluated according to the Knodell score[19]. The diagnosis and grading of liver allograft rejection were made according to the Banff international consensus[20].

HCV immunohistochemistry

Five-micron-thick sections of liver tissue were obtained and stored at -80 ℃. HCV immunohistochemistry (IHC) was performed as previously described[21-23]. Reaction positivity was graded according to the percentage of positive cells divided by the total number of hepatocytes (at least 200 cells/high magnification field)[21-23].

Definition

A rapid virological response was defined as HCV-RNA decrease of at least 2 log UI/mL or an undetectable level after 1 mo of treatment. A SVR was defined as a persistently undetectable serum HCV-RNA 6 mo after the end of treatment. The presence of fibrosing cholestatic hepatitis (FCH) or F4 fibrosis on enrollment was considered to be severe, recurrent HCV. A histological response was defined as an improvement or stabilization of liver fibrosis.

Statistical analysis

The data are expressed as the mean ± SE. Group comparisons were calculated using the χ^2 test, the Mann-Whitney test, the Wilcoxon test, t tests (both independent sample t-test and paired t-test), and an ANOVA when appropriate. Clinical events (SVR, and death) were analyzed using Kaplan Meier curves. Logistic regression was used to detect variables that were independently related to the clinical events. Statistical analysis was performed using the MedCalc package v.11.5 for Windows.

RESULTS

Patient characteristics

Forty-six patients (30 males, 57.9 ± 1.28 years old) with post-transplant HCV recurrence were prospectively enrolled; the patients' baseline characteristics are described in Table 1. Eleven patients presented with severe liver disease; 3 patients had FHC, and 8 patients had F4 fibrosis. Thirty-five patients presented with chronic HCV hepatitis with F1-3 fibrosis. Thirty-six patients had HCV genotype 1 or 4, while 10 patients had HCV genotype 2 or 3.

Treatment

The delay between LT and the initiation of treatment was 26.3 ± 5.1 mo. All of the patients (n = 46) received *tiw* IFN plus RBV treatment for the first month; then, 30 patients received 3 MU IFN daily plus RBV (Group A), while 16 continued *tiw* IFN + RBV treatment (Group B). The mean ribavirin dose during the treatment period was 8.4 ± 0.7 mg/kg per day; there was no difference in the RBV dose between Groups A and B.

Virological response

Among the entire population, seventeen patients (37%) achieved undetectable HCV-RNA levels during therapy and continued IFN+RBV treatment for 12 mo after viral clearance (mean 20.7 ± 2.5 mo); 4 of them relapsed after discontinuing treatment and were included in the long-term treatment group. Thirteen patients achieved an SVR: 8 of 30 patients (26.7%) in Group A and 5 of 16 (31.2%) in Group B. No difference between the groups was observed (P > 0.05).

The SVR rate was significantly higher for those with HCV genotype 2 or 3 than genotype 1 or 4 (70.0% *vs* 16.7%, respectively P = 0.0007); the overall SVR rate was 28.3%. Nine patients were rapid virological responders; among them, 7 had HCV genotype 2 or 3, and 2 had genotype 1 or 4. The variables from the univariate

Table 2 Variables associated with sustained virological response according to univariate analysis

Variable	*r*	95%CI	*P*-value
BMI > 25 kg/m^2	0.30	0.01455-0.5458	0.0400
Genotypes 2-3 *vs* 1-4	0.37	0.09103-0.5974	0.0100
RVR	0.54	0.2992-0.7194	0.0001
HCV-RNA clearance during treatment	0.82	0.6948-0.8967	0.0001
CyA *vs* FK immunosuppression	0.41	0.09597-0.6519	0.0120

RVR: Rapid virological response; HCV: Hepatitis C virus; CyA: Cyclosporine; FK: Tacrolimus; BMI: Body mass index.

Table 3 Histological response after treatment (mean ± SE)

	Grading			Staging		
	Before	After	*P*-value	Before	After	*P*-value
Sustained virological response (n = 9)	7.2 ± 0.8	2.6 ± 0.6	0.0039	2.1 ± 0.3	1.0 ± 0.1	0.0031
Long-term treated (n = 19)	7.9 ± 0.7	4.7 ± 0.6	0.0001	2.7 ± 0.3	2.5 ± 0.3	0.0001
Drop out (n = 7)	7.4 ± 1.1	6.0 ± 0.8	NS	2.6 ± 0.6	3.0 ± 0.6	NS

NS: Not significant.

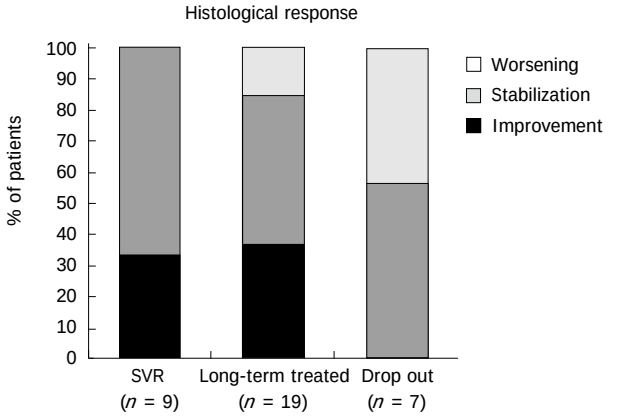

Figure 1 Histological response. Histological response of patients with sustained virological response, long-term treated and drop-out. SVR: Sustained virological response.

analysis associated with an SVR are shown in Table 2; in the multivariate analysis, a rapid virological response (OR = 99.6, 95%CI: 3.1-3190.0, P = 0.0093), a cyclosporine-based immunosuppressive regimen (OR = 685.4, 95%CI: 1.5-314392.9, P = 0.036) and the presence of severe, recurrent HCV (OR = 0.91 95%CI: 0.82-0.99, P = 0.04) were independently associated with a SVR. Eight patients withdrew therapy after 15.2 ± 2.0 mo, one because of moderate-severe anemia and seven because of non-compliance to therapy. Finally, 25 HCV-RNA-positive patients (21 non-responders and 4 relapsers) entered the long-term treatment group and were treated for a mean of 32.4 ± 2.8 mo.

Biochemical response

A significant improvement in ALT was observed in the 13 patients who achieved an SVR (186.1 ± 40.4 IU/L before enrollment *vs* 21.4 ± 2.2 IU/L after treatment, P = 0.0028) and in the 25 long-term treatment patients (154.0 ± 26.6-37.2 ± 4.7 U/L, P = 0.0003). Also, those patients who discontinued therapy (n = 8) showed a biochemical improvement (ALT 169.5 ± 42.7-58.1 ± 3.6 U/L, P = 0.0389). At the end of follow-up, the patients who achieved an SVR had a significantly lower ALT than the other patients (P < 0.05). We also observed that the ALT levels were lower in long-term treatment patients compared to the patients who had to stop treatment (P < 0.05).

Histological response

Among the entire population, 35 patients (9 sustained virological responders, 19 long-term treatment patients and 7 who stopped treatment) underwent a second liver biopsy after 30.3 ± 2.7 mo; seven patients refused the paired biopsy, while four died before the scheduled follow-up. The mean grade and stage are shown in Table 3. The post-treatment grade was significantly lower for the patients who achieved a SVR and received long-term treatment (P = 0.0039 and 0.0001, respectively), while the grade was unchanged for the patients who discontinued therapy.

Liver fibrosis improved (at least 1 stage) in ten of the 35 (28.6%) patients, remained stable in 19 patients (54.3%) and worsened in six patients (17.1%). The histological response in the three groups (responders, long-term treatment and discontinued treatment) is shown in Figures 1 and 2. Liver fibrosis remained stable or improved (histological response) in all of the patients who achieved a SVR (9 of 9, 100%); in this group, the mean post-treatment fibrosis appeared to be significantly lower (P = 0.0031). Interestingly, in the non-sustained virological responders, the histological response was higher in long-term treatment patients (16 of 19) than in the patients who stopped treatment (4 of 7) (84% *vs* 57%, P > 0.05). In the long-term treatment patients, the mean post-treatment fibrosis values were unchanged (2.7 ± 0.3 *vs* 2.5 ± 0.3).

Liver immunohistochemistry

HCV IHC (Figure 3) was performed for all of the liver biopsy (46/46) and paired liver biopsy samples (35/35). The median number of immunoreactive hepatocytes before treatment was 50% (95%CI: 38.9-60.0), and there was no significant difference among the responders, long-term treatment patients and patients who stopped treatment (median 60.0%, from 35.3% to 70.0%; median 45.0%, from 17.6% to 68.3%; median 40.0%, from 4.4% to 80.0%, respectively; P > 0.05).

After treatment, all of the patients who achieved a SVR (9 of 9) had no (0%) immunoreactive hepatocytes in the liver samples (P = 0.0002). Interestingly, the non-responders who received long-term treatment had a significant reduction (P = 0.001) in immunoreactive hepatocytes (before treatment: median 45%, from 17.6% to 68.3%; after treatment: median 0.0%, from 0.0% to

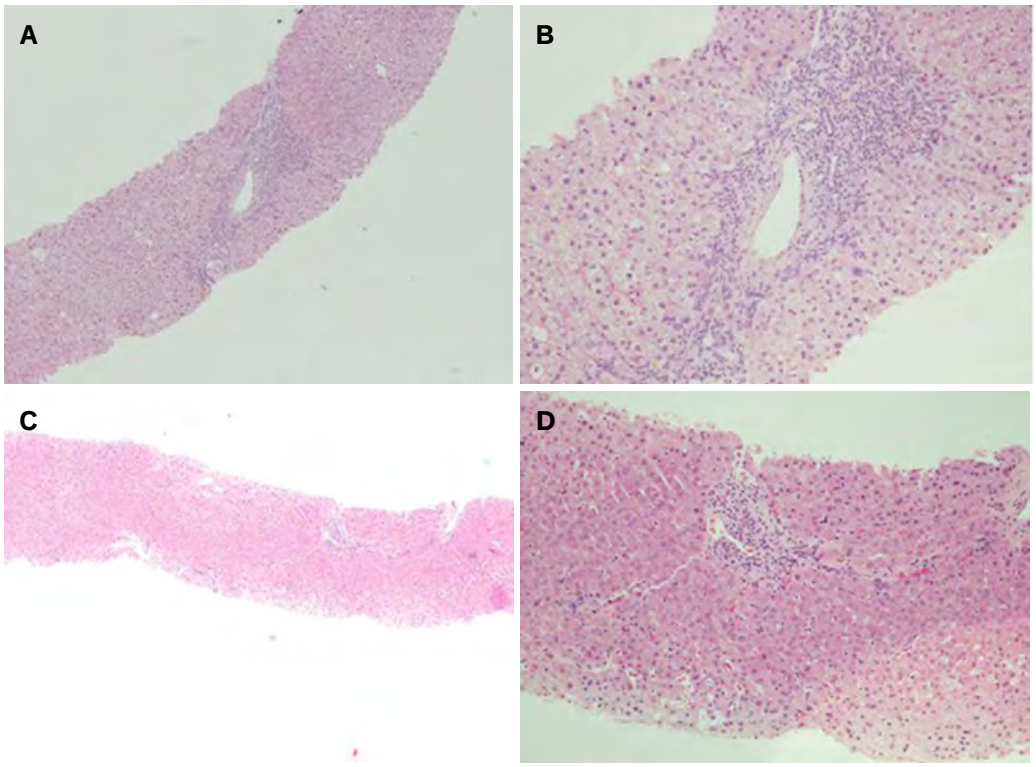

Figure 2 Liver histology. Liver histology before and after treatment in sustained virological response and Long-term treated patients: liver histology from a patient before treatment (A, B) and liver histology of the same patient (non responder) after long-term treatment (C, D). A, C: Hematoxylin and eosin (HE), × 10; B, D: HE, × 20.

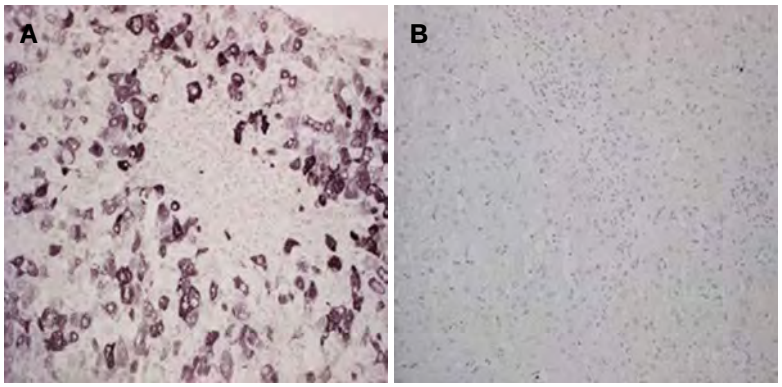

Figure 3 Hepatitis C virus immunohistochemistry. Lobular areas from serial biopsies of the same patient, showing the cytoplasmic positivity of hepatocyte for hepatitis C virus antigens before (A) and after treatment (B). Immunohistochemistry, × 20.

14.1%). No significant difference was observed before and after treatment in the patients who stopped treatment (before: median 40.0%, from 4.4% to 80.0%; after: median 20.0%, from 10.0% to 33.1%; $P > 0.05$).

Tolerability

Treatment was generally well tolerated; 29 (63%) patients, during combination therapy, required growth factors with 28 (61%) patients receiving erythropoietin for anemia and thirteen (28%) receiving G-CSF for neutropenia. Despite the use of grow factors, one patient withdrew from treatment due to moderate-severe anemia (no need for blood transfusion or hospitalization). No patient developed autoimmune disease or graft rejection.

Survival

Six patients died during the study period (follow-up 40.6 ± 7.7 mo). Two patients with a severe HCV recurrence (FCH), one who was a non-responder and another who stopped therapy, died because of a severe infection (encephalitis and cholangitis). Two patients (basal fibrosis F4), who were non-responders (1 long-term treatment and 1 who stopped treatment), died from liver decompensation. One patient with FCH on enrollment received long-term treatment for 40 mo and died 44 mo after enrollment due to liver decompensation. One patient with a mild HCV recurrence died due to a myocardial infarction.

The presence of diabetes (OR = 0.38, 95%CI: 0.08-0.59;

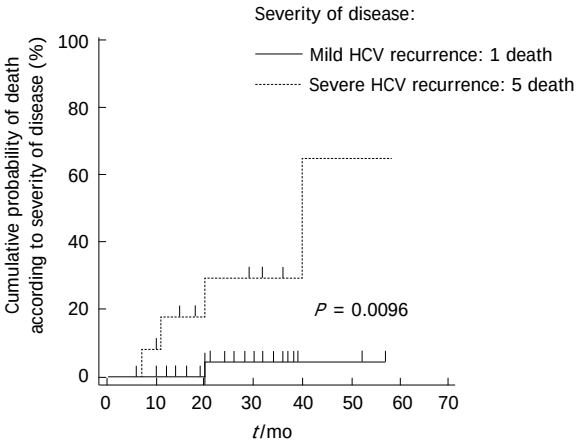

Figure 4 Survival analysis (Kaplan-Maier curve) according to presence of severe hepatitis C virus recurrence. HCV: Hepatitis C virus.

$P = 0.01$), leukopenia (OR = 0.54, 95%CI: 0.03-0.57; $P = 0.03$) or severe HCV recurrence (OR = 0.51, 95%CI: 0.25-0.69; $P = 0.0003$) were associated with survival; in a multivariate analysis, the presence of a severe HCV recurrence (OR = 29.6, 95%CI: 2.4-371.2; $P = 0.0086$) was the only variable to be independently associated with death. Kaplan-Maier analysis (curve shown in Figure 4) demonstrated an increased risk of death ($P = 0.0096$) for patients with a severe, recurrent HCV compared to patients with a mild recurrence. No difference between the survival of patients undergoing long-term treatment and those who discontinued treatment was observed.

DISCUSSION

Recurrent HCV hepatitis is associated with a significant increase in the morbidity and mortality of transplanted patients due to the early development of graft cirrhosis. In the post-transplant setting, the goals of antiviral treatment are to induce viral eradication and to slow disease progression.

Previous studies[5,24-27] have reported biochemical and necro-inflammatory improvement in transplanted patients who achieved a virological response after a course of antiviral treatment; however, the response rate is still unsatisfactory (17%-30% with IFN plus RBV; 18%-45% with PEG-IFN plus RBV)[5-9]. Among the non-responders, a significant number of patients will develop of graft cirrhosis and liver-related death in a few years[2,3]. To slow disease progression, the efficacy of antiviral maintenance therapy was evaluated in two studies[17,18], which showed preliminary evidence of benefit. Our study aimed to evaluate the efficacy of long-term treatment with ln-α-IFN plus ribavirin.

We reported an overall SVR rate of 28.3%; our response rate was similar to those observed with IFN-based and PEG-IFN-based regimens[5-9]. We did not observe any different virological outcomes between patients receiving daily and *tiw* IFN; this result could be due to variation in the HCV genotype distribution. Six patients of 16 (37.5%) in the Group B had a favorable genotype (2 or 3).

The role of cyclosporine in patients with HCV recurrence is still controversial. Our previous experience has demonstrated that the type of immunosuppression during antiviral treatment may predict the SVR[28], but a meta-analysis failed to demonstrate a significant difference in clinical outcome (graft survival and mortality)[29]. Our results showed that a cyclosporine-based immunosuppressive regimen correlated with an increased SVR rate, although there was a small number of CyA-treated patients.

The potential efficacy of pegylated IFN-based antiviral treatments in the transplant setting is limited by poor tolerability and a high rate of adverse events (hematological, autoimmune and rejection) leading to dose reduction and/or therapy discontinuation. Moreover, in our center, we previously experienced several (9 of 44 patients) *de novo* cases of autoimmune hepatitis during PEG-IFN plus ribavirin treatment[12]; therefore, in this study, to reduce adverse events and increase patient tolerability, we used a natural IFN-based regimen. As expected, we observed a good safety and tolerability profile; no patient developed autoimmune disease or graft rejection, while only one (2.2%) stopped treatment due to anemia.

Histological analysis from paired liver biopsy samples showed a reduction in necro-inflammatory activity in the patients who cleared HCV-RNA and those who received a long-term course of therapy. As in the non-transplant setting, achievement of complete viral eradication led to an improvement in liver inflammation, biochemically and histologically. Also, we observed a significant decrease in activity scores (from 7.9 ± 0.7 to 4.7 ± 0.6; $P = 0.0001$) in the non-responders who received long-term treatment with IFN plus RBV.

To evaluate the anti-viral and anti-inflammatory effects of IFN plus RBV treatment, we tested, on paired liver tissue samples, hepatocyte expression of viral proteins using IHC analysis as previously described by Ballardini *et al*[21,22]. As expected, our results showed that patients who cleared HCV did not have HCV-positive hepatocytes in their liver biopsies, while the non-responders who had interrupted antiviral treatment did not have reduced HCV protein expression. Interestingly, the non-responders who received a long-term course of therapy had a significantly reduced percentage of HCV-positive hepatocytes (median: 45%-0%), leading to a significant reduction in liver inflammation. To exclude sampling error, liver HCV-RNA was quantified in those cases; in all of the liver biopsies, HCV-RNA was detected. We hypothesized that the effect of IFN treatment, even in the non-responders, could reduce liver tissue inflammation by reducing the degree of hepatitis C viral antigen staining[30].

The role of antiviral treatment on disease progression is still debated. Patients who achieve an SVR have been shown to have delayed fibrosis progression[24], while some authors have also observed fibrosis regression[16]. A pivotal role for antiviral therapy was demonstrated by a previous study that reported treatment was the only variable to be independently associated with histological

improvement or stabilization among patients with HCV recurrence[16].

In our experience, nine patients who cleared HCV had a significantly reduced staging score on liver biopsy; moreover, a significant percentage (84%) of long-term treatment patients had a histological response despite the lack of viral clearance. Although there was a small number of patients, these results suggest the efficacy of long-term antiviral treatment on disease progression independent of a virological response.

The presence of severe HCV recurrence (histological cirrhosis, cholestatic hepatitis or FCH) is associated with a worse clinical outcome; as in the non-transplant setting[31,32], patients with advanced disease have a reduced SVR rate, due to increased averse events and therapy discontinuation. Moreover, we found that severe HCV recurrence is the only variable to be independently related to the risk of death. These findings suggest that recurrent HCV hepatitis should be treated at the onset of biochemical and histological signs to improve virological and clinical outcomes.

In conclusion, long-term treatment with ln-α-IFN plus ribavirin was able to improve histological staging in SVR patients, slow disease progression in non-responders, and demonstrate a good safety and tolerability profile. These findings suggest the importance of long-term treatment for HCV recurrence; this treatment seems to be able to reduce liver decompensation, graft failure and liver-related death.

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COMMENTS

Background

Hepatitis C virus (HCV) graft re-infection after liver transplantation is almost universal, leading to accelerated, severe liver disease; moreover, antiviral therapy in this setting is less effective. Only patients who achieve a virological response have an improvement in biochemical and necro-inflammatory activity, while the effect of antiviral treatment on disease progression in non-responders is still controversial.

Research frontiers

The use of long-term maintenance therapy in transplanted patients who do not achieve a virological response is still debated. Some authors have reported that antiviral maintenance treatment could prevent disease progression, leading to an improvement in long-term survival.

Innovations and breakthroughs

The results support the concept that long-term antiviral treatment leads to a better histological outcome even in patients who do not achieve viral clearance; therefore, long-term antiviral treatment improves disease progression, leading to a better clinical outcome.

Applications

New direct antiviral agents are changing the approach to HCV treatment, including in transplanted patients; however, the management of patients who do not achieve a viral response will be a future clinical challenge. The results supported the safety and tolerability of long-term treatment with interferon and ribavirin in patients who did not respond to therapy. We demonstrated that, even in non-responders, long-term treatment improves clinical and histological outcomes.

Peer review

As a retrospective clinical study on patients with HCV recurrence after liver transplantation, the results proved the efficacy of long-term antiviral treatment with leukocyte natural α-interferon and ribavirin on disease progression and showed a good safety and tolerability profile.

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BRIEF ARTICLE

Somatic molecular changes and histo-pathological features of colorectal cancer in Tunisia

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Abstract

AIM: To determine correlations between family history, clinical features and mutational status of genes involved in the progression of colorectal cancer (CRC).

METHODS: Histo-pathological features and molecular changes [*KRAS*, *BRAF* and *CTNNB1* genes mutations, microsatellite instability (MSI) phenotype, expression of mismatch repair (MMR) and mucin (MUC) 5AC proteins, mutation and expression analysis of *TP53*, *MLH1* promoter hypermethylation analysis] were examined in a series of 51 unselected Tunisian CRC patients, 10 of them had a proven or probable hereditary disease, on the track of new tumoral markers for CRC susceptibility in Tunisian patients.

RESULTS: As expected, MSI and MMR expression loss were associated to the presence of familial CRC (75% *vs* 9%, $P < 0.001$). However, no significant associations have been detected between personal or familial cancer history and *KRAS* (codons 12 and 13) or *TP53* (exons 4-9) alterations. A significant inverse relationship has been observed between the presence of MSI and TP53 accumulation (10.0% *vs* 48.8%, $P = 0.0335$) in CRC tumors, suggesting different molecular pathways to CRC that in turn may reflect different environmental exposures. Interestingly, MUC5AC expression was significantly associated to the presence of MSI (46.7% *vs* 8.3%, $P = 0.0039$), MMR expression loss (46.7% *vs* 8.3%, $P = 0.0039$) and the presence of familial CRC (63% *vs* 23%, $P = 0.039$).

CONCLUSION: These findings suggest that MUC5AC expression analysis may be useful in the screening of Tunisian patients with high risk of CRC.

Key words: DNA mismatch repair; *KRAS*; *TP53*; Mucin 5AC

Core tip: This study reports, for the first time in Tunisia, the value of various histo-pathologic features and

somatic molecular changes [*BRAF*, *KRAS*, *CTNNB1*, *TP53*, mismatch repair (MMR) expression, microsatellite instability (MSI), MLH1 promoter methylation] in distinguishing patients with hereditary non polyposis colorectal cancer. Our results revealed that MUC5AC expression was significantly associated with the presence of MSI (46.7% *vs* 8.3%, *P* = 0.0039), MMR expression loss (46.7% *vs* 8.3%, *P* = 0.0039) and the presence of familial colorectal cancer (63% *vs* 23%, *P* = 0.039). These findings suggest that mucin 5AC expression analysis may be useful in the screening of Tunisian patients with high risk of colorectal cancer.

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INTRODUCTION

Colorectal cancer (CRC) is a complex biological process involving many genes. Intensive screening for genetic alteration in CRC led to the identification of at least two different molecular mechanisms implicated in CRC carcinogenesis: chromosomal (CIN) and microsatellite instabilities (MSI). The CIN pathway is found in about 80% of sporadic CRC and in familial adenomatous polyposis[1]. It involves chromosomal allelic losses[2,3]. The MSI pathway is found in most cases of hereditary non-polyposis colorectal cancer (HNPCC) and in 12% of sporadic CRC. It involves inactivation of DNA mismatch repair (*MMR*) genes. The presence of MMR deficiency leads to the accumulation of mutations in mononuclear tracts in the coding region of genes controlling cell cycle[4]. Although CRC shows genetic heterogeneity, the same four different signalling pathways could be implicated in tumor progression. The WNT/Wingless pathway could be activated through an *APC* mutation in CIN tumors or through a *CTNNB1* stabilizing mutation in MSI tumors[5]. *CTNNB1* and *APC* mutations were observed as early as the adenomatous stage of CRC neoplasia. The transforming growth factor beta (TGFβ) pathway is driven by *SMAD2* or *SMAD4* inactivating mutation in CIN tumors[6] or by a frame-shift mutation in the TGFβ type Ⅱ receptor in MSI tumors[7]. The RAS-MAP kinase pathway is activated by *KRAS* mutations in CIN[8] or by *BRAF* mutations in sporadic MSI tumors. Alteration of these genes correlated closely with the progression of the adenoma to cancer. The TP53 pathway is inactivated by *TP53* mutations in CIN tumors or by *BAX* inactivating mutation in MSI tumors. These alterations contribute to the adenoma-carcinoma transition. More recently, the existence of a third phenotype was suggested. The main alteration associated with this group of tumors is the hypermethylation of the promoter region of numerous genes, leading to their inactivation[9,10]. Activating somatic mutation of *BRAF* gene has been reported in 15% of sporadic tumors with MSI due to *MLH1* hypermethylation and never in tumors from HNPCC families with *MLH1* and *MSH2* germline mutations[11]. MMR germline mutations detections is an important supplement to HNPCC clinical diagnosis. It enables at-risk and mutation-positive relatives to be informed about their cancer risks and to benefit from intensive surveillance programs that have been proven to reduce the incidence of CRC[12]. However, germline tests are time-consuming and costly due to heterogeneity of mutations. In addition, MMR germline mutations are not always detected in Amsterdam positive families (sensibility, 50%-78%)[13]. The difference in somatic mutation status between sporadic CRC and HNPCC-related cancers may prove helpful in distinguishing HNPCC patients. In this study, we analysed for the first time in Tunisia the value of various histo-pathologic features and somatic mutations of 51 CRC cases in predicting CRC susceptibility.

MATERIALS AND METHODS

Patients and tissue specimens

Fifty-one formalin-fixed, paraffin embedded primary colorectal carcinomas and paired normal bowel of 51 different patients who had undergone colonic resection for the treatment of CRC were retrieved by retrospective review of the pathology archives. Ten of these patients were previously characterized for MMR germline mutations associated to Lynch syndrome[14]. Patients were evaluated according the revised Bethesda guidelines for the identification of HNPCC patients[15]. MSI testing, immunohistochemistry and somatic mutational analysis were performed in all patients regardless of age, personal or family history of cancer, and tumor characteristics.

DNA preparation

DNA was extracted from paraffin-embedded tissue samples of primary CRC and paired normal bowel using the DNeasy® tissue kit (Qiagen, Courtaboeuf, France).

MSI analysis

MSI was assessed using a set of five mononucleotide markers (BAT25, BAT26, NR21, NR22, NR24)[15,16].

Expression of MMR proteins

MMR was assessed by immunohistochemistry as previously described[16]. Immunohistochemistry for mucin (MUC) 5AC and TP53. Tumor sections were analysed using mouse monoclonal antibody against p53 (clone DO-7, Dakocytomation) and MUC5AC (clone CLH2, Novocastra). For TP53, a tumor was scored as TP53 overexpression-positive if nuclear staining was seen in more than 20% of the neoplastic cells in the absence of staining in the tumor adjacent cells. For MUC5AC, which is never expressed in normal colon mucosa[17], expression was interpreted as positive if more than 10% of tumor

Table 1 Clinical and histo-pathological characteristics of the 51 colorectal cancer patients *n* (%)

Characteristic	Patient
Age of onset of the first cancer (range) (yr)	51 (17-85)
⩽ 50	25 (49.0)
> 50	26 (51.0)
Sex	
Male	30 (58.8)
Female	21(41.2)
Site of the first CRC	
Right colon	14 (27.5)
Left colon	16 (31.4)
Rectum	21 (41.2)
TNM tumor stage	
Ⅰ	3 (5.9)
Ⅱ	24 (47.1)
Ⅲ	20 (39.2)
Ⅳ	3 (5.9)
Others	1 (2.0)
Degree of differentiation	
Well	33 (64.7)
Moderate	14 (27.5)
Poor	2 (3.9)
Mucinous CRC	2 (3.9)
Mucinous carcinoma type	
⩾ 50%	14 (27.5)
⩽ 50%	37 (72.5)
Signet ring cell carcinoma	2 (3.9)
Tumor infiltrating lymphocyte	
Crohn's–like reaction	2 (3.9)
Intra epithelial lymphocytes	1 (2.0)
Lymphoïde peritumoral reaction	10 (19.6)
Synchronous CRC	3 (5.9)
Metachronous CRC and HNPCC related cancer	3 (5.9)
Fulfillment of guidelines	
Amsterdam	3 (5.9)
Revised Bethesda	22 (43.1)
B1	22
B2	3
B3	11
B4	1
B5	1

CRC: Colorectal cancer; HNPCC: Hereditary nonpolyposis colorectal cancer; TNM: Tumor node metastasis.

cells displayed cytoplasmic staining in the absence of staining in the tumor adjacent cells.

TP53 mutations screening

Primers were designated for the coding regions and exon-intron boundaries of exons 5 to 8. Exons 4 and 9 were only analysed on those samples negative for mutations in exons 5-8. Primer sequences and polymerase chain reaction (PCR) conditions are available on request.

Mutation screening for KRAS, CTNNB1 and BRAF genes

KRAS (codon 12, 13), *BRAF* (exon 15) and *CTNNB1* (β-catenin) (exon 3) were screened in each CRC cancer using direct sequencing in forward and reverse orientations. Primer sequences and PCR conditions are available on request.

MLH1 promoter methylation assay

Genomic DNA obtained from paraffin-embedded tissue section was modified with sodium bisulfite using the EZ DNA Methylation kit (Zymo Research) according to the specifications of the manufacturer. Primer sequences for methylation-specific PCR were modified from Grady *et al*[18].

Statistical analysis

Continuous variables are described as mean and range (min-max) and categorical variables as frequencies and percentages. The association between the different measured parameters was tested using non parametric tests. The difference between two independent groups was determinate by Mann-Whitney *U*-test and the significance of differences between more than two groups was calculated using Kruskal-Wallis test. Categorical data were compared by χ^2 appropriate or Fisher exact tests. A *P*-value < 0.05 was considered as statistically significant. Statistical analyses were performed with SPSS version 15.0 (SPSS, Chicago, IL, United States).

RESULTS

Clinical and pathological features

Demographic, clinical and tumor-related characteristics of the study group are summarised in Table 1. Twenty-five (49.0%) probands were under 50 years of age, including 12 (23.5%) under 40 years of age and 5 (9.8%) under 30 years of age; 26 (51%) aged more than 50 years, including 20 (39.2%) aged more than 60 years. Six patients (11.8%) had a personal history of synchronous/metachronous CRC tumors (4 cases) or previous primary CRC and HNPCC-related extracolonic tumors (2 cases). In 8 cases (16%), the proband was found to have at least one first-degree relative with CRC and/or HNPCC-related extracolonic cancers. In total, 25 (49%) of the 51 CRC patients belonged to families fulfilling the Amsterdam Criteria[19] for the clinical definition of HNPCC or fulfilled at least one criterion of the revised Bethesda criteria for the identification of HNPCC patients[15]. Criterion 1 was the most commonly satisfied Bethesda criterion (22/51, 43.1%). Clinical data analysis revealed that CRC was essentially right sided for patients having at least one first- or second-degree relative with CRC; whereas cancer was more frequently left sided or rectal for patients without a familial history of CRC ($P = 0.039$) (Table 2). However, no significant difference in tumor site was seen when Bethesda criteria where considered. The Bethesda-positives CRC tumors same to be associated to a more advanced stage of the disease ($P = 0.050$) (Table 2). However, no statistical difference has been seen when familial history was considered (Table 2).

Pattern and frequency of MSI

MSI-high (MSI-H) phenotype was detected in 10 (19.6%) of the 51 tested tumors. All the MSI tumors showed instability in all 5 analysed markers. Eight patients (8/25; 32%) were Bethesda-positives and only 2 (2/26; 8%) were Bethesda-negatives (Table 2). For the remaining 41 cases, the tumors were microsatellite stable (MSS) including 1 (1/3, 33%) Amsterdam I-positive patient and 16 (16/25,

Table 2 Statistical analysis of clinicopathological parameters of the 51 colorectal cancer studied tumors as a function of tumoral phenotype *n* (%)

	Mutation MMR - (*n* = 6)	Mutation MMR + (*n* = 4)	Family history of colorectal cancer		*P*	Ams - and Beth - (*n* = 26)	Ams - and Beth + (*n* = 22)	*P*	Ams (- or +) and Beth + (*n* = 25)	*P*
			Yes (*n* = 8)	No (*n* = 43)						
Mutation geminale										
MMR+			4	0		0	2		4	
MMR-			3	3		0	5		6	
Site of tumor (CCR)					NS			NS		NS
Right colon	1	3	5 (63)	10 (23)		5 (19)	9 (41)		10 (40)	
Left colon	2	0	1 (13)	14 (33)		8 (31)	7 (32)		7 (28)	
Rectum	3	1	2 (25)	19 (44)		13 (50)	6 (27)		8 (32)	
Left colon + rectum	5	1	3 (38)	33 (77)	0.039	21 (81)	13 (59)	NS	15 (60)	NS
Right + left colon	3	3	6 (75)	24 (56)	NS	13 (50)	16 (73)	NS	17 (68)	NS
TNM Stage					NS			NS		NS
Ⅰ	0	1	1 (14)	2 (5)		2 (8)	0 (0)		1 (4)	
Ⅱ	1	0	2 (29)	23 (53)		16 (62)	9 (41)		9 (38)	
Ⅲ	4	3	4 (57)	15 (35)		7 (27)	11 (50)		12 (50)	
Ⅳ	0	0	0 (0)	3 (7)		1 (4)	2 (9)		2 (8)	
Ⅰ / Ⅱ	1	1	3 (43)	25 (58)	NS	18 (69)	9 (41)		10 (42)	
Ⅲ / Ⅳ	4	3	4 (57)	18 (42)		8 (31)	13 (59)	NS	14 (58)	0.050
Microsatellite instability					< 0.001			NS		0.038
MSI (MSI-L or MSI-H)	1	4	6 (75)	4 (9)		2 (8)	6 (27)		8 (32)	
MSS (MSI-L or MSS)	5	0	2 (25)	39 (91)		24 (92)	16 (73)		17 (68)	
Somatic mutations										
TP53	6	0	3 (38)	25 (64)	NS	14 (61)	13 (62)	NS	14 (58)	NS
KRAS	1	2	2 (25)	14 (33)	NS	9 (35)	5 (23)	NS	7 (28)	
BRAF	0	0	0 (0)	1 (2)		1 (4)	0 (5)		0 (0)	
CTNNB1	0	0	0 (0)	1 (2)		0 (0)	1 (5)		1 (4)	
Immunohistochemistry										
Loss of MMR	1	4	5 (50)	5 (50)	NS	2 (8)	6 (27)	NS	8 (32)	0.038
Overexpression of p53	5	0	2 (25)	19 (44)	NS	9 (35)	11 (50)	NS	12 (48)	NS
Overexpression of MUC5AC	2	3	5 (63)	10 (23)	0.039	6 (23)	8 (36)	NS	9 (36)	NS

Ams: Extented Amsterdam Ⅱ criteria; NS: Not statistically significant; Beth: Revised Bethesda Guidelines; MMR: Mismatch repair; CCR: Colorectal cancer; MSI: Microsatellite instability; MSS: Microsatellite stable; MUC: Mucin.

64%) Bethesda-positives patients. Six (27%) of the 22 Bethesda-positives Amsterdam-negatives patients showed MSI in tumor tissue. Hence, the sensitivity and the specificity of the Bethesda criteria in the prediction of MSI were 80% and 60%, respectively. MSI was also observed in 3 sporadic CRC cases (3/49, 6%). Other laboratories have demonstrated a frequency of MSI between 10% and 20% amongst sporadic CRC cases. Therefore, our results are comparable with results from other series[20,21]. CRC was diagnosed before 50 years of age in 80% (8/10) of the patients with MSI (Table 3). The mean age at tumor diagnosis in MSS patients was higher than in MSI patients [56.2 years (range 17-85 years) *vs* 42.4 years (range 18-72 years)] (Table 3). Two of the 10 MSI patients (20%) had synchronous/metachronous colorectal cancer and no one had additional extracolonic cancer. Mucinous colloid component was significantly more important in MSI-H tumors ($P = 0.0178$) (Table 3). No significant associations were observed between MSI phenotype and sex, tumor site and tumor node metastasis (TNM) stage (Table 3). However, MSI-H tumors have been reported to be more frequent in the proximal colon[22]. A *KRAS* somatic mutation was detected in 4 (4/10, 40%) MSI-H tumors (Table 4): 3 were located at codon 13 (*p*.Gly13Asp) and 1 was at codon 12 (*p*.Gly12Asp). No significant association has been detected between MSI and *KRAS* alterations (Table 4). However, an inverse correlation came to exist between MSI-H phenotype and TP53 overexpression ($P = 0.0335$) (Table 4). On the other hand, MUC5AC abnormal expression was significantly more frequent in MSI tumors compared to MSS tumors ($P = 0.0039$) (Table 4). This result was in accordance with data reported by Biemer-Hüttmann *et al*[23].

MMR protein expression

Forty-one of the 51 (80.4%) analysed CRCs exhibited normal MMR protein expression. Of the remaining 10 (19.6%) CRCs, 8 (80%) showed a combined MLH1 and PMS2 proteins expression loss suggesting an *MLH1* deleterious mutation, 1 (10%) showed a combined MSH2 and MSH6 proteins expression loss, hardly suggesting an *MSH2* deleterious mutation (or *MSH6*, eventually), while just 1 (10%) demonstrated loss of only MSH6 protein, suggesting an *MSH6* deleterious mutation. Two (2/10, 20%) of these patients were Amsterdam-positives whereas 8 (8/10, 80%) where Amsterdam-negatives (Table 2). Five patients with MMR proteins expression loss had a family history of cancer. Of the 8 cases with MLH1 expression loss, 2 (2/8, 25%) had an Amsterdam-positive family history. For MSH2 and/or MSH6 none of the 2 cases with expression loss had a cancer family history. The 10 tumors with MMR expression loss corresponded

Table 3 Comparison of the somatic phenotype and genotype as a function of the patient's clinical characteristics

	MSI (*n* = 10)	*KRAS* mutations (*n* = 16)	*TP53* mutations (*n* = 28)	TP53 overexpression (*n* = 21)	MUC5AC overexpression (*n* = 15)
Mean age at diagnosis (range), yr	45 (18-72)	51.5 (18-85)	48.5 (18-79)	49.5 (24-75)	50 (24-76)
Sex					
Males	80.00%	75.00%	57.10%	52.40%	66.70%
Females	20.00%	25.00%	42.90%	47.60%	33.30%
Tumor site[1]					
Proximal	50.00%	18.80%	21.40%	28.60%	33.30%
Distal	50.00%	81.30%	78.60%	71.40%	66.70%
TNM stage[2]					
Ⅰ	10.00%	6.30%	0.00%	0.00%	0.00%
Ⅱ	40.00%	43.80%	48.10%	38.10%	40.00%
Ⅲ	50.00%	50.00%	44.40%	47.60%	53.30%
Ⅳ	0.00%	0.00%	7.40%	9.50%	6.70%

[1]Proximal, right colon; distal, left colon + rectum; [2]Tumor, node, metastasis (TNM) stage was unknown for one patient; MSI: Microsatellite instability; MUC: Mucin.

Table 4 Comparison of the microsatellite instability phenotype as a function of tumoral parameters

	MSI-H tumors (*n* = 10)	MSS tumors (*n* = 41)	*P*
MMR expression	100.00%	0.00%	< 0.0001[1]
KRAS mutations	40.00%	29.30%	NS
TP53 mutations	30.00%	67.60%	NS
TP53 surexpression	10.00%	48.80%	0.0335[1]
MUC5AC surexpression	70.00%	19.50%	0.0039[1]

[1]Fisher exact test. NS: Not significant; MSI: Microsatellite instability; MUC: Mucin; MMR: Mismatch repair; MSS: Microsatellite stable.

Table 5 Comparison of *TP53* somatic mutations as a function of tumoral parameters

	Presence of *TP53* mutations (*n* = 28)	Absence of *TP53* mutations (*n* = 19)	*P*
MSI	10.70%	36.80%	NS
MMR expression loss	10.70%	36.80%	NS
KRAS mutations	25.00%	47.40%	NS
TP53 surexpression	60.70%	21.10%	0.0090[1]
MUC5AC surexpression	21.40%	47.40%	NS

[1]Fisher exact test. NS: Not significant; MSI: Microsatellite instability; MUC: Mucin; MMR: Mismatch repair.

to the 10 MSI-H tumors. All the MSS tumors showed normal MMR proteins expression. Hence, immunohistochemical analysis had 100% sensitivity for the detection of tumors with high MSI. Of note, germline deleterious mutations in *MMR* genes had been reported in our previous studies[14,24] in 4 patients with MSI-H CRC tumors and MMR protein expression loss.

TP53 protein expression analysis

TP53 positive nuclear immunostaining was observed in the CRC tumors from 21 (21/51, 41%) patients. No significant association has been detected between TP53 overexpression and selection criteria (Amsterdam or Bethesda) or the presence of relatives with CRC (Table 2). Overall, no association has been detected between TP53 overexpression and the different clinical parameters, including age at tumor diagnosis, gender, TNM stage or tumor location (Table 3). All but one (20/21, 95.2%) of the CRC tumors with TP53 overexpression were MSS and showed normal MMR proteins expression. The remaining tumor was of MSI-H phenotype associated to a combined MLH1 and PMS2 proteins expression loss, suggesting an MMR deficiency. On other hand, we have noted a close correlation between *TP53* mutations and TP53 protein level ($P = 0.0090$) (Table 5), as previously reported[25,26]. The absence of mutation in the 4 tumors overexpressing TP53 may be due to a lack of sensibility of the utilized sequencing technique, which requires greater than 15%-20% of neoplastic cells burden in the analysed specimens. In addition, mutations may be located outside the screened exons (exons 4-9), which represent less than 5% of the *TP53* detected mutations[27].

Expression analysis of MUC5AC

Abnormal MUC5AC expression was identified in 15 CRC tumors (15/51, 29.41%), 6 of them showed mucinous colloid component ⩾ 50%. In 3 tumors, the stained area was limited to the focal glands. MUC5AC expression was significantly associated to the presence of personal and family history of CRC ($P = 0.039$) (Table 2). It is very interesting to note that abundant MUC5AC expression was seen in the tumor of 3 HNPCC subjects with deleterious germline *MMR* mutations[14]. However, we didn't detect any other significant association between MUC5AC expression and clinico-pathological characteristics (Table 6). Interestingly, MUC5AC expression was significantly associated to MSI phenotype and MMR proteins expression loss ($P = 0.0039$) (Table 6). In contrast, no significant association was detected between MUC5AC expression and *TP53* or *KRAS* genes mutations (Table 6).

TP53 mutations analysis

The *TP53* mutation analysis was possible in the CRC

Table 6 Comparison of mucin 5AC expression as a function of tumoral parameters

	MUC5AC expression (n = 15)	Absence of MUC5AC expression (n = 36)	P
MSI phenotype (n = 10)	46.70%	8.30%	0.0039[1]
MMR expression loss (n = 10)	46.70%	8.30%	0.0039[1]
TP53 mutations (n = 28)	40.00%	68.80%	NS
TP53 overexpression (n = 21)	33.30%	44.40%	NS
KRAS mutations (n = 16)	26.70%	33.30%	NS

[1]Fisher exact test. NS: Not significant; MSI: Microsatellite instability; MUC: Mucin; MMR: Mismatch repair.

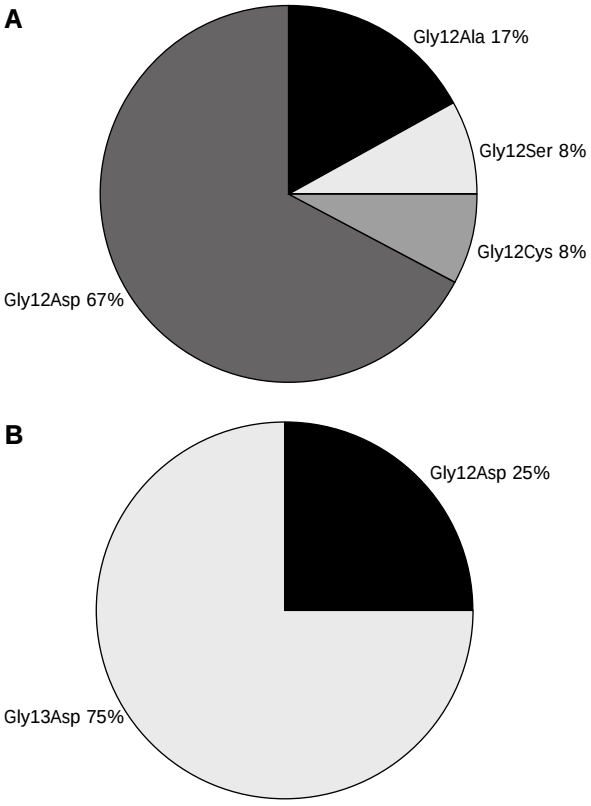

Figure 1 *KRAS* mutation spectrum as a function of tumoral microsatellite instability phenotype. A: Microsatellite stable (MSS); B: Microsatellite instability-high (MSI-H).

tumors of 47 patients. In total, a deleterious somatic mutation has been detected in 28 patients (28/47, 59.6%). Overall, there were no significant association of *TP53* mutations with Bethesda criteria, cancer family history (Table 2) or patient's clinical and histo-pathological data (Table 3). Particularly, we didn't detect any association between the presence of *TP53* mutations and tumor site (P = 0.0658) (Table 3). This was in contrast with data reported by other groups which showed that *TP53* mutations where more frequent in left-sided and rectal tumors[28-30]. A *KRAS* somatic mutation was identified in 7 (7/28, 25%) of the CRC tumors with *TP53* mutations (Table 5). All these mutations were G>A transitions

Table 7 Comparison *KRAS* somatic mutations as a function of tumoral parameters

	Presence of *KRAS* mutations (n = 16)	Absence of *KRAS* mutation (n = 35)	P
MSI	25.00%	17.10%	NS
MMR expression loss	25.00%	17.10%	NS
TP53 mutations	46.70%	65.60%	NS
TP53 overexpression	31.30%	45.70%	NS
MUC5AC overexpression	25.00%	31.40%	NS

NS: Not significant; MSI: Microsatellite instability; MUC: Mucin; MMR: Mismatch repair.

in codon 12 (5 mutations were p.Gly12Asp and 1 was p.Gly12Ser) and no mutation has been detected in codon 13.

KRAS somatic mutations

A *KRAS* mutations was identified in 16 (16/51, 31.5%) of all the CRC tumors. There was no significant association of *KRAS* mutations with Bethesda criteria, cancer family history (Table 2) and patient's clinical and histo-pathological data (Table 3). In addition, no significant association has been detected between *KRAS* mutations and the other tumoral parameters (Table 7). However, the mutation spectra same to be different between MSS and MSI tumors and more varied mutations have been detected in MSS tumors (Figure 1). Some amino acid changes were detected only in MSS tumors (Figure 1). Whereas, the *KRAS* mutation p.Gly13Asp have been detected only in MSI-H tumors in the absence of *TP53* mutations or TP53 overexpression (Figure 1).

BRAF mutations

The *BRAF* activating mutation c.1796A>T, p.Val600Glu was found in only 1 (1/51, 2%) stage Ⅱ non-mucinous and non-invasive tumor of the proximal colon of a 79 years old man with no cancer family history. This mutation was shown to be specific to sporadic CRC tumors due to *MLH1* promoter hypermethylation and absent in CRC tumors with MSS phenotype[31] and patients with *MLH1* or *MSH2* germline mutations[11]. Because we didn't examine the entire *BRAF* gene, we cannot rule out the presence of other mutations in the remaining CRC tumors. This tumor showed peritumoral lymphatic reaction, MSS phenotype, normal MMR proteins expression and abnormal MUC5AC expression. In addition, no somatic mutations in *KRAS* or *TP53* genes or overexpression of TP53 protein were detected in this tumor. According to some authors, this tumoral phenotype characterized CRC tumors arising from serrated polyps[32].

CTNNB1 mutations

Only 1 putative pathogenic somatic mutation was detected in an MSI-H CRC tumor (1/51, 2%). The change was a typical missense mutation causing alteration of serine at codon 45 (c.134C>T, p.Ser45Phe). This patient

was operated of TNM stage Ⅱ well differentiated and mucinous adenocarcinoma of the sigmoid at 36 years of age and didn't have any relatives with cancer. Tumor analysis detected a p.Gly13Asp *KRAS* mutation and a combined MLH1 and PMS2 proteins expression loss. These findings were in accordance with the specific affinity of *CTNNB1* mutation to MSI-H CRC tumors[5,33]. In addition, normal TP53 and MUC5AC proteins expression was detected in this tumor in the absence of *TP53* or *BRAF* somatic alterations. According to Young *et al*[34], this tumoral phenotype characterises CRC in Lynch syndrome, highlighting the presence of this syndrome in this patient.

MLH1 promoter methylation

Further analysis of *MLH1* promoter methylation status in the tumor of 4 patients showing MSI-H phenotype and MLH1 protein expression loss in the absence of *MLH1* deleterious somatic mutation by MLPA or sequencing, did not detect aberrant methylation discarding the hypothesis of sporadic cancers due to epigenetic inactivation of *MLH1* gene.

DISCUSSION

The detection of subjects at high risk of CRC remains problematic. It was essentially based on the family history of patients. Nevertheless, MSI testing and MMR protein expression analysis still the major screening tool for identifying HNPCC. In the present report, we have studied the phenotype and the genetic characteristics of the CRC tumors of 51 Tunisian non related patients selected according to the revised Bethesda criteria in order to compare the tumor phenotype due to MMR deficiency with somatic alterations in genes implicated in CRC tumorigenesis. Our aim was to define for each tumor the pathway of carcinogenesis and to identify new tumoral markers which may help in the diagnosis of CRC susceptibility and easy to use in medical practice. Clinical data analysis showed that CRC was essentially right sided in patients with first or second degree CRC relatives, whereas CRC was mostly distal (left colon and rectum) in patients without cancer family history. These findings are in accordance with data published[35,36]. As expected, genetic characteristics analysis of the 51 tumors showed that MSI phenotype and MMR expression loss were significantly associated to the presence of a CRC family history ($P < 0.001$). *TP53* mutations have been detected in 59.6% of the analysed patients. This finding was in agreement with previous studies in CRC, which reported *TP53* mutation frequencies between 50% and 70%[28-30]. Our study shows statistically inverse relationships between MSI and *TP53* alterations in CRC ($P = 0.0335$). This finding was in accordance with that reported by Samowitz *et al*[29]. This data highlights the hypothesis that MMR deficient CRC tumors evolve through a pathway that is independent of *TP53* gene. *KRAS* mutations were identified in 31.5% of all CRC tumors. This is consistent with previous reports that have identified *KRAS* mutations in 30%-45% of CRC tumors[8,29,37]. No significant association had been detected between MSI phenotype and *KRAS* alterations. However, the mutation spectrum was different between MSS and MSI-H tumors. In spite of our reduced number of tumors this finding was in accordance with that reported[8]. On the other hand, abnormal MUC5AC expression was found to be significantly associated to MSI phenotype ($P = 0.0039$) and CRC personal and family history ($P = 0.039$). In contrast, no significant association was detected between MUC5AC expression and *KRAS* or *TP53* genes mutations.

In conclusion, we suggest that MUC5AC expression analysis of CRC tumors may be useful in the screening of patients with high risk of CRC.

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COMMENTS

Background

This paper tend to make correlations between family history, clinical features and mutational status of genes involved in the progression of colorectal cancer (CRC) on the track of new tumoral markers for CRC susceptibility in Tunisian patients.

Research frontiers

Authors have screened 51 CRC tumors containing mixed hereditary non-polyposis colorectal cancer (HNPCC) and sporadic CRC Tunisian cases for somatic changes in *KRAS*, *BRAF* and *CTNNB1*, for microsatellite instability, for expression of mismatch repair (MMR) and mucin (MUC) 5AC, for mutation and expression of *TP53* and for *MLH1* promoter hypermethylation. They have also compared these molecular findings with clinical, pedigree and pathological data, regardless of age, personal or family history of cancer, and tumor characteristics.

Innovations and breakthroughs

In this study, authors report for the first time in Tunisia the value of various histo-pathologic features and somatic molecular changes in distinguishing patients with HNPCC from those with sporadic colorectal cancer in the aim to identify new tumoral markers of colorectal cancer susceptibility easy to use in the design of diagnostic, therapeutic and preventive strategies in Tunisia.

Applications

MUC5AC expression was significantly associated to the presence of the presence of familial CRC, microsatellite instability and MMR expression loss. This finding suggests that MUC5AC expression analysis may be useful in the screening of patients with high risk of CRC in Tunisia.

Peer review

Screening the subjects at risk of CRC is important. In this manuscript, the authors retrospectively reviewed the histo-pathological features, molecular changes and family history of 51 Tunisian CRC patients. Among many genetic and clinical variables, MUC5AC expression was concluded to be useful in the screening of patients at high risk of CRC susceptibility.

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BRIEF ARTICLE

Investigation of genome instability in patients with non-alcoholic steatohepatitis

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Abstract

AIM: To evaluate the occurrence of micronucleus (MN), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) in the mitogen-stimulated lymphocytes of patients with non-alcoholic steatohepatitis (NASH).

METHODS: The study was performed in 25 (9 females, 16 males) patients newly diagnosed with NASH, and 25 healthy subjects of similar ages and genders were used as a control group. None of the controls was known to be receiving any drugs for medical or other reasons or using alcohol. Hepatosteatosis was further excluded by abdominal ultrasound imaging in the control group. The numbers of MN, NPBs and NBUDs scored in binucleated (BN) cells were obtained from the mitogen-stimulated lymphocytes of patients and control subjects. Statistical comparisons of the numbers of BN cells with MN, NPBs and NBUDs and ages between the patients with NASH and control subjects were performed.

RESULTS: The mean ages of the patients and the control group were 41.92 ± 13.33 and 41.80 ± 13.09 years ($P > 0.05$), respectively. The values of the mean body mass index (BMI), HOMA-IR, hemoglobin, creatinin, aspartate aminotransferase, alanine aminotransferase, triglyceride, high density lipoprotein, and low density lipoprotein were 31.19 ± 4.62 kg/m^2 *vs* 25.07 ± 4.14 kg/m^2, 6.71 ± 4.68 *vs* 1.40 ± 0.53, 14.73 ± 1.49 g/dL *vs* 14.64 ± 1.30 g/dL, 0.74 ± 0.15 mg/dL *vs* 0.80 ± 0.13 mg/dL, 56.08 ± 29.11 U/L *vs* 16.88 ± 3.33 U/L, 92.2 ± 41.43 U/L *vs* 15.88 ± 5.88 U/L, 219.21 ± 141.68 mg/dL *vs* 102.56 ± 57.98 mg/dL, 16.37 ± 9.65 mg/dL *vs* 48.72 ± 15.31 mg/dL, and 136.75 ± 30.14 mg/dL *vs* 114.63 ± 34.13 mg/dL in the patients and control groups, respectively. The total numbers and frequencies of BN cells with MN, NPBs and NBUDs, which were scored using the CBMN cytome assay on PHA-stimulated lymphocytes, were evaluated in the patients with NASH and control group. We found significantly higher numbers of MN, NPBs and NBUDs in the BN cells of patients with NASH than in those of the control subjects (21.60 ± 9.32 *vs* 6.88 ± 3.91; 29.28 ± 13.31 *vs* 7.84 ± 3.96; 15.60 ± 5.55 *vs* 4.20 ± 1.63, respectively, $P < 0.0001$).

CONCLUSION: The increased numbers of MN, NPBs and NBUDs observed in the lymphocytes obtained from patients with NASH may reflect genomic instability.

Key words: Non-alcoholic steatohepatitis; Micronucleus; Nucleoplasmic bridges; Nuclear buds

Core tip: We aimed to evaluate the micronucleus, nucleoplasmic bridges and nuclear buds in the mitogen-

stimulated lymphocytes of patients with non-alcoholic steatohepatitis (NASH). Genomic instability may be a stage in the development of hepatic carcinogenesis. NASH is a major cause of so-called cryptogenic liver cirrhosis and can result in hepatocellular carcinoma (HCC). Our results support this suggestion; although none of the patients had liver cirrhosis in our study, there is high genomic instability in their mitogen-stimulated lymphocytes. Further prospective studies are needed to further clarify this topic, especially among patients with HCC, cirrhosis and NASH.

Karaman H, Karaman A, Donmez-Altuntas H, Bitgen N, Hamurcu Z, Oguz A, Karakukcu C. Investigation of genome instability in patients with non-alcoholic steatohepatitis. *World J Gastroenterol* 2013; 19(32): 5295-5301 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5295.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5295

INTRODUCTION

Non-alcoholic steatohepatitis (NASH) is an underdiagnosed liver disease characterized by steatosis and necroinflammation with hepatocyte injury (ballooning), with or without fibrosis. Non-alcoholic fatty liver (NAFL) is characterized by steatosis without inflammation and fibrosis[1]; its prevalence is 10%-30% in adults[2]. NASH is a major cause of so-called cryptogenic liver cirrhosis[3] and cause hepatocellular carcinoma[4,5].

The use of the cytokinesis-blocked micronucleus (CBMN) assay on peripheral blood lymphocytes is one of the most well-validated cytogenetic tests for measuring DNA damage, genome instability and cancer risk[6]. The CBMN assay allows once-divided cells to be recognized by their binucleated (BN) cell appearances after the inhibition of cytokinesis by cytochalasin B[7]. This method was initially proposed for the evaluation of the micronucleus (MN) in BN cells. However, the CBMN assay has more recently been considered a multipurpose test because it can analyze the proliferation index (a measure of cytostasis), cell death (a measure of cytotoxicity) and DNA damage[8-10]. It is often called a cytome assay[11,12]. The events of DNA damage are scored specifically in once-divided BN cells. The frequency of BN cells with MN, nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) provides a measure of genome instability or DNA damage. MN is formed through different processes, such as chromosome breakage or complete chromosome loss that lags behind anaphase in cell division. NPBs originate from asymmetrical chromosome rearrangements and/or telomere end fusions. NBUDs are considered biomarkers of gene amplification[9,12].

In this study, our objective was to determine the spontaneous number of MN, NPBs and NBUDs in the phytohemagglutinin (PHA)-stimulated lymphocytes of patients with NASH.

MATERIALS AND METHODS

Patients and controls

This study was conducted between August 2012 and September 2012 in Kayseri Educational and Research Hospital Department of Gastroenterology. Written informed patient consent was obtained from each patient before the procedure, and the study was approved by the Ethics Committee of Kayseri Educational and Research Hospital. The study was performed on 25 (9 females, 16 males) patients newly diagnosed with NASH and on 25 healthy controls of similar ages and genders. None of the participants was known to be receiving any drugs for medical or other reasons or using alcohol. In addition, hepatosteatosis was excluded by abdominal ultrasound imaging in the control group.

Inclusion and exclusion criteria

Each subject in the NASH group had a history of chronic serum alanine aminotransferase (ALT) elevation, which was defined as an ALT > 40 U/L that occurred on two separate occasions separated by at least 3 mo (90 d). No patients or control subjects had an alcohol habit. The subjects also underwent a work-up for other causes of chronic hepatitis of unknown etiology, including serological evaluation for alpha-1-antitrypsin, hepatitis B surface antigen, hepatitis C antibody, copper, ceruloplasmin, antinuclear antibody, anti-smooth muscle antibody, anti-liver kidney microsomal antibody, and total immunoglobulin G. None of the patients, controls, or any of their first degree relatives had diabetes mellitus. Subsequently, the NASH subjects underwent a standard-of-care liver biopsy to identify the etiology and severity of NASH. To be included in the study, the biopsy had to show macrovesicular fat in a minimum of 5% of the hepatocytes, the absence of other etiologies identifying the presence of fat, and a pattern of injury consistent with NASH, as determined by a pathologist[13].

Our patients and control subjects were asked about and examined for conditions affecting MN frequency, including malnutrition, occupational or environmental exposure to known genotoxic agents, smoking, and tea or coffee drinking. None of the patients were receiving medication.

Body mass index (BMI), homeostasis model assessment insulin resistance (HOMA-IR), aspartate aminotransferase (AST), ALT, triglyceride (TG), low density lipoprotein (LDL), high density lipoprotein (HDL), hemoglobin (Hb) and creatinin were measured or calculated for both groups.

Lymphocyte cultures for CBMN assay

Three milliliter blood samples were collected in heparinized tubes from the antecubital vein after informed consent had been obtained from all patients and control subjects. Approximately 0.4 mL of heparinized whole blood samples was cultured for 72 h at 37 ℃ in 5 mL of

Table 1 Demographic and laboratory parameters of the patient and control groups

Parameter	*n*	mean	SD	*P*
Age (yr)				0.977
Patient	25	41.92	13.33	
Control	25	41.80	13.09	
BMI (kg/m^2)				0.001
Patient	25	31.19	4.62	
Control	25	25.07	4.14	
HOMA-IR				0.001
Patient	25	6.71	4.68	
Control	25	1.40	0.53	
Hb (g/dL)				0.80
Patient	25	14.73	1.49	
Control	25	14.64	1.30	
Creatinine (mg/dL)				0.12
Patient	25	0.74	0.15	
Control	25	0.80	0.13	
AST (U/L)				0.001
Patient	25	56.08	29.11	
Control	25	16.88	3.33	
ALT (U/L)				0.001
Patient	25	92.20	41.43	
Control	25	15.88	5.88	
TG (mg/dL)				0.001
Patient	25	219.21	141.68	
Control	25	102.56	57.98	
HDL (mg/dL)				0.52
Patient	25	46.37	9.65	
Control	25	48.72	15.31	
LDL (mg/dL)				0.02
Patient	25	136.75	30.14	
Control	25	114.63	34.13	

BMI: Body mass index; HOMA-IR: Homeostasis model assessment insulin resistance; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TG: Triglyceride; HDL: High density lipoprotein; LDL: Low density lipoprotein; Hb: Hemoglobin.

peripheral blood karyotyping medium that was supplemented with 1.5% phytohemagglutinin-M to stimulate T-lymphocytes (all from Biological Industries, Kibbutz Beit Haemek, Israel).

In our study, two parallel cultures were prepared simultaneously for each patient and control subject to determine their intra-individual differences. Different slides of two parallel cultures were prepared[14].

Forty-four hours after the initiation of the cultures, the cells were blocked from entering cytokinesis by the addition of cytochalasin-B to each culture tube at a final concentration of 3 μg/mL (Sigma-Aldrich)[14]. The cultures were stopped at 72 h after initiation, treated with hypotonic solution (0.1 mol/L KCl) for 4 min and fixed in two changes of methanol:acetic-acid (3:1)[15]. The fixed cells were spread onto glass slides and stained with 5% Giemsa (Merck) in Sorensen's buffer for 10 min.

CBMN cytome assay

Different slides of two parallel cultures from each patient and control subject were prepared and evaluated. All slides were evaluated blindly using a Nikon Alphaphot-2 light optical microscope. For each sample (patient and control subject), 1000 BN cells were scored for the numbers of micronucleus (MN), NPBs, and nuclear buds (BUDs) in the lymphocytes of the patients and control subjects. The published criteria for the determinations of BN cells, MN, NPBs and NBUDs were followed[12].

Statistical analysis

Statistical comparisons of the number of BN cells with MN, NPBs, NBUDs and the ages of the patients with NASH with those of the control subjects were performed using a non-parametric Mann-Whitney *U* test for two independent samples. Spearman's rho correlation analysis was used to determine the relationships among age and the numbers of MN, NPBs and NBUDs.

RESULTS

The mean ages of the patients and control group were 41.92 ± 13.33 and 41.80 ± 13.09 years, respectively ($P > 0.05$). The demographic and laboratory parameters of the patient and control groups are shown in Table 1. The total numbers and frequencies of BN cells with MN, NPBs and NBUDs scored using a CBMN cytome assay in PHA-stimulated lymphocytes from patients with NASH are shown in Table 2, and those of the control group are shown in Table 2. We found significantly higher numbers of MN, NPBs and NBUDs in the BN cells of patients with NASH than in those of the control subjects (21.60 ± 9.32 *vs* 6.88 ± 3.91; 29.28 ± 13.31 *vs* 7.84 ± 3.96; 15.60 ± 5.55 *vs* 4.20 ± 1.63, respectively, $P < 0.0001$) (Table 3).

DISCUSSION

In the present study, the numbers of MN, NPBs and NBUDs in the lymphocytes of patients with NASH showed a significant increase compared to the control group. Considering the lack of data in the literature related to our values obtained for MN, NPBs and NBUDs, it was not possible to make direct comparisons with other studies. However, the formation of nuclear anomalies, including MN, NPBs and NBUDs, have previously been reported as events commonly observed in the early stages of carcinogenesis[6]. Therefore, we believe that the increased presence of DNA damage biomarkers, including MN, NPBs and NBUDs, in the lymphocytes of patients with NASH may be associated with an increased risk of developing liver cancer.

Oxidative stress, genetic defects in cell cycle checkpoints, defects in DNA repair genes or environmental/dietary factors can each cause the formation of MN *via* chromosomal rearrangements, altered gene expressions or aneuploidy, all of which are associated with a chromosome instability phenotype that is observed primarily in cases of cancer[16,17].

Some previous studies have discussed the association between the induction of MN and the development of cancer. In untreated cancer patients and in subjects with cancer-prone congenital diseases, such as Bloom

Table 2 Total numbers and frequencies of binucleated cells with micronucleus, nucleoplasmic bridges and nuclear buds scored using the cytokinesis-blocked micronucleus cytome assay in phytohemagglutinin-stimulated lymphocytes from patients with non-alcoholic steatohepatitis and the control subjects

ID	Age (yr)	Sex	No. of MN in BN cells[1]	Distribution of BN cells with					No. of BN cells with NPBs	No. of BN cells with NBUDs
				1MN	2MN	3MN	4MN	5MN		
Patients with non-alcoholic steatohepatitis										
1	45	M	19	15	-	-	1	-	25	19
2	60	M	24	19	1	1	-	-	46	11
3	50	M	33	24	1	1	2	-	33	22
4	66	M	41	39	1	-	-	-	20	22
5	22	M	8	8	-	-	-	-	32	17
6	43	M	33	29	2	-	-	-	25	14
7	36	F	14	14	-	-	-	-	21	17
8	51	F	9	9	-	-	-	-	16	10
9	35	M	21	16	-	-	-	1	25	14
10	42	M	11	11	-	-	-	-	12	15
11	30	F	28	24	2	-	-	-	50	20
12	22	M	11	7	2	-	-	-	17	15
13	31	M	12	10	1	-	-	-	21	16
14	41	M	15	13	1	-	-	-	18	25
15	33	M	10	10	-	-	-	-	30	10
16	44	F	22	17	1	1	-	-	58	21
17	47	M	20	13	2	1	-	-	40	17
18	20	M	25	16	1	1	-	1	14	10
19	22	M	24	20	-	-	1	-	32	8
20	46	F	37	27	2	2	-	-	25	10
21	68	F	36	26	5	-	-	-	63	28
22	48	M	17	15	1	-	-	-	28	6
23	60	M	22	18	-	-	1	-	36	19
24	45	F	24	20	2	-	-	-	25	14
25	41	M	24	19	1	1	-	-	20	10
The control subjects										
1	45	M	5	5	-	-	-		11	3
2	60	M	8	6	1	-	-		10	5
3	50	M	7	7	-	-	-		6	5
4	66	M	11	9	1	-	-		12	6
5	22	M	2	2	-	-	-		2	4
6	43	M	6	4	1	-	-		1	4
7	36	F	3	3	-	-	-		9	4
8	51	F	9	5	2	-	-		5	3
9	35	M	4	2	1	-	-		4	1
10	42	M	6	6	-	-	-		7	5
11	30	F	8	8	-	-	-		1	4
12	22	M	2	2	-	-	-		8	4
13	31	M	2	2	-	-	-		10	5
14	41	M	7	5	1	-	-		16	8
15	33	M	7	7	-	-	-		12	6
16	44	F	11	8	-	1	-		5	6
17	47	M	9	7	1	-	-		4	2
18	20	M	1	1	-	-	-		8	4
19	22	M	4	4	-	-	-		9	1
20	46	F	11	7	2	-	-		8	3
21	65	F	17	15	1	-	-		9	5
22	48	M	12	10	1	-	-		14	6
23	60	M	6	6	-	-	-		13	3
24	45	F	11	9	1	-	-		5	3
25	41	M	3	3	-	-	-		7	5

The numbers of micronucleus (MN), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) were scored on 1000 binucleated (BN) cells per subject. [1]Total number of MN: (1MNX1) + (2MNX2) + (3MNX3) + (4MNX4) + (5MNX5). M: Male; F: Female.

Syndrome, an increased frequency of MN has been shown[16,18]. Clinical chemoprevention trials on oral premalignancies have used MN in the oral mucosa as a surrogate endpoint of cancer[19,20]. Another piece of corroborating evidence concerning the association between the MN frequency and the development of cancer is the correlation between genotoxic MN-inducing agents, such as ionizing and ultraviolet radiation, and carcinogenesis[21,22].

Bonassi *et al*[6] evaluated the MN frequency in a total of 6718 subjects selected from the database of Human

Table 3 The numbers of micronucleus, nucleoplasmic bridges and nuclear buds in phytohemagglutinin-stimulated lymphocytes from patients and controls (means ± SD)

Group	Age (yr)	No. of MN in BN cells	No. of BN cells with NPBs	No. of BN cells with NBUDs
Patients (n = 25)	41.92 ± 13.33	21.60 ± 9.32	29.28 ± 13.31	15.60 ± 5.55
Controls (n = 25)	41.80 ± 13.09	6.88 ± 3.91	7.84 ± 3.96	4.20 ± 1.63
P value	0.977	< 0.0001[1]	< 0.0001[1]	< 0.0001[1]
Z value	0.029	5.413	5.953	6.016

The numbers of micronucleus (MN), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) were scored on 1000 binucleated (BN) cells per subject. [1]Patients with non-alcoholic steatohepatitis exhibited statistically higher numbers of MN, NPBs and NBUDs in 1000 BN cells than controls, according to the two-tailed nonparametric Mann-Whitney *U*-test for the comparison of the means of independent variables.

Micronucleus Projects, and they followed the subjects for cancer incidence or mortality. After a median duration for follow-up of 8 years, 219 incident cancers and 56 cancer deaths were detected. The subjects in the medium/high MN frequency groups demonstrated a significant correlation between overall cancer incidence and MN frequency (the P values were 0.001 and 0.03, respectively). The risks associated with specific cancer sites were also tested. All cancer sites except for the hepatobiliary and pancreas primaries (RR = 0.163; 0.27-1.44) were shown to have higher relative risks in the medium/high MN groups. The most prominent risk increase was found for bladder and kidney cancers (RR = 8.23; 1.08-63.0). The group concluded that MN frequency in PBL is predictive of cancer risk[6].

The MN technique provides a convenient and reliable index of both chromosome breakage and chromosome loss[18,23]. No studies have been conducted regarding MN formation in the lymphocytes of patients with NASH. In our study, a significant increase in the number of MN was found in the stimulated lymphocytes of patients with NASH. These results strongly support the theory that genomic impairment is elevated in the lymphocytes of patients with NASH.

In addition, the number of MN may be related to other factors, such as micronutrients (folate and riboflavin concentration), occupational or environmental exposure, genetic polymorphisms, lifestyle, smoking and tea or coffee drinking[24-26]. Our patients and control subjects were free from any conditions affecting their MN frequency, such as malnutrition, occupational or environmental exposure. The smoking, tea and coffee habits of the patients and control subjects were similar.

It has been reported that patients who are affected by familiar cutaneous malignant melanoma[27] or cancer-prone congenital diseases, *e.g.*, Bloom syndrome or ataxia telangiectasia, have abnormally high MN frequencies[28]. Moreover, Karaman *et al*[29] observed a significant increase in the MN levels of the lymphocytes of patients with colorectal adenocarcinomas and neoplastic polyps. Hamurcu *et al*[15] showed a clear increase in the frequency of MN in the peripheral lymphocytes of untreated cancer patients. In our previous study, we reported high MN, NPB and NBUD ratios in patients with ulcerative colitis[30]. Additionally, increased MN frequency has been reported in patients with diseases with high cancer risks, such as acromegaly[31] and polycystic ovary syndrome[32].

There are some reports about NASH-related hepatocellular carcinoma (HCC)[4,33-35]. Takuma *et al*[36] reviewed the literature and reported 105 cases (11 of them were their patients) of NASH-associated HCC. They reported that patients with non-cirrhotic NASH may be a high-risk group for HCC. Our results support this suggestion; although none of the patients had liver cirrhosis in our study, there was high genomic instability in their mitogen-stimulated lymphocytes.

Further studies are required to understand the importance of MN, NPBs and NBUDs on NASH-related genomic damage and hepatocellular carcinoma.

COMMENTS

Background

Non-alcoholic steatohepatitis (NASH) is an underdiagnosed liver disease characterized by steatosis and necroinflammation with hepatocyte injury (ballooning), with or without fibrosis. NASH is a major cause of so-called cryptogenic liver cirrhosis and can result in hepatocellular carcinoma. The use of the cytokinesis-blocked micronucleus assay on peripheral blood lymphocytes is one of the most well-validated cytogenetic tests for measuring DNA damage, genome instability and cancer risk. Authors evaluated the risk of genomic instability in patients with NASH in this study.

Research frontiers

The micronucleus (MN) technique provides a convenient and reliable index of both chromosome breakage and chromosome loss. The technique is simple and inexpensive, but it provides important knowledge about genomic instability and DNA damage.

Innovations and breakthroughs

This is the first study that investigates DNA damage in patients with NASH using this method.

Applications

Patients with NASH show genomic instability, but further studies investigating genomic instability in patients with cirrhosis and hepatocellular carcinoma are necessary.

Terminology

MN is formed through several different processes, such as chromosome breakage or complete chromosome loss, that lag behind anaphase in cell division. Nucleoplasmic bridges originate from asymmetrical chromosome rearrangements and/or telomere end fusions; nuclear buds are considered biomarkers of gene amplification.

Peer review

This is a good study that investigates the micronucleus frequency in patients with NASH. A suggestion to the authors is that the patients with high micronucleus ratios should be followed to observe whether they develop hepatocellular carcinoma.

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BRIEF ARTICLE

High level of preoperative carbohydrate antigen 19-9 is a poor survival predictor in gastric cancer

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Abstract

AIM: To assess the clinical significance and the prognostic value of preoperative serum carbohydrate antigen 19-9 (CA 19-9) level in gastric cancer.

METHODS: Between January 2005 and December 2006, 1960 patients underwent surgery for histologically confirmed gastric cancer. Of these, 163 patients had elevated serum levels of CA 19-9 preoperatively, and 1628 patients had normal serum levels of CA 19-9 preoperatively. For this study, 325 patients were selected from the group of 1628 patients by age, sex, and cancer stage to serve as controls. Statistically significant differences in survival rates were calculated using the log-rank test. A P value less than 0.05 was considered statistically significant and was determined using SAS software.

RESULTS: The baseline characteristics showed some differences between the two groups with regard to histology. Overall survival (OS) in the elevated and non-elevated group was 37.90 and 68.67 mo, respectively (P < 0.001). N stage (P = 0.001) was a significant predictor of disease-free survival by multivariate analysis. Also, N stage (P < 0.001), and the presence of peritoneal metastasis (P < 0.001) remained independent factors in predicting OS by multivariate analysis. Additionally, preoperative serum CA 19-9 levels were significantly associated with OS in univariate (P = 0.009) and multivariate (P = 0.021) analyses.

CONCLUSION: Serum CA 19-9 can be considered an independent prognostic factor in predicting OS in patients anticipating surgery for gastric cancer.

Key words: Gastric cancer; Carbohydrate antigen 19-9; Disease-free survival; Overall survival

Core tip: The exact functions of preoperative carbohydrate antigen (CA) 19-9 in stomach cancer have yet to be uncovered. We sought to assess the clinical significance of preoperatively high levels of CA 19-9 in patients with gastric cancer and aimed to investigate the relationship between serum levels of CA 19-9 and disease-free survival and overall survival (OS). We conclude that OS in gastric cancer patients with elevated CA 19-9 levels was lower than that in patients with non-elevated levels. Serum CA 19-9 can be considered an independent prognostic factor in predicting OS in patients anticipating surgery for gastric cancer.

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INTRODUCTION

Gastric cancer is one of the most common malignancies and the cause of many cancer-related deaths worldwide. Although a single tumor marker is limited for the diagnosis of cancer, it can be used in various clinical aspects, including assessment of clinical status, monitoring of treatment response, prediction of prognoses, and as a surveillance marker for recurrence[1-6]. Carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 19-9 are tumor markers that are commonly used for the early diagnosis and prognostic evaluation of gastric cancer[7-9], potentially reflecting tumor biology[10]. Additionally, the relatively new marker cancer antigen, CA 72-4 provides prognostic information in gastric cancer[11,12].

Recent clinical studies have shown that CEA and CA 19-9 are recognized as poor prognostic factors for gastric cancer[13-15] and are related to its recurrence[6,14]. The prognostic relevance of such tumor markers in patients with gastric cancer is not comparable with those markers used in other carcinomas[5,16-18]. Specifically, CA 19-9 has been reported to be elevated in certain forms of gastric cancer[11,19,20]. However, because little research on the prognoses of gastric cancer patients with elevated preoperative CA 19-9 levels has been performed, the clinical significance of preoperative CA 19-9 levels has not been fully verified[5,13].

Therefore, it is important to interpret the prognostic value of CA 19-9 levels in patients with gastric cancer, especially in patients anticipating surgery for postoperative survival. Thus, we sought to assess the clinical significance of preoperatively high levels of CA 19-9 in patients with gastric cancer and aimed to investigate the relationship between serum levels of CA 19-9 and disease-free survival (DFS) and overall survival (OS).

MATERIALS AND METHODS

Patients

Between January 2005 and December 2006, 1960 patients underwent surgery for histologically confirmed gastric cancer at Severance Hospital, Seoul, South Korea. Sixty-nine patients who did not have preoperative serum CA 19-9 were excluded. Of the remaining 1891, 163 patients had elevated serum levels of CA 19-9 preoperatively, and 1628 patients had normal serum levels of CA 19-9 preoperatively. For this study, 325 patients were selected from the group of 1628 patients by age, sex, and cancer stage to serve as controls. A separate group of 488 patients who received surgery as a treatment modality for confirmed gastric cancer was included and analyzed retrospectively in this study.

Classification of gastric cancers

The endoscopic findings of early gastric cancer were classified according to the criteria of the Japanese Research Society for Gastric Cancer (JRSGC) as follows: elevated (types I or ⅡA), flat (type ⅡB), depressed (types ⅡC, ⅡC + Ⅲ, or ⅡA + ⅡC), and mixed. Advanced gastric cancers were categorized according to Borrmann's classification. Histological evaluation was performed according to the Japanese General Rules for Gastric Cancer Study in Surgery and Pathology from the JRSGC[21].

In addition, patients were classified into three groups, which were based on the location of the primary lesion: upper third, middle third, and lower third. The upper third-designated cancer developed in the gastric cardia and fundus, the middle third-designated cancer developed in the gastric body, and the lower third-designated cancer was found in the antrum and pylorus[22].

Treatment modalities

Surgical treatments were considered curative and palliative according to the Union for International Cancer Control criteria[23]. The standard surgical treatment was radical total or subtotal gastrectomy with D2 lymph node dissection in accordance with JRSGC rules[21]. Curative resection (R0) was defined as the absence of tumor either macroscopically or microscopically after surgery. In selected inoperable cases, palliative gastrectomy was performed when necessary.

Initial work-up and follow-up

A follow up period was started on January 1st, 2005 and ended on August 22th, 2008. Initial evaluation included complete medical history and physical examination, paying special attention to symptoms often associated with stomach cancer. Chest radiography and laboratory tests were performed, including complete blood cell count, blood urea nitrogen and creatinine levels, and liver function tests. Serum CA 19-9 concentrations were measured using a commercial chemiluminescent enzyme immunoassay with a normal upper limit of 37 U/mL[24]. Serum CA 19-9 levels were routinely measured immediately before surgery. The entire study population underwent esophagogastroduodenoscopy and computed tomography of the abdomen. After surgery, esophagogastroduodenoscopy, computed tomography of the abdomen and laboratory tests performed during the initial work-up were repeated at each follow-up visit.

Statistical analysis

This study was based on matched pair data considering age, sex and cancer stage. In the mixed model, comparisons between patients with elevated CA 19-9 levels and those with normal levels based on age, sex, cancer stage, and survival were performed. Categorical data were evaluated by the χ^2 test or Fisher's exact test, and all continuous variables were expressed as the median (range) and analyzed using the Mann-Whitney *U* test. Multivariate analysis of survival was performed using the Cox proportional hazards model. OS was defined from the date of surgery until death or the date of last follow-up. DFS was defined as the interval from the operation date to the date of confirming recurrence, death from any cause other than cancer, or last visiting date. Paired Kaplan-Meier

Table 1 Baseline characteristics with a comparison of patients with elevated serum carbohydrate antigen 19-9 levels and normal serum carbohydrate antigen 19-9 levels

Characteristic	Elevated group (*n* = 163)	Non-elevated group (*n* = 325)	*P* value
Sex			0.963
Male	111 (68.1)	222 (68.3)	
Female	52 (31.9)	103 (31.7)	
Mean age, yr (range)	60.70 ± 12.00 (29-84)	59.71 ± 12.40 (27-85)	0.295
Number of lesions			0.436
Single	145 (89.0)	281 (86.5)	
Multiple	18 (11.0)	44 (13.5)	
Size (cm)			
Horizontal	6.38 ± 3.51	5.78 ± 3.16	0.060
Vertical	5.15 ± 2.79	4.66 ± 2.60	0.053
Endoscopic findings			0.135
EGC			
Elevated	10 (6.1)	23 (7.1)	
Flat	3 (1.8)	15 (1.6)	
Depressed	10 (6.1)	18 (5.5)	
AGC			
Borrmann Ⅰ	9 (5.5)	8 (2.5)	
Borrmann Ⅱ	25 (15.3)	38 (11.7)	
Borrmann Ⅲ	89 (54.6)	203 (62.5)	
Borrmann Ⅳ	17 (10.4)	20 (6.2)	
Histology			0.033
Well differentiated	19 (11.7)	23 (7.1)	
Moderately differentiated	57 (35.0)	86 (26.5)	
Poorly differentiated	54 (33.1)	145 (44.6)	
Signet ring cell cancer	33 (20.3)	71 (21.9)	
Location			0.404
Lower	108 (66.3)	210 (61.6)	
Middle	16 (9.8)	49 (15.1)	
Upper	31 (19.0)	52 (16.0)	
Diffuse	8 (1.9)	14 (4.3)	
T stage			0.843
T0-2	55 (36.7)	117 (37.6)	
T3, 4	95 (63.3)	194 (62.4)	
N stage			0.908
N0, 1	79 (52.7)	162 (52.1)	
N2, 3	71 (47.3)	149 (47.9)	
Mean metastatic Lymph nodes (N)	9.88 ± 13.03	10.09 ± 11.94	0.893
TNM stage			> 0.999
0	1 (0.6)	2 (0.6)	
Ⅰ	26 (16.0)	52 (16.0)	
Ⅱ	21 (12.9)	42 (12.9)	
Ⅲ	57 (35.0)	114 (35.1)	
Ⅳ	58 (35.6)	115 (35.4)	
Operation			0.114
For radical	139 (85.3)	293 (90.2)	
For palliative	24 (14.7)	32 (0.8)	
Lymphovascular invasion			0.225
Positive	98 (76.0)	190 (70.1)	
Negative	31 (24.0)	81 (29.9)	
Peritoneal metastasis			0.899
Positive	10 (6.1)	19 (5.8)	
Negative	153 (93.9)	306 (94.2)	
Hepatic metastasis			0.853
Positive	5 (3.1)	11 (3.4)	
Negative	158 (96.9)	314 (96.6)	
Neoadjuvant chemotherapy			0.217
Positive	6 (3.7)	6 (1.8)	
Negative	157 (96.3)	319 (98.2)	
Mean serum CA 19-9 (range)	575.74 ± 518.09 (37.4-12800)	8.45 ± 8.42 (0-36.8)	< 0.001
Mean serum CEA (range)	6.00 ± 21.86 (0.01-260.27)	5.49 ± 17.30 (0.01-189.21)	0.777
Mean serum CA 72-4 (range)	9.31 ± 20.52 (0.33-164)	14.42 ± 68.61 (0.2-600)	0.376

Data are expressed as mean ± SD or *n* (%). AGC: Advanced gastric cancer; CA: Carbohydrate antigen; CEA: Carcinoembryonic antigen; EGC: Early gastric cancer; TNM: Tumor-node-metastasis.

curves and Cox regression analyses using robust standard error for survival analysis were performed. Statistically significant differences in survival rates were calculated using the log-rank test. A *P* value less than 0.05 was considered statistically significant and was determined using SAS software (version 9.1.3, SAS Institute Inc., Cary, NC, United States).

RESULTS

Patient characteristics

We compared the outcomes and clinicopathologic characteristics of 163 patients with elevated preoperative serum CA 19-9 levels (elevated group) with those of 325 patients with non-elevated preoperative serum CA 19-9 levels (non-elevated group), which are summarized in Table 1. Baseline characteristics did not show a significant statistical relationship between the two groups except for histology and serum CA 19-9 levels, which revealed a significantly higher proportion of less differentiated adenocarcinoma in patients with elevated preoperative serum CA 19-9 levels and mean serum CA 19-9 values of 575.74 ± 518.09 U/mL in the elevated group and 8.45 ± 8.42 U/mL in the non-elevated group. However, there were no significant differences in baseline characteristics with regard to other variables, such as sex, age, endoscopic findings, and other serum tumor markers. In 56 patients, surgery was performed as a palliative treatment. Of these, gastrojejunostomy was performed for bypass in 27 patients. Hepatic and peritoneal metastases were appraised by radiological findings, histological examination, and/or intraoperative observation.

Survival outcome according to preoperative CA 19-9 levels

The median OS was 58.433 mo (95%CI: 43.07-70.90). A significantly longer median OS was observed in the non-elevated group than in the elevated group (68.67 mo *vs* 37.90 mo, 95%CI: 25.07-56.13; $P < 0.001$; Figure 1A). Because the majority of patients died near the end of the study, the upper limit of the confidence interval in the non-elevated group was not calculated.

As survival curves for DFS did not reach 50% after Kaplan-Meier analysis, a median DFS was not defined. A longer DFS was seen in the non-elevated group than in the elevated group, but the difference was not statistically significant ($P = 0.099$; Figure 1B).

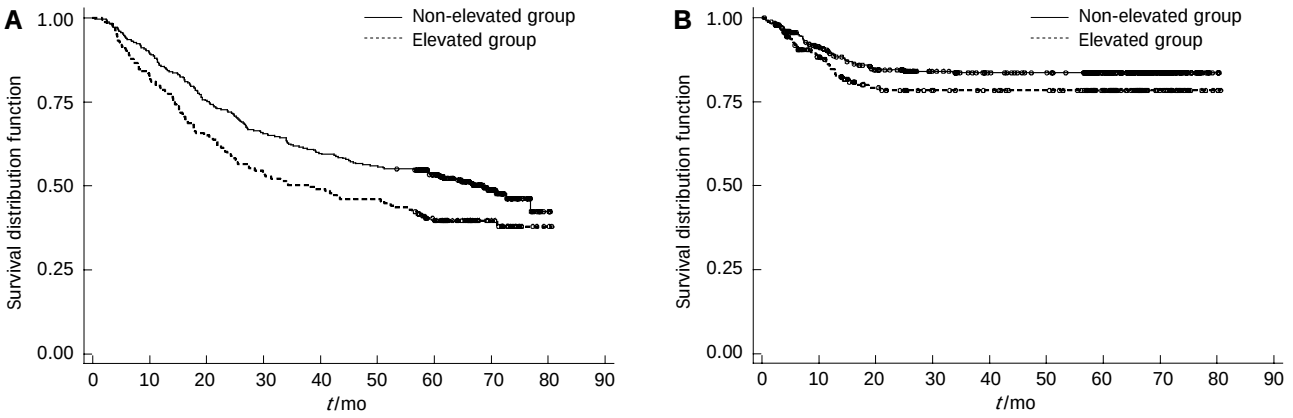

Figure 1 Kaplan-Meier curves. A: Overall survival, *P*-value by log-rank test < 0.001; B: Disease free survival in patients with elevated serum carbohydrate antigen 19-9 (CA 19-9) levels (*n* = 163) and those with normal serum CA 19-9 levels (*n* = 325), *P*-value by log-rank test = 0.099.

Table 2 Univariate and multivariate analysis of factors associated with disease-free survival

Variable	Univariate analysis		Multivariate analysis	
	Hazard ratio (95%CI)	*P* value	Hazard ratio (95%CI)	*P* value
Disease-free survival				
Age (< 60 *vs* ≥ 60 yr)	0.737 (0.475-1.143)	0.737	0.620 (0.379-1.015)	0.053
Sex (male *vs* female)	1.140 (0.720-1.806)	0.575	0.777 (0.454-1.331)	0.359
Lesions (single *vs* multiple)	1.350 (0.746-2.446)	0.322	0.930 (0.458-1.892)	0.842
T staging (T 0-2 *vs* T 3, 4)	2.469 (1.442-4.228)	0.001	1.841 (0.961-3.527)	0.066
N staging (N 0, 1 *vs* N 2, 3)	4.069 (2.445-6.772)	< 0.001	2.993 (1.587-5.646)	0.001
Differentiation (well *vs* poorly)	1.378 (0.863-2.201)	0.179	1.419 (0.786-2.563)	0.246
Histology (adenocarcinoma *vs* signet ring cell cancer)	0.979 (0.573-1.674)	0.940	0.599 (0.315-1.142)	0.119
Lymphovascular invasion (negative *vs* positive)	3.054 (1.514-6.161)	0.002	1.15 (0.495-2.674)	0.745
Peritoneal metastasis (negative *vs* positive)	0.956 (0.301-3.034)	0.939	0.507 (0.069-3.704)	0.503
Hepatic metastasis (negative *vs* positive)	0.048 (0.000-29.505)	0.354	0.000 (0.000-1.254)	0.962
CA19-9 (< 37.0 U/mL *vs* ≥ 37.0 U/mL)	1.385 (0.883-2.172)	0.156	1.179 (0.710-1.958)	0.525
Overall survival				
Age (< 60 *vs* ≥ 60 yr)	1.047 (0.816-1.342)	0.719	1.218 (0.905-1.639)	0.193
Sex (male *vs* female)	0.979 (0.755-1.269)	0.874	0.995 (0.732-1.355)	0.977
Lesions (single *vs* multiple)	1.144 (0.804-1.626)	0.455	1.163 (0.769-1.761)	0.474
T staging (T 0-2 *vs* T 3, 4)	2.498 (1.846-3.382)	< 0.001	1.437 (0.986-2.094)	0.059
N staging (N 0, 1 *vs* N 2, 3)	3.577 (2.713-4.715)	< 0.001	2.817 (1.984-4.001)	< 0.001
Differentiation (well *vs* poorly)	1.595 (1.227-2.073)	< 0.001	1.408 (0.990-2.002)	0.057
Histology (adenocarcinoma *vs* signet ring cell cancer)	1.171 (0.880-1.560)	0.279	1.000 (0.702-1.424)	0.999
Lymphovascular invasion (negative *vs* positive)	2.527 (1.739-3.673)	< 0.001	1.004 (0.643-1.569)	0.985
Peritoneal metastasis (negative *vs* positive)	4.620 (3.098-6.6887)	< 0.001	3.213 (1.792-5.762)	< 0.001
Hepatic metastasis (negative *vs* positive)	2.294 (1.285-4.097)	0.005	2.114 (0.916-4.880)	0.079
CA19-9 (< 37.0 U/mL *vs* ≥ 37.0 U/mL)	1.395 (1.087-1.791)	0.009	1.414 (1.053-1.898)	0.021

CA: Carbohydrate antigen.

Prognostic factors

Potential prognostic variables for DFS are presented in Table 2. In univariate analysis, DFS was significantly associated with T stage ($P = 0.001$), N stage ($P < 0.001$), and the presence of lymphovascular invasion ($P = 0.002$). Other factors, including age, sex, number of lesions, differentiation, histology, peritoneal metastasis, and hepatic metastasis, were not significantly associated with DFS. Only N stage ($P = 0.001$) remained significantly linked with DFS in multivariate analysis. Neither univariate nor multivariate analyses revealed that preoperative serum CA 19-9 levels affected DFS.

Potential prognostic variables are shown in Table 2. Focusing on OS, univariate analysis demonstrated an association with T stage ($P < 0.001$), N stage ($P < 0.001$), differentiation ($P < 0.001$), the presence of lymphovascular invasion ($P < 0.001$), the presence of peritoneal metastasis ($P < 0.001$), and hepatic metastasis ($P = 0.005$). Multivariate analysis showed that N stage ($P < 0.001$), and the presence of peritoneal metastasis ($P < 0.001$) remained independent factors in predicting OS. Additionally, preoperative serum CA 19-9 levels were significantly associated with OS in univariate ($P = 0.009$) and multivariate ($P = 0.021$) analyses. Cox proportional hazards regression analysis indicated that patients with elevated levels of CA 19-9 had a 1.4-fold higher risk of worse OS

than patients with low levels of this marker.

DISCUSSION

Gastric cancer is one of the most common cancers worldwide with approximately 989600 new cases and 738000 deaths per year, accounting for approximately 8% of new cancers[25]. Thus, gastric cancer continues to be a global health problem. However, gastric cancer-specific tumor markers have not yet been identified. The tumor markers currently in use have limited clinical utility due to insufficient specificity and poor sensitivity[14,26,27].

Nevertheless, current serum tumor markers are primarily used for the preoperative staging of neoplasms, postoperative monitoring of treatment effectiveness, and early diagnosis of recurrence, as they can be easily and cost-effectively identified[28]. Specifically, tumor markers, including alpha-fetoprotein (AFP), CEA, CA 19-9, CA 50, and CA 72-4, have been reported to be elevated in certain gastric cancer patients[11,19,20]. AFP, a marker commonly used for germ cell and hepatocellular carcinoma, is elevated in AFP-producing gastric cancer, often presenting as liver metastasis and leading to a poor prognosis. However, the value of these tumor markers in gastric cancer is still controversial[29-31]. In the case of CEA, preoperative serum CEA levels have been reported to be useful for determining or predicting gastric cancer prognosis[11,32,33]. However, some authors have indicated that CEA positivity is not a prognostic factor in gastric cancer[15]. In addition, earlier studies have reported that CA 72-4 is more relevant than other tumor markers for gastric cancer, but this has not been verified[13,33-36]. Of currently used markers, CA 19-9 is known to have a positive correlation with depth of invasion, nodal involvement, and peritoneal metastasis in gastric cancer[15,17,37]. However, CA 19-9 can be elevated in endometrial, lung, breast, and pancreatic cancers as well as benign conditions, including cholecystitis, cholangitis, acute pancreatitis, and liver cirrhosis[38,39]. An association between CA 19-9 levels and prognosis has not yet been established and remains controversial[5,13]. Thus, none of these markers are used alone to diagnose, monitor disease, or predict prognosis. Although prognosis is mainly determined by tumor stage at the time of gastric cancer surgery, recent studies have assessed the usefulness of preoperative tumor marker levels to predict invasiveness and prognosis[5,6,18,32,33,40].

Therefore, we focused on the prognostic significance of high preoperative levels of CA 19-9 in patients with gastric cancer. The results of our study indicate that measurements of preoperative serum CA 19-9 levels may be useful in the prediction of survival and prognoses in patients with gastric cancer, confirming its association with OS and DFS. OS is thought to be influenced by preoperative CA 19-9 levels.

Previous studies identified the usefulness of postoperative CA 19-9 levels to predict prognosis and recurrence of gastric cancer after gastrectomy[6,37]. In many studies, however, preoperative CA 19-9 levels were neither prognostic nor were associated with survival. According to studies by Dilege *et al*[17] and Ishigami *et al*[41], preoperative CA 19-9 levels were only significantly correlated with lymph node metastasis; patient survival did not correlate with preoperative CA 19-9 levels. In addition, Ucar *et al*[15] showed that CA 19-9 was only significantly related to lymph node metastasis and peritoneal carcinomatosis, but prognosis and survival were not relevant. These authors suggested that CA 72-4 was an independent prognostic factor for risk of death in this study.

These studies did not provide any predictive information for preoperative CA 19-9 levels on prognosis or survival in gastric cancer patients. One study showed by univariate analysis that preoperative CA 19-9 levels could predict specific clinical outcomes such as DFS. Another study showed poor OS in CA 19-9-positive patients by the log-rank test[5]. However, the independent prognostic value of CA 19-9 on OS by Cox regression multivariate analysis was not shown[5]. The association between CA 19-9 levels and stage of disease, lymph node metastasis, and depth of invasion has been reported, but none have been assigned an independent prognostic value by multivariate analysis[5,16,41]. In contrast, our study showed survival outcome according to preoperative CA 19-9 levels and prognostic factors for OS and DFS. There were significant differences regarding OS between the non-elevated group and the elevated group. Preoperative CA 19-9 levels were a reliable prognostic factor for OS in our study. With respect to DFS, despite an insignificant prognostic value for CA 19-9 levels, we can see possibilities for future research, as our findings are confined to the limited data presented here.

A recent study by Jo *et al*[42] showed that an elevated CA 19-9 concentration before chemotherapy was significantly associated with shorter survival especially in metastatic or recurrent gastric cancer. The patients in this study had metastatic or recurrent gastric cancer. However, our study was designed to analyze treatment-naïve patients who were planning to undergo gastrectomy. Therefore, we have superiority and originality compared to previous studies due to the differences in the subject and focus of study.

We aimed to determine whether the preoperative tumor marker CA 19-9 could provide useful information on clinical outcome and postoperative prognosis similar to other common prognostic factors. Unlike the study by Marrelli *et al*[14], we analyzed not only 432 R0 resection cases, but also 56 palliative gastrectomy cases; of these, 27 cases of bypass surgery were also included. We cannot exclude the possibility that survival rates will appear low due to inclusion of the latter cases and influence tumor progression. These factors might affect the tumor burden and predominance of advanced cancer among marker-positive patients[18].

Our study has limitations associated with its retrospective nature, single-center design and relatively small sample numbers. In addition, preoperative CA 19-9 sampling was not performed at the same time before surgery

due to the retrospective design.

Also, analyses regarding the presence of chemotherapy or radiation therapy after surgery were not performed in this study. Twelve patients who had neoadjuvant chemotherapy were included in this study. However, for patients who had adjuvant chemotherapy or other therapies after surgery, analyses were not performed. Although neither adjuvant nor neoadjuvant chemotherapy showed any clear significant survival benefit in gastric cancer[18], these factors might be crucial in influencing survival, therefore further study is necessary. In the future, it would be interesting to measure CA 19-9 consistently during the preoperative examination before surgery and analyze the effects of additional therapies such as adjuvant chemotherapy or radiation therapy. Therefore, multi-center and prospective studies should be designed to certify the prognostic significance of CA 19-9.

We conclude that OS in gastric cancer patients with elevated CA 19-9 levels was lower than that in patients with non-elevated levels. Serum CA 19-9 can be considered an independent prognostic factor in predicting OS in patients anticipating surgery for gastric cancer.

COMMENTS

Background

Gastric cancer is one of the most common cancers worldwide with approximately 989600 new cases and 738000 deaths per year, accounting for approximately 8% of new cancers. Thus, gastric cancer continues to be a global health problem. However, gastric cancer-specific tumor markers have not yet been identified. The tumor markers currently in use have limited clinical utility due to insufficient specificity and poor sensitivity.

Research frontiers

Recent clinical studies have shown that carcinoembryonic antigen and carbohydrate antigen 19-9 (CA 19-9) are recognized as poor prognostic factors for gastric cancer and are related to its recurrence. The prognostic relevance of such tumor markers in patients with gastric cancer is not comparable with those markers used in other carcinomas. Specifically, CA 19-9 has been reported to be elevated in certain forms of gastric cancer. However, because little research on the prognoses of gastric cancer patients with elevated preoperative CA 19-9 levels has been performed, the clinical significance of preoperative CA 19-9 levels has not been fully verified.

Innovations and breakthroughs

In most current research related to preoperative CA 19-9 levels in gastric cancer, discussions on survival have only focused on overall survival (OS). The association between CA 19-9 levels and stage of disease, lymph node metastasis, and depth of invasion has been reported, but none have been assigned an independent prognostic value by multivariate analysis. This study showed survival outcome according to preoperative CA 19-9 levels and prognostic factors for OS and disease-free survival (DFS). There were significant differences regarding OS between the non-elevated group and the elevated group. Preoperative CA 19-9 levels were a reliable prognostic factor for OS in our study. With respect to DFS, despite an insignificant prognostic value for CA 19-9 levels, we can see possibilities for future research, as the findings are confined to the limited data presented here.

Applications

Prior to surgery, CA 19-9 levels could be used to predict poor prognosis and to suggest adjuvant therapies in early stages of the disease when more aggressive surgical approaches are warranted.

Terminology

Serum CA 19-9 concentrations were measured using a commercial chemiluminescent enzyme immunoassay with a normal upper limit of 37 U/mL. Serum CA 19-9 levels were routinely measured immediately before surgery.

Peer review

The authors have focused on the prognostic significance of high preoperative levels of CA 19-9 in patients with gastric cancer. The results of the study indicate that measurements of preoperative serum CA 19-9 levels may be useful in the prediction of survival and prognoses in patients with gastric cancer, confirming its association with OS and DFS. OS is thought to be influenced by preoperative CA 19-9 levels.

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BRIEF ARTICLE

Can trans-anal reinforcing sutures after double stapling in lower anterior resection reduce the need for a temporary diverting ostomy?

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Abstract

AIM: To evaluate trans-anal reinforcing sutures in low anterior resection using the double-stapled anastomosis technique for primary rectal cancers performed at a single institution.

METHODS: The data of patients who received trans-anal reinforcing sutures were compared with those of patients who did not receive them after low anterior resection. Patients who underwent laparoscopic low anterior resection and the double-stapled anastomosis technique for primary rectal cancer between January 2008 and December 2011 were included in this study. Patients with no anastomosis, a hand-sewn anastomosis, high anterior resection, or preoperative chemoradiation were excluded. The primary outcomes measured were the incidence of postoperative anastomotic complications and placement of a diverting ileostomy.

RESULTS: Among 110 patients, the rate of placement of a diverting ileostomy was significantly lower in the suture group (SG) compared with the non-suture control group (CG) [SG, *n* = 6 (12.8%); CG, *n* = 19 (30.2%), *P* = 0.031]. No significant difference was observed in the rate of anastomotic leakage [SG, *n* = 3 (6.4%); CG, *n* = 5 (7.9%)].

CONCLUSION: Trans-anal reinforcing sutures may reduce the need for diverting ileostomy. A randomized prospective study with a larger population should be performed in the future to demonstrate the efficacy of trans-anal reinforcing sutures.

Key words: Anastomotic leak; Low anterior resection; Rectal neoplasms; Double-stapled anastomotic technique; Reinforcement sutures

Core tip: We have performed trans-anal reinforcing sutures after the double-stapled anastomotic technique to intensify the anastomotic line and to reduce leakage. As a result, we found that the rate of placement of a diverting ileostomy was significantly reduced in cases of performing the trans-anal reinforcing sutures although there was no significant decrease of anastomotic leakage.

Baek SJ, Kim J, Kwak J, Kim SH. Can trans-anal reinforcing sutures after double stapling in lower anterior resection reduce the need for a temporary diverting ostomy? *World J Gastroenterol* 2013; 19(32): 5309-5313 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5309.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5309

INTRODUCTION

Anastomotic leakage is a major problem in patients who undergo rectal cancer surgery. This complication is associated with reoperation, prolonged hospital stay, and high morbidity and mortality. In addition, it can adversely in-

fluence functional and oncologic outcomes[1-4]. An anastomotic leakage rate of 2.5%-12% has been reported[5-8]. Leakage can be the result of a combination of technical, local, and systemic factors. Several risk factors, including old age, male sex, smoking, diabetes, obesity, preoperative chemotherapy, and a more distal tumor location, are associated with anastomotic leakage after rectal cancer surgery[9-12]. In particular, the technical aspects of anastomosis are also very important. Leakage rates have also been used as an indicator of surgical quality[13,14].

Since being introduced by Griffen *et al*[15] and Knight *et al*[16], the double-stapled anastomotic technique has been widely used in colorectal surgery because it allows the anastomosis to be made very low in the pelvis and preserves the anal sphincter[17]. However, this technique creates stapled corners known as "dog ears", which are made by crossing at least two staple lines and become potentially vulnerable areas[18]. The staple line may also be weakened through friction created by hard stools, increasing the risk of anastomotic failure[19].

To address these problems, various methods, such as the single-stapled, double-pursestring method, and bioabsorbable staple-line reinforcement, have been suggested[18,20]. The trans-anal reinforcing suture is another such improvement that has been proposed. We hypothesized that placing the sutures along the staple line, including the corners, can reinforce the anastomosis and reduce anastomotic leakage. Therefore, we are currently using trans-anal reinforcing sutures for low anterior resection. The aim of this study was to determine the effect of trans-anal reinforcing sutures in terms of anastomotic complications and diverting stoma placement.

MATERIALS AND METHODS

Between January 2008 and December 2011, patients who underwent rectal resection at Korea University Anam Hospital for primary rectal cancer were enrolled in this study. The patients who underwent laparoscopic low anterior resection and double stapled anastomosis and had an anastomotic line located within 5-6 cm of the anal verge where trans-anal suturing is possible were included. The exclusion criteria were as follows: intersphincteric resection and coloanal anastomosis, total abdominal colectomy and ileo-rectal anastomosis, abdominoperineal resection, Hartmann's operation, transanal resection and high anterior resection, and a history of receiving chemoradiotherapy preoperatively.

We have been utilizing trans-anal reinforcing sutures since January 2010. A schematic view of the procedure and trans-anal view are shown in Figures 1 and 2. After rectal division using an endo-linear cutter (Echelon, Ethicon), end-to-end anastomosis is performed using a circular stapler (CDH 29 mm, Ethicon), and trans-anal reinforcing sutures are used via the anal canal. Six to eight interrupted sutures are placed along the staple line circumferentially, and two corners made by crossing circular and linear staple lines are always included. An air leakage test is performed for all patients after anastomosis and

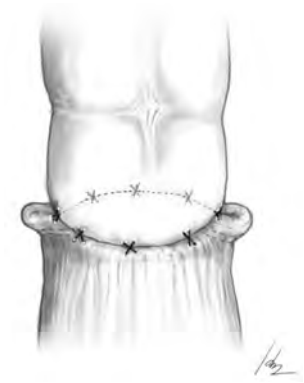

Figure 1 Schematic view of the trans-anal reinforcing sutures. Six to eight interrupted sutures are placed circumferentially along the anastomotic line located within 5-6 cm of the anal verge *via* the anal canal, including the two corners.

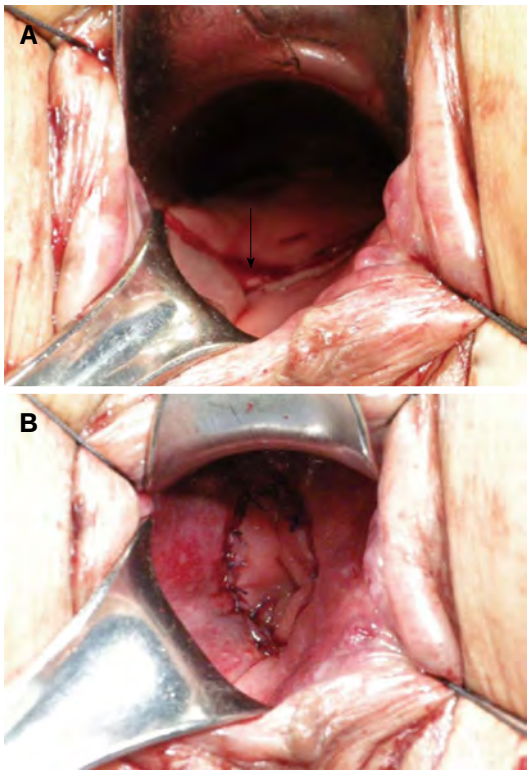

Figure 2 Trans-anal view. A: Crossing point (arrow); B: Reinforcing sutures.

trans-anal reinforcing suture, if done. Temporary diverting ileostomy is considered in cases with several operative or preoperative risk factors such as: a positive air leakage test, insufficient vascular supply at colonic section, several stapling for rectal division, incomplete circular stapling donut, underlying cardiovascular disease, rectal wall muscle injury, and stool spillage. We do not perform ostomy in all male patients.

Clinical anastomotic leakage is defined in the event of clinical symptoms of sepsis, including abdominal pain, tenderness, fever, or leukocytosis. All patients diagnosed with anastomotic leakage in this study were identified within 30 d. Clinical leakage signs were discharge of gas, pus, or feces through the abdominal drain, rectum, or vagina, fecal peritonitis, abscess at the level of the anastomosis, and fluid/air bubbles surrounding the anastomosis on computed tomography (CT). Asymptomatic anastomotic leakages were not considered because routine contrast

Table 1 Patient demographics, tumor characteristics, and operative records

	Suture group (n = 47)	Control group (n = 63)	P value
Sex			0.196
Male	29 (61.7)	31 (49.2)	
Female	18 (38.3)	32 (50.8)	
Age (yr) (range)	64.1 ± 9.8 (39-80)	61.4 ± 11.0 (42-82)	0.199
BMI (kg/m^2) (range)	24.1 ± 3.1 (18.5-33.7)	23.5 ± 2.7 (17.9-28.8)	0.272
Tumor level (cm above AV) (ranges)	9.7 ± 3.9 (2-15)	9.7 ± 3.6 (4-15)	0.974
Diverting ileostomy	6 (12.8)	19 (30.2)	0.031
Length of the operation (min) (ranges)	198.3 ± 75.7 (90-477)	212.1 ± 65.0 (75-335)	0.305
Estimated blood loss (mL) (ranges)	174.5 ± 348.0 (0-2000)	188.4 ± 301.5 (0-1500)	0.823

Data are expressed as absolute numbers (percentage) or mean ± SD. BMI: Body mass index; AV: Anal verge.

Table 2 Postoperative courses

	Suture group (n = 47)	Control group (n = 63)	P value
Flatus (d) (range)	1.5 ± 0.9 (0-4)	1.5 ± 1.2 (0-7)	0.809
Stool (d) (range)	4.1 ± 2.5 (0-10)	3.8 ± 1.7 (1-7)	0.675
Feed (d) (range)	2.8 ± 1.1 (1-6)	2.3 ± 1.8 (1-13)	0.103
Postoperative HS (d), (range)	11.0 ± 5.6 (4-36)	9.8 ± 6.7 (5-44)	0.321
Complications	4 (8.5)	7 (11.1)	0.656
Anastomotic leakage	3 (6.4)	5 (7.9)	0.759
Conservative management	1	3	
Reoperation	2	2	
Intra-abdominal bleeding	0	1 (1.6)	0.390
Postoperative ARF	1 (2.1)	1 (1.6)	0.390

Data are expressed as absolute numbers (percentage) or mean ± SD. HS: Hospital stay; ARF: Acute renal failure.

enemas were not performed after surgery. Patients who developed leakage were treated conservatively with antibiotics, received CT or ultrasonography guided drainage, or were treated with reoperation under general anesthesia.

All data were prospectively collected in a database and analyzed under the approval of the Institutional Review Board. Patient demographics, tumor characteristics, operative records, and postoperative courses were compared between patients who had trans-anal reinforcing sutures and those who did not. Statistical analysis was performed using SPSS version 12.0 (Chicago, IL). Student's t-test was used to compare continuous variables. χ^2 test was used to compare discrete variables. $P < 0.05$ was considered statistically significant.

RESULTS

In total, 110 patients underwent laparoscopic low anterior resection with double-stapled anastomosis for primary rectal cancer [47 in the suture group (SG), and 63 in the non-suture control group (CG)]. Relevant patient characteristics and surgical histories are shown in Table 1. No significant difference was observed in sex, age, or body mass index (BMI) between groups. There was also no difference in mean tumor level (9.7 cm *vs* 9.7 cm from the anal verge, $P = 0.974$), mean length of operation (198.3 min *vs* 212.1 min, $P = 0.305$) or estimated blood loss (174.5 mL *vs* 188.4 mL, $P = 0.823$) between groups. The number of temporary diverting ileostomies performed was significantly higher in the control group [SG, $n = 6$ (12.8%); CG, $n = 19$ (30.2%), $P = 0.031$].

The postoperative courses are outlined in Table 2. No significant differences were observed in the time to postoperative flatus (1.5 d *vs* 1.5 d, $P = 0.809$), stool passage (4.1 d *vs* 3.8 d, $P = 0.675$), feeding (2.8 d *vs* 2.3 d, $P = 0.103$), or postoperative hospital stay (11.0 d *vs* 9.8 d, $P = 0.321$). The incidence of anastomotic leakage, which was not significant between groups ($P = 0.759$), was 6.4% in the SG ($n = 3$) and 7.9% in the CG ($n = 5$). Two patients in each group required reoperation for anastomotic leakage, while others were treated conservatively. There were no differences in other complications between the two groups.

DISCUSSION

The occurrence of anastomotic leakage is a major concern in rectal cancer surgery. The consensus is that the main causes of anastomotic leakage are ischemia and tension. Among the risk factors for anastomotic leakage, the technical aspects of surgery are very important as they are the only known factors that may be corrected. In the double-stapled anastomotic technique, at least two staple lines cross each other, creating vulnerable corners. Some reports have concluded that the anastomotic technique used is not an important factor in anastomotic leakage, however some controversy still exists[21].

Various attempts to modify the technical aspects in order to reduce the problem of the double-stapled anastomotic technique have been attempted. Marecik *et al*[18] used the single-stapled, double pursestring technique for colorectal anastomosis in 160 patients who underwent anterior resection of the upper- or mid-rectum, which resulted in an extremely low rate of anastomotic leakage (0.6%). Mukai *et al*[22] reported good results in two cases in which trans-anal reinforcing sutures after double-stapling for lower rectal cancer were used. Gadiot *et al*[19] compared 76 patients who received anti-traction sutures and 77 who did not, and found that the need for placement of a diverting ostomy was significantly lower in patients who received sutures.

In our study, there was no significant difference in anastomotic leakage between those who received trans-anal reinforcing sutures and those who did not. How-

ever, the need for temporary diverting ileostomy was significantly lower in the suture group, which is the most important outcome in this study. Although some controversy exists as to whether or not proximal diversion affects leak rates[12,23,24], diverting ileostomy may play a role in moderating symptoms or signs of anastomotic leakage to subclinical levels. Consequently, leakage rates may be underestimated in patients who undergo diverting ileostomy. Thus, the actual rate of anastomotic leakage in the control group, which had more ileostomies, was possibly higher than presented.

Meanwhile, trans-anal reinforcing sutures could reduce the need for placement of a diverting ileostomy. It may be due to the decrease in positive air leakage although we cannot present absolute numbers because we believe that the other risk factors for anastomotic leakage were similar between groups. Less air leakage means that trans-anal reinforcing sutures can reduce potential anastomotic leakage by serving as a mechanical safety mechanism. We believe this procedure can be a useful method for the prevention of mechanical failure by reducing anastomotic tension. Therefore, the need for less ileostomy in the suture group is clinically meaningful.

In addition, this procedure can provide emotional stability to surgeons. The placement of stoma usually depends on the surgeon's subjectivity. Apart from the cases where stoma definitely need to be made, many diverting stoma are made due only to the surgeon's insecurity. Although the trans-anal reinforcing sutures may not prevent definite major anastomotic leakage or may not reduce diverting stoma made due to the evident risk, it is believed that this procedure has a positive effect in that it decreases the number of unnecessary stoma by indirectly enhancing surgeons' emotional stability.

While diverting ileostomy is an important procedure for patients at risk for anastomotic leakage, it also carries the potential for many complications and is inconvenient for patients[25-27]. Complications related to ostomy include herniation, retraction, prolapse, stenosis, stoma ischemia, mucocutaneous suture line, and skin problems such as irritant contact dermatitis, inflammatory damage, or allergic reaction. Moreover, systemic complications such as dehydration may occur. In addition, surgery is required at least once more, which can impact patient quality of life and may result in poor cosmesis[28]. Therefore, unnecessary placement of an ileostomy should be avoided. If a simple procedure such as trans-anal reinforcing sutures can reduce the incidence of ileostomies, its use should be considered.

In our results, there was no significant difference between the suture group and the non-suture group in terms of operation time as it takes about 5-0 min to perform the trans-anal reinforcing sutures. Considering that the main disadvantages of using the single-stapled technique include the extra time needed and the potential for pelvic contamination[18], the trans-anal reinforcing suture method is easy and efficacious without additional time or complexity. As this procedure is not different from the one used at the time of trans-anal excision or hemorrhoid surgery, thus it is very familiar to surgeons and a specific learning curve for it may not be necessary even in male patients with narrow pelvises. The only precaution that may need to be taken concerns a risk of vaginal fistula in cases of deep sutures of the female anterior part. This risk should be kept in mind.

Another advantage of trans-anal reinforcing sutures is that anastomotic bleeding can be prevented. Anastomotic bleeding may occur at the staple line and sometimes requires hemostasis with endoscopy or surgery. Thus, routine trans-anal inspection and suturing could aid in the detection of anastomotic bleeding and thereby prevent the increase in rectal pressure due to blood collection.

Our study has several limitations. First, there may have been selection bias in the decision to place a diverting ileostomy since the decision for ileostomy is solely the surgeon's. Our results showed that the incidence of temporary diverting ileostomy was significantly lower in the suture group. Even so, one advantage of this procedure is that it may reduce the number of unnecessary diverting ileostomies made due to the surgeon's excessive anxiety. Second, this study was not randomized, and there was a difference between the two groups when the surgeries were performed. The time difference may be the result of bias due to the surgeon's experience and may have affected the results of the procedures or the postoperative courses. However, the effects of this bias may not be significant since the surgeon performing the procedures in this study was very experienced and had performed a large volume of cases prior to the study period. Third, the sample size was relatively small. Thus, a randomized prospective study should be conducted in a larger population in the future.

In conclusion, our study demonstrates that trans-anal reinforcing sutures can be performed easily and safely in patients undergoing low anterior resection using the double-stapled anastomosis technique for primary rectal cancer. This procedure may reduce the number of diverting ileostomies performed. A prospective randomized trial is necessary to evaluate the effect of trans-anal reinforcing sutures on anastomotic leakage as well as the necessity of the placement of stomas.

COMMENTS

Background

Anastomotic leakage is a major problem in patients who undergo rectal cancer surgery. This complication is associated with reoperation, prolonged hospital stay, and high morbidity and mortality. In addition, it can adversely influence functional and oncologic outcomes. Leakage can be the result of a combination of technical, local, and systemic factors. Several risk factors, including old age, male sex, smoking, diabetes, obesity, preoperative chemotherapy, and a more distal tumor location, are associated with anastomotic leakage after rectal cancer surgery.

Research frontiers

To address these problems, various methods, such as the single-stapled, double-pursestring method, and bioabsorbable staple-line reinforcement, have been suggested. The trans-anal reinforcing suture is another such improvement that has been proposed.

Innovations and breakthroughs

This study was conducted to determine the effect of trans-anal reinforcing su-

tures in terms of anastomotic complications and diverting stoma placement.

Applications

This study demonstrates that trans-anal reinforcing sutures can be performed easily and safely in patients undergoing low anterior resection using the double-stapled anastomosis technique for primary rectal cancer.

Peer review

This paper addresses an important issue which is of interest to most surgeons. Anastomotic breakdown carries a major morbidity and mortality. Any procedure that attempts to reduce this is welcome.

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BRIEF ARTICLE

HEF-19-induced relaxation of colonic smooth muscles and the underlying mechanisms

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Abstract

AIM: To investigate the relaxant effect of chromane HEF-19 on colonic smooth muscles isolated from rabbits, and the underlying mechanisms.

METHODS: The relaxant effect and action mechanisms of HEF-19 were investigated using descending colon smooth muscle of the rabbits. Preparations 1 cm long were mounted in 15-mL tissue baths containing Tyrode's solution, maintained at 37 ± 0.5 ℃ and aerated with a mixture of 5% CO_2 in oxygen (Carbogen). The tension and amplitude of the smooth muscle strips were recorded after adding HEF-19 (10^{-6}, 10^{-5} and 10^{-4} mol/L). After cumulative administration of four antispasmodic agents, including acetylcholine chloride (Ach) (10^{-4} mol/L), histamine (10^{-4} mol/L), high-K^+ (60 mmol/L) and $BaCl_2$ (8.2 mmol/L), HEF-19 (3×10^{-7}-3×10^{-4} mol/L) was added to investigate the relaxant effect of HEF-19. $CaCl_2$ (10^{-4}-2.5×10^{-3} mol/L) was added cumulatively to the smooth muscle preparations pretreated with and without HEF-19 (1×10^{-6} or 3×10^{-6} mol/L) and verapamil (1×10^{-7} mol/L) to study the mechanisms involved. Finally, phasic contraction was induced with ACh (15×10^{-6} mol/L), and $CaCl_2$ (4×10^{-3} mol/L) was added to the smooth muscle preparations pretreated with and without HEF-19 (3×10^{-6} mol/L or 1×10^{-5} mol/L) and verapamil (1×10^{-7} mol/L) in calcium-free medium to further study the underlying mechanisms.

RESULTS: HEF-19 (1×10^{-6}, 1×10^{-5} and 1×10^{-4} mol/L) suppressed spontaneous contraction of rabbit colonic smooth muscles. HEF-19 (3×10^{-7}-3×10^{-4} mol/L) relaxed in a concentration-dependent manner colonic smooth muscle preparations pre-contracted with $BaCl_2$, high-K^+ solution, Ach or histamine with respective EC_{50} values of 5.15 ± 0.05, 5.12 ± 0.08, 5.58 ± 0.16 and 5.25 ± 0.24, thus showing a spasmolytic activity. HEF-19 (1×10^{-6} mol/L and 3×10^{-6} mol/L) shifted the concentration-response curves of $CaCl_2$ to the right and depressed the maximum response to $CaCl_2$. The two components contracted by Ach were attenuated with HEF-19 (3×10^{-6} mol/L or 10^{-5} mol/L) in calcium-free medium.

CONCLUSION: HEF-19 inhibited rabbit colonic smooth muscle contraction, probably through inhibiting opening of voltage-dependent Ca^{2+} channels. HEF-19 reduced inflow and intracellular release of Ca^{2+} ions.

Key words: Colonic smooth muscle; Smooth muscle relaxation; Ca^{2+} channels

Core tip: This is a good descriptive study in which authors found a new L-calcium-antagonist relaxing rabbit colonic smooth muscles and analyzed its possible mechanism. It provides an opportunity to search for a new drug highly selective to the gastrointestinal tract, effectively relieving pain, diarrhea and intestinal discomfort, but without significant adverse effects on irritable bowel syndrome patients.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a frequent gastrointestinal disease, characterized by a combination of several symptoms including abdominal pain or discomfort, flatulence, and problems related to bowel habits (constipation and/or diarrhea)[1]. Abnormal contraction of intestinal smooth muscle may be importantin producing the main IBS symptoms. thus, modifying the contractility is often the major aim in the treatment of IBS[2,3]. Calcium channel blockers have a good effect on IBS patients with abdominal pain and diarrhea[4].Calcium channel blockers have received increasing attention in the treatment of IBS. 3.4-Dihydro-7-[3-(diethylamino) propoxy] chroman hydrochloride (HEF-19) is a compound with a relaxant effect on colonic smooth muscles.

The present study investigated the relaxant effect of HEF-19 on isolated descending colon smooth muscle from rabbits, and the underlying mechanisms (Figure 1).

MATERIALS AND METHODS

Animals

New Zealand rabbits of either sex (2.0-2.5 kg) were obtained from the Experimental Animal Center of Shenyang Pharmaceutical University (Certificate number: SCXK20030011). All care and handling of animals were approved by the Institutional Animal Ethical Committee.

Chemicals and reagents

Normal Tyrode's solution contained: NaCl 136.86 mmol/L, KCl 2.68 mmol/L, $NaHCO_3$ 11.9 mmol/L, $MgCl_2$ 1.05 mmol/L, KH_2PO_4·H_2O 0.41 mmol/L, $CaCl_2$ 1.8 mmol/L, and glucose 5.6 mmol/L. A high-K^+ solution (KCl, 60 mmol/L) was obtained by equimolar replacement of NaCl by KCl in Tyrode's solution[5]. Ca^{2+}-free Tyrode solution was the solution in which $CaCl_2$ was omitted and ethylenediaminetetra-acetic acid (EDTA, 0.1 mmol/L) was added[6]. Ca^{2+}-free high-K^+ solution was the Ca^{2+}-free and high-K^+ Tyrode solution. All chemicals were dissolved in distilled water. All solutions were stored at 4 ℃ and fresh dilutions were made daily.

HEF-19 (> 99.5% purity) was provided by Organic Chemistry Laboratory of Shenyang Pharmaceutical University and dissolved in distilled water. KCl was from Shenyang Chemical Reagent Factory, Shenyang, China, $CaCl_2$ from Tianjin Bodi Chemical Co., Tianjin, China, $BaCl_2$ from Shenyang Xingdong Reagent Factory, Shenyang, China, verapamil injection from Tianjin Heping Pharmaceutical Plant, Tianjin, China, and acetylcholine chloride (Ach) and histamine were from Sigma, United States.

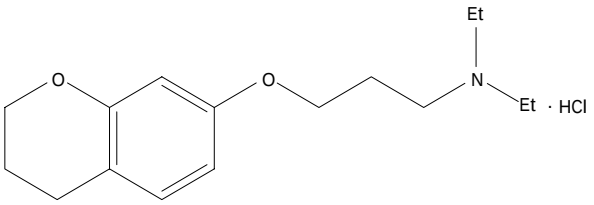

Figure 1 3.4-dihydro-7-[3-(diethylamino) propoxy] chroman hydrochloride.

Preparation of colonic smooth muscles

The animals had free access to water but were fasted for 24 h before the experiments. The animals were killed by a blow to the head. The descending colon portion was isolated, washed, and freed from the mesentery. Preparations 1 cm long were mounted in 15-mL tissue baths containing Tyrode's solution maintained at 37 ± 0.5 ℃ and aerated with a mixture of 5% CO_2 in oxygen. A preload of 3 g was applied and the tissues were kept undisturbed for an equilibrium period of 60 min. During that time, the nutrient solution was changed every 20 min. Changes in isometric tension were measured with a force-displacement transducer (Chengdu Instrument Plant, Chengdu, China) and recorded by an RM6240B Multichannel Physiological Signal Collection and Handling System (Chengdu Instrument Plant)[7].

Effect of HEF-19 on spontaneous contraction of rabbit descending colon

The normal tension and amplitude of the descending colonic smooth muscle strips were recorded after the contraction reached a stable plateau. HEF-19 (1×10^{-6}, 1×10^{-5} and 1×10^{-4} mol/L) and vehicle were added to the tissue baths containing Tyrode's solution.

Relaxant effect of HEF-19 on contraction induced by $BaCl_2$, high-K^+ solution, Ach or histamine

The isolated colon smooth muscle preparations were contracted with Ach (1×10^{-4} mol/L), histamine (1×10^{-4} mol/L) High-K^+ (60 mmol/L) or $BaCl_2$ (8.2 mmol/L), after the contraction reached a stable plateau, and cumulative concentrations of HEF-19 (3×10^{-7} mol/L-3×10^{-4} mol/L) were added. The relaxant effect was expressed as a percentage of relaxation and the EC_{50} (concentration to produce a 50% maximal relaxation) was calculated using a multichannel physiological system.

Inhibition of $CaCl_2$-induced cumulative contractions

The isolated preparations were allowed to stabilize in normal Tyrode's solution and were replaced with Ca^{2+}-free Tyrode's solution for 30 min, and then K^+-rich and Ca^{2+}-free Tyrode's solution. After 15 min incubation, Ca^{2+} was added in a cumulative fashion (1×10^{-4}-2.5×10^{-3} mol/L) to obtain control concentration-response curves. The results were expressed as the percentage of the maximum contractile tension to $CaCl_2$ before and after pretreatment

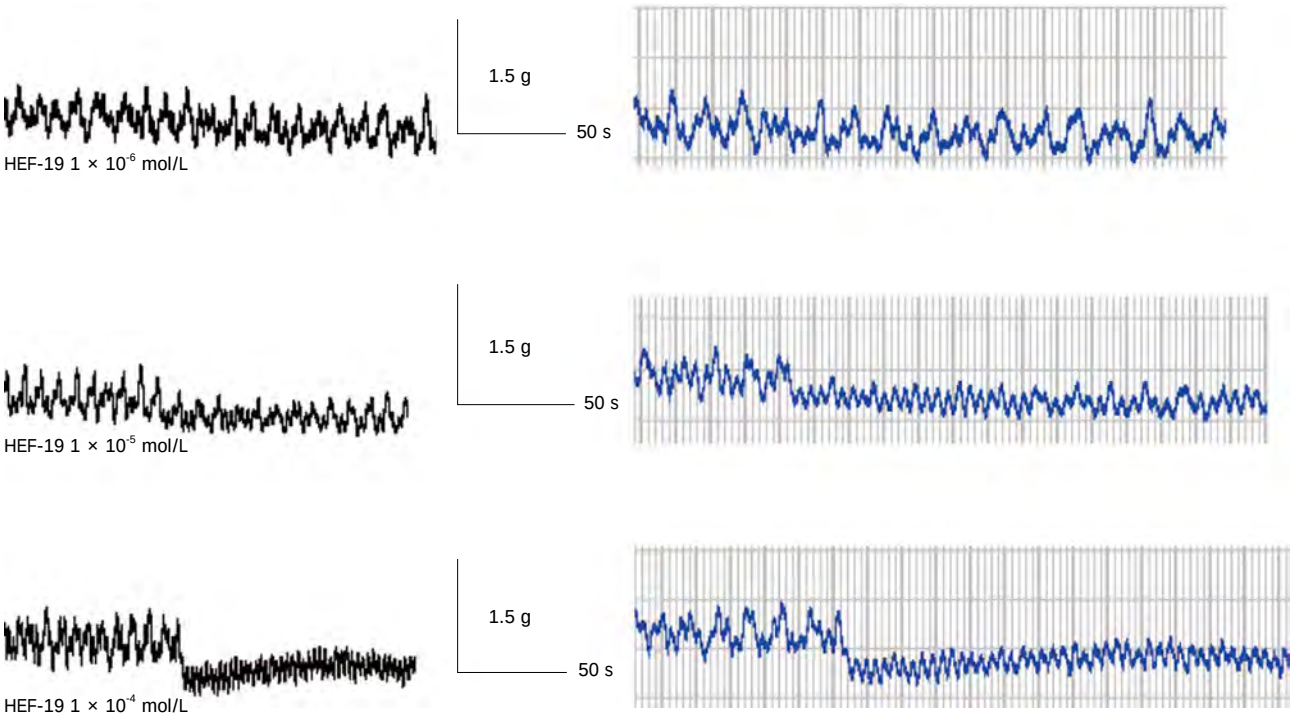

Figure 2 Effects of HEF-19 on spontaneous tension and amplitude of isolated rabbit descending colonic smooth muscle.

Table 1 Effects of HEF-19 on tension and amplitude of spontaneous contraction of descending colonic smooth muscles

Group	After administration (%)	
	Tension	Amplitude
Vehicle	97.98 ± 2.37	103.69 ± 10.13
HEF-19 (mol/L)		
10^{-6}	89.87 ± 2.60	81.96 ± 13.90[a]
10^{-5}	75.98 ± 3.2[b]	48.40 ± 6.07[b]
10^{-4}	55.05 ± 18.13[b]	37.77 ± 2.54[b]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* vehicle. Values are mean ± SD, $n = 6$.

with HEF-19 (1×10^{-6} or 3×10^{-6} mol/L) and verapamil (1×10^{-7} mol/L) respectively[8].

Inhibition of HEF-19 on biphasic contraction induced by ACh

After the equilibration period, normal Tyrode's solution was replaced with Ca^{2+}-free Tyrode's solution for 20 min. The phasic contraction caused by ACh (15×10^{-6} mol/L) was obtained, and tonic contraction was induced by further addition of $CaCl_2$ (4×10^{-3} mol/L). After washing with normal Tyrode's solution, the experiments were repeated with incubation for 10 min with HEF-19 (3×10^{-6} mol/L or 1×10^{-5} mol/L) and verapamil (1×10^{-7} mol/L) respectively[8,9].

Statistical analysis

Statistical evaluation of the data was performed using Student's *t* test when appropriate. The data were expressed as mean ± SD or mean ± SEM and $P < 0.05$ was considered statistically significant.

RESULTS

Effect of HEF-19 on spontaneous contraction of rabbit descending colon

HEF-19 (1×10^{-6}, 1×10^{-5} and 1×10^{-4} mol/L) significantly suppressed the tension and amplitude of *spontaneous contraction*, in a concentration-dependent manner. Figure 2 is print screen about tension and amplitude of spontaneous contraction of descending colonic smooth muscles. Tension is *y*-axis. Time is *x*-axis. Amplitude is difference between the peaks and troughs. The data of Figure 2 showed in Table 1.

Relaxant effects of HEF-19 in contraction induced by $BaCl_2$, high-K^+ solution, Ach or histamine

The maximum responses of the cumulative concentration-response curves to $BaCl_2$, high-K^+ solution, Ach or histamine were depressed by HEF-19 in a dose-dependent manner (3×10^{-7}-3×10^{-4} mol/L). EC_{50} values were 5.15 ± 0.05, 5.12 ± 0.08, 5.58 ± 0.16 and 5.25 ± 0.24 (Figure 3).

Inhibition of $CaCl_2$-induced contraction

The maximum cumulative concentration-response curves for $CaCl_2$-induced contraction were depressed by HEF-19 (1×10^{-6} and 3×10^{-6} mol/L) in a concentration-dependent manner. These results indicated that HEF-19 showed non-competitive antagonism (Figure 4).

Inhibitory effect of HEF-19 on biphasic contraction induced by ACh

The phasic and tonic contraction induced by ACh was decreased by HEF-19 (3×10^{-6} and 1×10^{-5} mol/L) in a

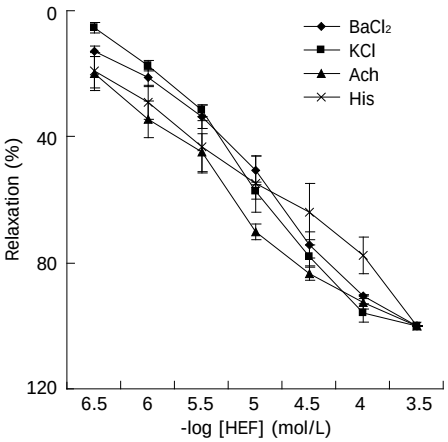

Figure 3 Relaxant effect of HEF-19 (3×10^{-7}-3×10^{-4} mol/L) on isolated rabbit descending colonic smooth muscle pre-contracted with Ach, histamine, high-K^+ solution or $BaCl_2$. Data are mean ± SE (n = 6).

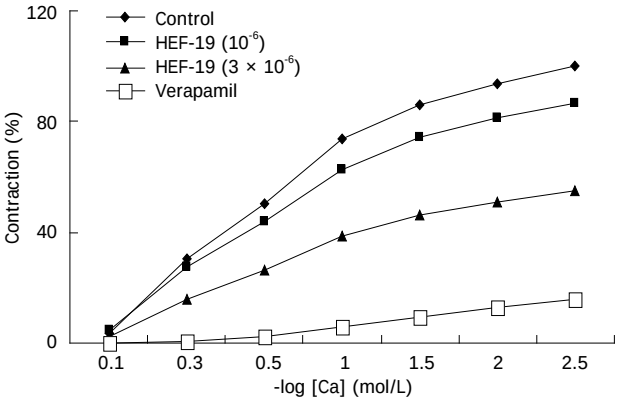

Figure 4 Effect of HEF-19 and verapamil on the contraction-response curve of $CaCl_2$ in descending colonic smooth muscle isolated from rabbits. Data are mean ± SE (n = 6) and are expressed as percentage of maximum contraction.

concentration-dependent manner after pretreatment in calcium-free medium with EGTA (Figure 5).

DISCUSSION

Excitation-contraction coupling in smooth muscle occurs through two main mechanisms. Many smooth muscles are activated by Ca^{2+} signaling cascades. In addition, there is a Rho/Rho kinase signaling pathway that acts by altering the Ca^{2+} sensitivity of the contractile system[10,11]. The predominant source of activator and intracellular Ca^{2+} has little role to play in mediating excitation-contraction coupling by agonists. Both tonic and phasic (rhythmic) contraction are regulated by intracellular Ca^{2+} concentration. Ca^{2+} originates from the intracellular Ca^{2+} store, the sarcoplasmic reticulum, and influx from the extracellular space. Phasic contraction is influenced by neurotransmitters, hormones, and drugs. In circular muscle, these agents can also increase calcium by releasing it from intracellular stores, thus inducing tonic contraction[12-19].

Smooth muscle has the automatic rhythmicity. Spontaneous contraction shows the basic rhythmic depolarization wave. HEF-19 suppressed the spontaneous contractile amplitude and tension of rabbit colonic smooth muscle in a concentration-dependent manner. It has been reported that extracellular Ca^{2+} participates in spontaneous activity and enters the cytosol by L-type voltage-dependent Ca^{2+} channels[20].

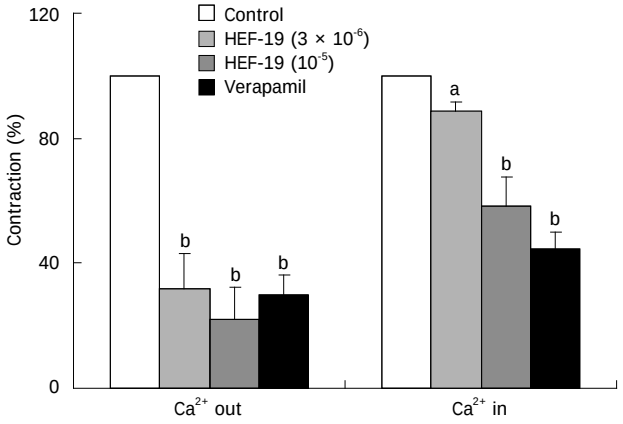

Figure 5 Effects of HEF-19 (3×10^{-6} and 10^{-5} mol/L) and verapamil (1×10^{-7} mol/L) on biphasic contraction induced by Ach in descending colonic smooth muscle isolated from rabbits. Data are mean ± SE (n = 6). $^aP < 0.05$, $^bP < 0.01$ *vs* the controls.

The contraction induced by $BaCl_2$, high-K^+ solution, Ach or histamine was relaxed by HEF-19. High-K^+ elicits an increase in intracellular Ca^{2+} and transient contractions[21,22]. ACh induces smooth muscle contraction via activating muscarinic receptors. Extracellular and intracellular Ca^{2+} participate in the Ach-induced contraction[23]. Histamine has a spasmogenic effect on the gastrointestinal tract through activating histaminergic receptors and increasing Ca^{2+} influx[24,25]. $BaCl_2$ causes cell membrane depolarization and intracellular Ca^{2+} release, and it can cross the cell membrane through the Ca^{2+} channels to bind with troponin directly[26].

HEF-19 depressed the maximum cumulative concentration-response curve for $CaCl_2$ in a non-competitive manner, similar to verapamil. The fact that HEF-19 inhibited $CaCl_2$-induced smooth muscle contraction indicated that it inhibited the voltage-dependent Ca^{2+} channels, because $CaCl_2$ can open these channels during high-K^+ depolarization[27,28].

There are biphasic responses, including fast and slow components, in the contraction induced by ACh. The fast (phasic) phase is due to the release of intracellular Ca^{2+} induced by ACh in Ca^{2+}-free medium[21], and the sustained (tonic) phase is largely dependent on the influx of external Ca^{2+} resulting from the reintroduction of $CaCl_2$ into the medium. HEF-19 decreased the phasic and tonic contraction. The results showed that HEF-19 eventually inhibited the Ca^{2+} channels to reduced release of intracellular Ca^{2+} and influx of external Ca^{2+}.

In conclusion, our results suggest that HEF-19 relaxed rabbit descending colonic smooth muscle by blocking voltage-dependent Ca^{2+} channels. HEF-19 inhibited

the inflow of extracellular Ca^{2+} into cells, and intracellular release of Ca^{2+} ions. Ca^{2+} channels blocking effect of HEF-19 is fewer than verapamil on colonic smooth muscle. Calcium channel blockers are also reported to be effective in the treatment of IBS[3]. However, the adverse effects on the cardiovascular system of these blockers limit their further application on IBS patients. HEF-19, a L-type calcium channel blocker with selectivity for the gastrointestinal tract, is expected to be a safe and effective drug for treatment of abdominal pain and diarrhea symptoms associated with IBS.

COMMENTS

Background

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder in which abdominal pain is associated with changes in bowel habits and abdominal distension. Abnormal contraction of intestinal smooth muscle may be important in producing the main symptoms of IBS, thus, modifying contractility is often the major aim of treatment. Traditional cholinolytic and opioid drugs have been reported to have much adverse reactions. Some enteric spasmolytics agents have been found to treat IBS by selectively blocking voltage-dependent Ca^{2+} channels.

Research frontiers

Current IBS pathophysiologic mechanisms are based on the abnormalities of brain-gut axis. With in-depth researches on various neurotransmitters, ion channel and receptors, designed as targets, new drugs are expected to appear against IBS. Since Pinaverium Bromide was developed and used clinically, there has been increasing concern to search for highly selective blockers of voltage-dependent Ca^{2+} channels to treat IBS patients with abdominal pain and diarrhea.

Innovations and breakthroughs

Chromane HEF-19 has a relaxant effect on colonic smooth muscles. It has previously been shown to have little activity on isolated vascular smooth muscle. The present study investigated the relaxant effect of HEF-19 on isolated descending colon smooth muscle of rabbits and the possible mechanisms. HEF-19 is expected to be a highly selective enteric spasmolytics agent through inhibition of opening of voltage-dependent Ca^{2+} channels in colonic smooth muscle. This is a potentially interesting study to find a drug for treatment of abdominal pain and diarrhea associated with IBS.

Applications

HEF-19 is expected to be a safe, effective and economic drug for treatment of abdominal pain and diarrhea symptoms associated with IBS.

Terminology

HEF-19: HEF-19, 3.4-dihydro-7-[3-(diethylamino) propoxy] chroman hydrochloride, is highly selective enteric spasmolytics agent. IBS is a functional gastrointestinal disorder in which abdominal pain is associated with changes in bowel habits and abdominal distension. People with a functional gastrointestinal (GI) disorder have frequent symptoms, but the GI tract is not damaged. IBS is a group of symptoms that occur together. The most common symptoms of IBS are abdominal pain or discomfort, often reported as cramping, along with diarrhea, constipation, or both.

Peer review

Very well written manuscript. In the manuscript entitled "HEF-19-induced relaxation of colonic smooth muscles and the underlying mechanisms", the authors investigated the relaxant effect of chromane HEF-19 on colonic smooth muscles isolated from rabbits. This is a good descriptive study on a hot topic. The research is well done. The result is well discussed.

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BRIEF ARTICLE

Prevalence of hepatitis C infection among intravenous drug users in Shanghai

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Author contributions: Tao YL and Tang YF carried out the screening tests for antibodies to hepatitis C virus, Recombinant immunoblot assay, and Qualitative tests for hepatitis C virus RNA; Qiu JP, Cai XF, Shen XT and Wang YX participated in the screening tests for antibodies to hepatitis C virus and sample assembly; Zhao XT designed this study, performed the statistical analysis and wrote the paper; all authors read and approved the final manuscript.
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Abstract

AIM: To characterize the prevalence of hepatitis C virus (HCV) infection among Chinese intravenous drug users (IDUs).

METHODS: A total of 432 adult IDUs (95 women and 337 men) in Shanghai were included in the study. The third-generation Elecsys Anti-HCV assay (Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305, Mannheim, Germany) was used to screen for antibodies against HCV. The RIBA strip, a supplemental anti-HCV test with high specificity, was performed on all of the samples that tested positive during the initial screening. All of the anti-HCV positive samples were analyzed with a Cobas TaqMan 48 Analyzer (Roche Diagnostics) for direct detection of HCV RNA. All of the HCV RNA-positive samples were sequenced for genotype determination.

RESULTS: The preliminary screening identified 262 (60.6%) subjects who were seropositive for HCV. Of the 62 females and 200 males seropositive subjects, 16 (16.7%) and 65 (19.3%), respectively, were confirmed by RIBA, yielding an overall HCV seropositive rate of 18.8%. Four female (6.5%) and 14 male (7.0%) subjects tested positive for HCV RNA, indicating an active infection rate of 4.2% for the entire study population. The 18 HCV RNA-positive serum samples were genotyped. Seven individuals were genotype 1b, and four were genotype 1a. One individual each was infected with genotypes 2a, 2b and 3a. Four subjects were co-infected with multiple strains: two with genotypes 1a and 2a, and two with genotypes 1b and 2a. The active infection rate among HCV-seropositive individuals was 22.2%, which was significantly lower than most estimates.

CONCLUSION: The prevalence of HCV is relatively low among IDUs in Shanghai, with a spontaneous recovery rate much higher than previous estimates.

Key words: Hepatitis C; Anti-hepatitis C virus antibodies; Prevalence of hepatitis C virus; Active infection rate; Intravenous drug users

Core tip: In this report, we examined the prevalence of anti-hepatitis C virus (HCV) antibodies, as well as chronic viremia, in 432 intravenous drug users (IDUs) in Shanghai, China. Our data will facilitate the characterization of the prevalence of HCV infection among Chinese IDUs and will complement our understanding of the natural course of HCV infections.

Tao YL, Tang YF, Qiu JP, Cai XF, Shen XT, Wang YX, Zhao XT. Prevalence of hepatitis C infection among intravenous drug users in Shanghai. *World J Gastroenterol* 2013; 19(32): 5320-5325

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INTRODUCTION

Hepatitis C virus (HCV) is an enveloped RNA virus with a diameter of approximately 50 nm, and it is classified as a *Hepacivirus* within the *Flaviviridae* family[1]. Humans are the primary reservoir of HCV; however, the virus has been transmitted experimentally to chimpanzees[2]. The HCV genome consists of a 9.6-kb single-stranded, positive-sense RNA molecule containing one long open reading frame (ORF). This single ORF encodes a large (approximately 3000 amino acids) polyprotein that undergoes co- and post-translational cleavage by host and viral proteases to yield individual viral proteins[1,2]. The N-terminal quarter of the genome encodes core and structural proteins; these proteins consist of a non-glycosylated nucleic acid-binding nucleocapsid protein (core) of 190 amino acids (approximately 21 kDa) and two membrane-associated glycoproteins (E1 and E2/NS1) of 190 and 370 amino acids, respectively (33 and 70 kDa, respectively, when glycosylated). The remaining three-quarters of the genome encode nonstructural proteins NS2-NS5. The NS2 (250 amino acids), NS3 (500 amino acids) and NS4A proteins interact to mediate processing of the presumed NS region of the polyprotein. NS3 is both a proteolytic cleavage enzyme and a helicase, which facilitates unwinding of the viral genome during replication. NS5b is the RNA-dependent RNA polymerase necessary for viral replication[3-5].

Seven HCV genotypes with several distinct subtypes have been identified worldwide[2]. HCV is the etiological agent of hepatitis C. HCV infections are often asymptomatic; however, chronic infection can result in the scarring of the liver, which can ultimately lead to cirrhosis[6-8]. Carriers who develop cirrhosis are at significantly greater risk for developing liver failure, liver cancer or life-threatening esophageal and gastric varices[8]. No effective anti-HCV vaccines are currently available[9,10]. The standard of care therapy for patients with HCV infection is the use of both peginterferon and ribavirin. These drugs are administered for either 48 wk (HCV genotypes 1, 4, 5 and 6) or 24 wk (HCV genotypes 2 and 3). These therapies induce a sustained virologic response (SVR) in infected individuals. SVR rates of 40%-50% are observed in patients with genotype 1 infections, and rates of > 80% are observed in those with genotype 2 and 3 infections[8]. Once achieved, SVRs are associated with the long-term clearance of HCV infection, as well as improved morbidity and mortality[8].

Two major advances have occurred in recent years: the development of direct-acting antiviral (DAA) agents; and the identification of several single-nucleotide polymorphisms associated with spontaneous and treatment-induced clearance of HCV infection[11-19]. Although peginterferon and ribavirin remain vital components of therapy, the emergence of DAAs has led to a substantial improvement in SVR rates, along with the option of abbreviated therapy for many patients with genotype 1 chronic HCV infections[8].

The World Health Organization (WHO) estimates that approximately 3% of the global population has been infected with HCV, including more than 170 million chronic carriers at risk of developing liver cirrhosis and/or liver cancer[2]. HCV transmission occurs primarily through exposure to infected blood[1-9]. Specific routes of infection include intravenous drug use, blood transfusions (before 1992), solid organ transplantation from an infected donor, unsafe medical practices, occupational exposure to infected blood, maternal-fetal transmission, sex with an infected person, high-risk sexual practices and possibly intranasal cocaine use[2]. In China, a nationwide HCV serological survey indicated the prevalence of anti-HCV antibodies to be > 0.5% among more than 80000 Chinese subjects. Furthermore, the rates of hepatitis C were much lower than the rates of hepatitis B among clinical inpatient and outpatient populations[20]. Beginning in the early 1990s, the strict screening of blood donors and precise control over the blood supply were implemented by the Chinese government, which effectively eliminated the transmission of many infectious diseases due to blood transfusions. The majority of HCV infections are now limited to specific subpopulations, such as intravenous drug users (IDUs) and patients with certain hemopathies.

Although the prevalence of HCV is greater among IDUs than in the general population, the infection rates of HCV and other diseases remain unknown among IDUs in China. Many hepatologists and virologists worldwide believe that as high as 40%-80% of individuals infected with HCV will develop chronic hepatitis C[2,8]; however, the true rate at which patients develop chronic hepatitis C remains is not known. This gap in understanding regarding the natural course of HCV infection could lead us to misjudge the true burden of HCV infection and might negatively impact clinical decision-making.

In this report, we examined the prevalence of anti-HCV antibodies, as well as chronic viremia, in 432 IDUs in Shanghai, China. Our data will facilitate the characterization of the prevalence of HCV infection among Chinese IDUs and will complement our understanding of the natural course of HCV infections.

MATERIALS AND METHODS

Study population

There are 17 districts in Shanghai, and each district contains one medical center that was established by the local government, where IDUs can receive diaminon therapy for heroin addiction on a regular basis. The total population of Shanghai is approximately 16 million, and Xuhui District is one of the central districts. The residential population of Xuhui District is approximately 1.2 million.

Our samples were collected from Xuhui District. There are approximately 500 IDUs in this district annually, who are treated at the medical center in Xuhui District, where they receive diaminon therapy. A total of 432 adult IDUs, primarily heroin users, were included in this study. Patient serum was collected every 6 mo to monitor HCV, HIV and *Treponema pallidum* (*T. pallidum*) subspecies *pallidum* infections. The participants reported no malaise, weakness, anorexia, jaundice or other symptoms of hepatitis, and they had not previously been diagnosed with viral hepatitis. Accordingly, all of the participants were negative for prior HCV therapy. All of the serum samples used in this study were collected in 2012. Written informed consent was obtained according to the guidelines of the National Ethics Regulation Committee, and the study was approved by the Internal Review Board of the Center for Disease Control and Prevention of Shanghai. The participants were informed of their right to withdraw consent. Consent could be withdrawn by participants, immediate relatives, caregivers or legal guardians.

Screening tests for antibodies to HCV

A third-generation Elecsys Anti-HCV assay (Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305, Mannheim, Germany) was used to screen for antibodies against HCV, according to the manufacturer's instructions. The assays were performed using a Cobas 411 e-analyzer. The cutoff index values used for determination of positive reactivity were set based upon the manufacturer's recommendation. Samples with a cutoff-index < 0.9 were considered non-reactive in the Elecsys Anti-HCV assay. Samples having a cutoff-index between $\geqslant 0.9$ and < 1.0 were considered borderline, whereas samples with a cutoff-index of $\geqslant 1.0$ were considered reactive.

Recombinant immunoblot assay

The recombinant immunoblot assay (RIBA) strip, a supplemental anti-HCV test with high specificity, was performed on all of the samples that tested positive during the initial screening. The assays were performed using an MP Diagnostics HCV BLOT 3.0 (MP Biomedicals, Solon, OH, United States), according to the manufacturer's instructions.

Qualitative tests for HCV RNA

A Cobas AmpliPrep Total Nucleic Acid Isolation Kit (Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim, Germany) was used to isolate HCV RNA from serum samples that tested positive for anti-HCV antibodies during the initial screening. Isolation was performed in accordance with the manufacturer's instructions. All of the samples were then analyzed using a Cobas TaqMan 48 analyzer (Roche Diagnostics) for direct detection of HCV RNA.

HCV genotyping

HCV RNA was extracted from 200 μL of EDTA-treated plasma for each HCV RNA-positive sample, using a QIAamp Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions. All of the primers were designed on the basis of consensus sequences, as reported by Duarte *et al*[21]. Two sets of primers were designed: one for the 5'-UTR region (for genotypes 1-6), and the other for the NS5B region (supplemental primers for genotypes 1a and 1b). Reverse transcription reactions were conducted with a Reverse Transcription Kit (Biovisualab, Shanghai, China). Multiplex PCR was then performed using a HiFiFast PCR high-fidelity DNA polymerase mix (Biovisualab). PCR was conducted in a Peltier Thermal Cycler (MJ96+/MJ966) under the following conditions: incubation at 94 ℃ for 5 min, followed by 35 cycles of denaturation at 94 ℃ for 10 s; annealing at 58 ℃ for 30 s; and extension at 72 ℃ for 20 s. There was then a final extension step at 72 ℃ for 3 min, and the reactions were held at 4 ℃ thereafter. For genotype determination, direct sequencing was performed bidirectionally using a Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA, United States) using 10 ng of QIAquick Spin-purified PCR product (Qiagen) and either the sense or antisense PCR primer, followed by detection on an ABI 310 automated sequencer (PE Applied Biosystems).

Statistical analysis

The results are expressed as the mean ± SD. The statistical analyses were performed using either Student's *t*-test or analysis of variance with *post hoc* Scheffe correction when appropriate. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Overview of study participants

A total of 432 adult IDUs, ranging from 23 to 63 years of age (mean age 44 ± 9 years old), were enrolled in the study. Of the study participants, 337 were male, and 95 were female. The average history of heroin use was 15 ± 5 years (ranging from 2 to 40 years). The majority of participants administered heroin by injection; all denied sharing syringes. All of the participants were seen at a medical center in Shanghai, where they receive diaminon therapy for heroin addiction on a regular basis. Blood samples were collected every 6 mo to screen for HCV, HIV and *T. pallidum* infections. The participants reported no malaise, weakness, anorexia, jaundice or other symptoms of hepatitis, and they had not previously been diagnosed with viral hepatitis. Accordingly, all of the participants were negative for prior HCV therapy.

Prevalence of antibodies against HCV

According to recommendations put forth by the United States Centers for Disease Control and Prevention (CDC), the detection of anti-HCV antibodies requires the use of a screening test with high sensitivity. In addition, reactions with low positivity should be verified by RIBA or

PCR to confirm the presence of viral RNA[22,23].

Preliminary screening tests for HCV were performed using Elecsys assays and a Cobas 411 e-analyzer. Of the 95 females subjects tested, 65.3% were positive for antibodies against HCV. Of the 337 males subjects, 59.3% were positive for antibodies against HCV. No significant differences in infection rates were observed between the men and women. The overall prevalence of anti-HCV antibodies was 60.6%. These results demonstrate a rate of HCV infection among IDUs that is substantially higher than that in the general population.

The sensitivity of the anti-HCV assay was significantly greater than that of the clinical measurements. For the 262 HCV-seropositive individuals, the cutoff index values ranged from 1.6 to 20.1, with an average of 5.7 ± 3.7, well above the standard 1.0 cutoff index value for positive reactivity.

HCV seropositivity and active infection rates confirmed by RIBA and PCR

To confirm the presence of viral RNA, we reanalyzed the 262 HCV-seropositive subjects using RIBA and PCR. Of the 62 females and 200 males subjects, 16 (16.7%) and 65 (19.3%) were confirmed to be true positives for anti-HCV antibodies, respectively. Therefore, the true HCV-positive rate of our study subjects was 18.8%. All of the RIBA-positive subjects were seropositive for core proteins. Eight subjects displayed a weak or no reaction to NS3-1, whereas 16 failed to display strong reactivity to NS3-2. Roughly half of the 81 subjects were positive for antibodies against NS4 and NS5.

To determine the current HCV infection rate, the sera from all 262 seropositive individuals were analyzed using the Cobas AmpliPrep/Cobas TaqMan HCV Test. Of the 62 females and 200 males subjects, 4 (6.5%) and 14 (7.0%), respectively, were positive for HCV RNA, indicating an active infection rate of 4.2% for the entire study population.

The 18 HCV RNA-positive sera were then genotyped. Seven individuals were genotype 1b, and four were genotype 1a. One individual each was infected with genotypes 2a, 2b and 3a. Four subjects were co-infected with multiple strains: two subjects with genotypes 1a and 2a, and two subjects with genotypes 1b and 2a.

HCV infection rates among HCV-seropositive subjects

HCV remains difficult to both treat and detect due to the high rate of mutation, which severely limits the efficacy of potential vaccines[2]. Current estimates suggest that as many as 70%-90% of infected individuals fail to clear the virus during the acute phase of the disease and therefore become chronic carriers[2,8]. However, the true rate of viral clearance is not known because neither the rate of HCV infection nor the rate of recovery has been established. Some insight into these questions can be drawn from our cohort of IDUs. Of the 16 females and 65 males subjects who tested positive for HCV antibodies, four and 14 subjects were also positive for HCV RNA, respectively (Table 1), yielding an overall clearance rate of 77.8%, which is substantially higher than most estimates[2,8,24,25].

Table 1 Hepatitis C virus current infection rates in anti-hepatitis C virus positive population

	NAT(+)	RIBA(+)	NAT(+)/RIBA(+) (%)
Female	4	16	25.00%
Male	14	65	21.50%
Total	18	81	22.20%

Hepatitis C virus (HCV) current infection rates in anti-HCV positive population were calculated as [number of individuals with nucleic acid testing (NAT)]/total studied population with anti-HCV positive. RIBA: Recombinant immunoblot assay.

DISCUSSION

Since HCV was identified in 1989[26], infection by means of blood transfusion has been virtually eliminated worldwide, limiting the spread of HCV to select populations, particularly IDUs[2]. In China, the prevalence of HCV in the general population is relatively low[20]. However, the number of IDUs is increasing, with the spread of numerous infectious diseases, including viral hepatitis, HIV and *T. pallidum*, subsequently increasing as well. In this report, 18.8% (81/432) of individuals were confirmed to be seropositive for HCV by RIBA testing. Among these individuals, 14 were also positive for HCV RNA, indicating an active infection rate of 4.2% for our cohort. In 1997, the WHO estimated that 3% of the world's population was infected with HCV[2]; however, a recent nationwide survey in China reported an HCV seropositive rate of < 0.5% among more than 80000 Chinese subjects[20], casting doubt on the WHO estimates. The active infection rate of 4.2% observed in our cohort of IDUs is low compared to other reports; however, it is markedly higher than in the general population. These results highlight the importance of studying at-risk populations, including IDUs.

The active infection rate among HCV-seropositive individuals was 22.2% in this study, which is significantly lower than most estimates[2,8,24,25]. Current estimates suggest that as many as 40%-80% of HCV infections will develop into chronic infections[2,8,24,25]. While these estimates are likely inaccurate, studying infection rates among high-risk populations remains difficult. In addition, the susceptibility and specificity of older detection methods, including anti-HCV and viral RNA tests, are low. The data presented here directly challenge assumptions regarding the rate of chronic infection. Our data indicate that as many as 77.8% of individuals were able to clear HCV infections without the need for anti-viral therapy.

A total of 262 (60.6%) subjects tested positive for anti-HCV antibodies during the initial screening stage, of whom only 81 (18.8%) were confirmed by RIBA, indicating that as many as 181 individuals were false positives detected by the Elecsys and Cobas e-analyzers. The ac-

curacy of our findings was supported by use of these automated systems, which remove biases caused by human error.

Despite improvements in technology, false-positive results for anti-HCV antibodies are a well-known problem. A number of conditions have been shown to induce false positives, including high gamma globulin levels, nephritic syndrome, liver diseases, autoimmune diseases, viral or parasitic infections and pregnancy[27,28]. The United States CDC estimates that for immunocompetent individuals, approximately 35% of the anti-HCV enzyme linked immunosorbent assay immunoassay results are false positives. Adjustments to cutoff indices have been insufficient to overcome these issues[27,28], highlighting the need for more accurate screening methods. Although the third-generation Elecsys Anti-HCV assay and RIBA test detect similar antigens, the RIBA test is capable of distinguishing among the antibodies against core, NS3-1, NS3-2, NS4 and NS5 proteins, and this method was used to confirm the Elecsys results.

Anti-HCV antibodies develop during acute infection, generally between 2 and 8 wk after evidence of liver injury[2,8]. Anti-HCV antibodies are generally not detectable in patients with initial signs or symptoms of hepatitis C, with some individuals not testing positive until 6-9 mo after the onset of illness[2,8]. In contrast, hepatitis C viremia can be detected by reverse transcription polymerase chain reaction within a few days after infection[2,8]. In this study, all of the HCV RNA-positive individuals were confirmed to be seropositive for HCV by RIBA, indicating that the rate of early infection was low.

The 18 HCV RNA-positive sera were genotyped. Seven individuals were genotype 1b, and four were genotype 1a. One individual each was infected with genotypes 2a, 2b and 3a. Four subjects were co-infected with multiple strains: two with genotypes 1a and 2a, and two with genotypes 1b and 2a. These data indicate that the genotype distribution in the population is complex.

The diagnosis of hepatitis is made by biochemical assessment of liver function. Initial laboratory evaluations include total and direct bilirubin, alanine aminotransaminase, aspartate aminotransferase, alkaline phosphatase, prothrombin time, total protein, albumin, globulin, complete blood count and coagulation studies[2,8]. In this study, we did not perform the above clinical evaluations. Further investigation and follow-up of affected individuals are ongoing.

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COMMENTS

Background

Since the discovery of hepatitis C virus (HCV) in 1989, strict screening measures have virtually eliminated viral transmission through blood transfusions, limiting the spread of HCV to select populations, particularly intravenous drug users (IDUs). The prevalence of HCV infection is relatively low among the general population in China. However, infection rates among high risk populations in China are unknown.

Research frontiers

Many hepatologists and virologists worldwide believe that as high as 40%-80% of individuals infected with HCV will develop chronic hepatitis C; however, the true rate at which patients develop chronic hepatitis C remains is not known. The gap in understanding regarding the natural course of HCV infection could lead us to misjudge the true burden of HCV infection and might negatively impact clinical decision-making.

Innovations and breakthroughs

In this report, the authors examined the prevalence of anti-HCV antibodies, as well as chronic viremia, in 432 IDUs in Shanghai, China. The active infection rate among HCV-seropositive individuals was 22.2%, which was significantly lower than most estimates.

Applications

The data will facilitate the characterization of the prevalence of HCV infection among Chinese IDUs and will complement our understanding of the natural course of HCV infections.

Terminology

The prevalence of anti-HCV antibodies indicates the prevalence of total antibodies against HCV. False positive results for anti-HCV antibodies are a well-known problem. The recombinant immunoblot assay test is capable of distinguishing between antibodies against core, NS3-1, NS3-2, NS4, and NS5 proteins, whereas this method was used to confirm Elecsys results. The active infection of HCV indicates that the HCV RNA could be detected in individual serum by reverse transcription polymerase chain reaction.

Peer review

The paper is well written and gives important epidemiology information of the HCV infection.

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BRIEF ARTICLE

Effects of Fufang Biejia Ruangan Pills on hepatic fibrosis *in vivo* and *in vitro*

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Abstract

AIM: To explore the protective effect and the relevant mechanisms of Fufang Biejia Ruangan Pills (FFBJRGP) on hepatic fibrosis *in vivo* and *in vitro*.

METHODS: Hepatic fibrosis was induced by carbon tetrachloride composite factors. Adult Wistar rats were randomly divided into four groups: normal control group; hepatic fibrosis model group; FFBJRGP-treated group at a daily dose of 0.55 g/kg; and colchicine-treated group at a daily dose of 0.1 g/kg. The effects of FFBJRGP on liver function, serum levels of hyaluronic acid (HA), type Ⅳ collagen (CⅣ), type Ⅲ procollagen (PC Ⅲ), laminin (LN), histopathology, and expression of transforming growth factor (TGF-β1) and Smad3 in hepatic fibrosis were evaluated *in vivo*. The effects of FFBJRGP on survival rate, hydroxyproline content and cell cycle distribution were further detected *in vitro*.

RESULTS: Compared with the hepatic fibrosis model group, rats treated with FFBJRGP showed a reduction in hepatic collagen deposition and improvement in hepatic lesions. Compared with those of the model group, the activities of alanine aminotransferase (62.0 ± 23.7 U/L) and aspartate aminotransferase (98.8 ± 40.0 U/L) in the FFBJRGP-treated group were decreased (50.02 ± 3.7 U/L and 57.2 ± 30.0 U/L, respectively, $P < 0.01$). Compared with those in the model group, the levels of PCⅢ (35.73 ± 17.90 μg/mL), HA (563.82 ± 335.54 ng/mL), LN (89.57 ± 7.59 ng/mL) and CⅣ (29.20 ± 6.17 ng/mL) were decreased to 30.18 ± 9.41, 456.18 ± 410.83, 85.46 ± 7.51 and 28.02 ± 9.45 ng/mL, respectively. Reverse-transcriptase polymerase chain reaction and Western blotting also revealed that expression of TGF-β1 and Smad3 were down-regulated *in vivo*. Cell proliferation was inhibited, the level of hydroxyproline was decreased compared with the control group ($P < 0.01$), and the cell cycle was redistributed when exposed to FFBJRGP *in vitro*.

CONCLUSION: FFBJRGP inhibits hepatic fibrosis *in vivo* and *in vitro*, which is probably associated with downregulation of fibrogenic signal transduction of the TGF-β-Smad pathway.

Key words: Fufang Biejia Ruangan Pill; Hepatic fibrosis; Transforming growth factor-Smad signaling

Core tip: Fufang Biejia Ruangan Pill (FFBJRGP) is the first anti-fibrosis drug approved by the China State Food and Drug Administration. It has been demonstrated that FFBJRGP has a better efficacy of anti-fibrosis. However, the underlying therapeutic mechanisms of FFBJRGP in hepatic fibrosis are still unclear. In our study, FFBJRGP showed a strong ameliorative effect in hepatic fibrosis *in vivo* and *in vitro*. It reduced production and deposition of collagen in liver tissues. FFBJRGP inhibited expression of transforming growth factor (TGF-β1) and Smad3, which implied that inhibition of TGF-β/Smad-mediated fibrogenesis may be a central mechanism by which FFBJRGP protects against liver injury.

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INTRODUCTION

Liver fibrosis represents the final common pathway of virtually all chronic liver diseases. It is characterized by the excessive accumulation of extracellular matrix (ECM) and activated hepatic stellate cells (HSCs) that are undergoing myofibroblast transition. Several studies have shown that hepatic fibrosis is a reversible disease, therefore, an effective treatment would probably prevent or reverse the fibrotic process in the liver[1]. In the long pathological progression of hepatic fibrosis to cirrhosis, transforming growth factor (TGF)-β1 is one of the strongest profibrotic cytokines[2,3], and TGF-β-Smad signaling is the main signal transduction pathway[4], which has been verified by several related studies. The downregulation of TGF-β expression and modulation of TGF-β-Smad signaling may be effective in preventing liver fibrosis[5].

Traditional Chinese medicine plays a unique role in the treatment of liver fibrosis. Fufang Biejia Ruangan Pill (FFBJRGP) has been demonstrated to have a better antifibrotic efficacy for its traditional Chinese medical effects of "softening and resolving hard masses, dissolving blood stasis and detoxication, replenishing Qi and Blood". Numerous clinical observations have confirmed that patients with hepatic fibrosis receiving FFBJRGP have a favorable outcome[6]. However, the underlying therapeutic mechanisms of FFBJRGP in hepatic fibrosis are still unclear. Thus, in the present study, we investigated the antifibrotic effect and potential mechanisms of action of FFBJRGP in hepatic fibrosis, in order to establish the clinical efficacy and make better application of FFBJRGP.

MATERIALS AND METHODS

Composition of FFBJRGP

The composition of FFBJRGP includes *Carapax Trionycis, Radix Paeoniae Rubra, Radix Angelicae Sinensis, Codonopsis Pilosula* and *Radix Astragali*.

In vivo study

Animals and experiment protocol: Healthy adult Wistar rats, female and male, weighing 237.8 ± 8.5 g, were obtained from the Experimental Animal Center of Academy of Medical Sciences of Chinese People's Liberation Army (Beijing, China). All animals were cared for according to the Guide for the Care and Use of Laboratory Animals (NIH Publications, No. 80-23, revised in 1996). Housed in a room with a 12-h light-dark cycle (temperature 22-24 ℃ and 50%-60% humidity), the rats were given *ad libitum* access to standard laboratory rodent chow and water. All processes conformed to international guidelines on the ethical use of animals.

The rats were subcutaneously injected with carbon tetrachloride (CCl_4) dissolved in peanut oil (CCl_4: peanut oil = 4:6, v/v), 0.5 mL/100 g body weight for the first time, and then 0.3 mL/100 g body weight twice weekly for 8 wk. In the first 2 wk, rats were raised with feedstuff I (80% corn meal, 20% lard, and 0.5% cholesterol). After 2 wk, they were raised with feedstuff Ⅱ (corn meal and 0.5% cholesterol). At the same time, 1 mL 30% alcohol was given orally to each rat every other day from the beginning.

The rats were randomly divided into the normal control group (n = 6); model group (n = 14); FFBJRGP treatment group (n = 12); and colchicine positive control group (n = 12). In the FFBJRGP treatment group, FFBJRGP was administered orally at 0.55 g/kg daily, which was equal to the dose in humans. The rats in the positive control group were given colchicine orally at a daily dose of 0.1 g/kg, which was also equal to the dose for humans. The rats in the normal control and model groups were administered the same volume of physiological saline as for the FFBJRGP group.

Liver laboratory tests: Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured using commercially available kits (Jiancheng Institute of Biotechnology, Nanjing, China) according to the manufacturer's instructions.

Serum levels of hyaluronic acid, type Ⅳ collagen, type Ⅲ procollagen and laminin: Serum levels of hyaluronic acid (HA), type Ⅳ collagen (CⅣ), type Ⅲ procollagen (PCⅢ) and laminin (LN) were determined by radioimmunoassay using commercially available kits (Beifang Institute of Biotechnology, Beijing, China) according to the manufacturer's instructions.

Histological examination

Liver tissues were collected from the left lobe of the liver of each rat, and fixed in 15% buffered paraformaldehyde, and dehydrated in a graded alcohol series. Specimens were embedded in paraffin blocks, cut into 5-μm-thick sections and placed on glass slides. The sections were stained with hematoxylin-eosin and Ponceau S[7]. Fibrosis was graded according to the method of Scheuer[8] as follows: stage 0: no fibrosis; stage 1: increase in collagen without formation of septa (small satellite expansion of the portal fields), expansion of portal tracts without linkage; stage 2: formation of incomplete septa not interconnecting with each other, from the portal tract to the central vein; stage 3: complete but thin septa interconnecting with each other, which divide the parenchyma into separate fragments; and stage 4: complete cirrhosis, similar to stage 3 with thicker septa.

Pathological examination was performed by the same pathologist who was blinded to the treatment assignment for the rats.

Determination of TGF-β1 mRNA level in liver tissues by real-time reverse transcriptase-polymerase chain reaction: Total RNA was extracted from liver tissues of each group with the tissue/cell total RNA isolation kit according to the manufacturer's protocol (Dalian TaKaRa Biotechnology Company, Dalian, China). The quantity and purity of RNA were detected by determining absorbance at 260/280 nm using a spectrophotometer. Total RNA was reversibly transcribed into cDNA using the cDNA synthesis kit according to the manufacturer's protocol (Dalian TaKaRa Biotechnology Company, Dalian, China). The ABI PRISM 7900 HT Real Time-polymerase chain reaction (PCR) System and real-time PCR kit were used according to the manufacturers' instructions. The specific primers for the target gene and β-actin were synthesized by Dalian TaKaRa Biotechnology Company (Dalian, China), as follows: TGF-β1: 5'-TGGCGTTACCTTGGTAACC-3' (forward); 5'-GGTGTT GAGCCCTTTCCAG-3' (reverse); β-actin: 5'-ACCCTTAAGGCCAACCGTGA AAAG-3' (forward); 5'-TCATGAGGTAGTCTGTCAGGT-3' (reverse).

The two-step PCR procedure was as follows: predenaturation for 30 s at 95 ℃, 1 cycle; 94 ℃ for 15 s and 56 ℃ for 40 s, 40 cycles. The final products were identified by electrophoresis in 1.5% agarose gel and melt curve analysis. Melt curve detection: 95 ℃ for 15 s, 60 ℃ for 15 s, and 95 ℃ for 15 s. The final results were described with the relative values ($2^{-\Delta\Delta Ct}$). The calculation and analysis were performed by Sequence Detection Software version 2.1 in the ABI PRISM 7900 HT Real Time PCR System.

Determination of Smad3 level in liver tissues by Western blotting: Total protein was extracted from liver tissues and analyzed with bicinchoninic acid protein concentration assay kit. Sample protein was separated by electrophoresis in 12% SDS-PAGE with a Bio-Rad electrophoresis system (Hercules, CA, United States). The primary antibodies (rabbit anti Smad3 antibody, 1:1000 dilution) were incubated at 4 ℃ overnight. The corresponding horseradish-peroxidase-conjugated secondary antibodies (anti-rabbit IgG, 1:5000 dilution) were incubated at room temperature. Immobilon Western chemiluminescent horseradish peroxidase substrate and Quantity ONE were used for revealing and quantitative analysis of the blots. β-actin was used as the internal control.

In vitro study

Drug serum preparation: The normal rats were administered with FFBJRGP and colchicine at a dose of 0.55 and 0.1 g/kg, respectively, for 2 d. At 2 h after the final administration, the sera were collected from the rats, mixed, and inactivated at 56 ℃ for 30 min. The blank control sera were collected from the normal rats.

Cell culture: HSC-LX-2 cells, an immortalized human HSC line, were cultured in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cultures were placed in a humidified atmosphere of 5% CO_2 at 37 ℃, and the medium was changed twice a week.

Cell viability test: HSC-LX-2 cells were seeded into 96-well plates at a density of 2×10^4 cells/well until 50% confluence. Cells treated with the above drug sera (20 μL/well) for 48 h were incubated with 5 mg/mL methyl thiazolyl tetrazolium (MTT) in DMEM for 4 h at 37 ℃. The supernatant was removed and 100 μL DMSO was added to each well to dissolve the formazan product. Absorbance at 570 nm was measured using a microplate reader.

Determination of hydroxyproline content: Collagen was determined by estimating the hydroxyproline content, an amino acid characteristic of collagen. HSC-LX-2 cells were lysed after treatment with the above drug sera. The lysates were used to measure hydroxyproline content using commercially available kits according to the manufacturer's instructions (Jiancheng Institute of Biotechnology, Nanjing, China).

Cell cycle analysis: For cell cycle analysis, HSC-LX-2 cells were synchronized by serum starvation in medium containing 0.4% serum for 24 h and induced to re-enter the cell cycle by an exchange of DMEM supplemented with 10% FBS. Drug sera of different groups were added (1 mL/bottle); the cells were cultured for 48 h and then harvested; washed and suspended in phosphate-buffered saline (PBS) twice; fixed in 80% ethanol for 48 h at 4 ℃; and suspended in 500 μL PBS containing RNase A for 30 min at 37 ℃. A total of 2×10^6 cells were harvested and resuspended in 0.5 mL of a solution containing 50 μg/mL propidium iodide, 1 mg/mL sodium citrate, 100 μg/mL RNase, and 0.1% Triton X-100. Flow cytometric analysis was made with a fluorescence-activated cell sorter. Forward light scatter characteristics were used to exclude cell debris from the analysis. The G0/G1 and S phases of the cell cycle were analyzed by diploid staining profiles.

Statistical analysis

All values were expressed as mean ± SD. Comparisons were analyzed by one-way ANOVA using the SPSS 12.0 statistical package. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Effect of FFBJRGP on liver function

There were significant differences in the ALT and AST

Table 1 Effect of Fufang Biejia Ruangan Pill on serum levels (mean ± SD)

Group	ALT(U/L)	AST(U/L)	PCⅢ (μg/mL)	HA (ng/mL)	LN (ng/mL)	CIV (ng/mL)
Control	23.8 ± 8.5[b]	30.0 ± 11.4[b]	15.16 ± 15.12[b]	205.30 ± 48.92[a]	82.02 ± 8.86	21.71 ± 1.76
Model	62.0 ± 23.7	98.8 ± 40.0	35.73 ± 17.90	563.82 ± 335.54	89.57 ± 7.59	29.20 ± 6.17
FFBJRGP-treated	50.02 ± 3.7	57.2 ± 30.0[b]	30.18 ± 9.41	456.18 ± 410.83	85.46 ± 7.51	28.02 ± 9.45
Colchicine-treated	46.1 ± 14.8	66.0 ± 33.2[a]	34.08 ± 9.19	313.17 ± 230.06[a]	88.61 ± 8.97	29.22 ± 7.95

[a]$P < 0.05$, [b]$P < 0.01$ *vs* model group. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PCⅢ: Type Ⅲ procollagen; HA: Hyaluronic acid; LN: Laminin; CIV: Type Ⅳ collagen; FFBJRGP: Fufang Biejia Ruangan Pill.

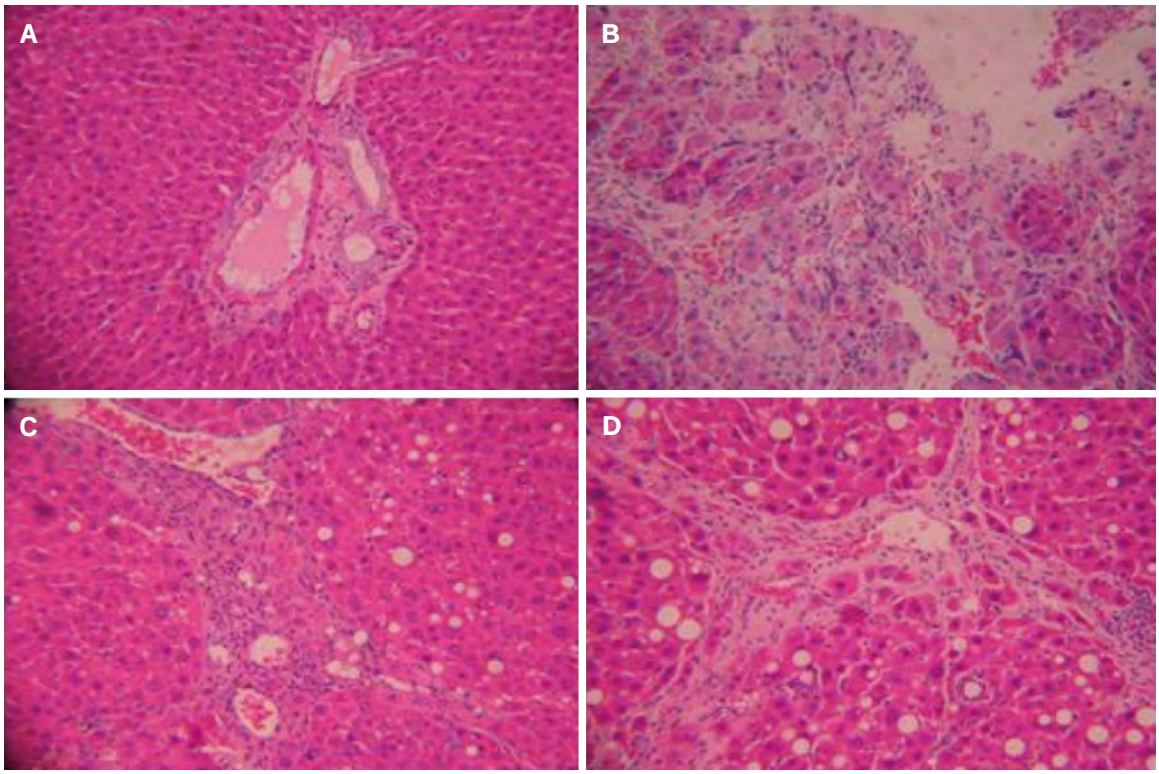

Figure 1 **Histological profiles of liver tissues in rats.** A: Normal rats; B: Rats with hepatic fibrosis; C: Fufang Biejia Ruangan Pill-treated rats; D: Colchicine-treated rats (stained with hematoxylin and eosin, × 100).

activities among the experimental groups. The ALT and AST activities in the model group were significantly higher compared with those in the normal control group ($P < 0.01$), while those in the FFBJRGP-treated group (0.55 g/kg) were significantly lower than in the model group ($P < 0.01$), and those in the colchicine-treated group (0.1 g/kg) were also lower than in the model group ($P < 0.05$) (Table 1).

Effect of FFBJRGP on serum levels of PCⅢ, HA, LN and CIV

The serum levels of PCⅢ, HA, LN and CIV were significantly increased in the model group, as serum markers of hepatic fibrosis, when compared with the normal control group. The FFBJRGP-treated (0.55 g/kg) and colchicine-treated (0.1 g/kg) groups had decreased serum levels of PCⅢ, HA, LN and CIV (Table 1).

Effect of FFBJRGP on hepatic histopathology

At the end of the study, normal hepatic lobules, without fibroplasia and inflammatory cell infiltration, were observed in normal rats (Figure 1A). Many inflammatory cells infiltrated the intra- and inter-lobular areas, and cell degeneration, focal necrosis and bile duct proliferation were found in rats with hepatic fibrosis (Figure 1B). The histological pattern of the livers treated by FFBJRGP showed a low level of infiltration of leukocytes, necrosis, and bile duct proliferation (Figure 1C). Similar trends were also observed in the colchicine group (Figure 1D).

Effect of FFBJRGP on hepatic collagen deposition

The rat liver was stained with Ponceau S, which showed the collagen fibers as red. Normal hepatic lobules without fibroplasia were observed in normal rats. Complete septa interconnecting with each other were formed, which divided the parenchyma into separate fragments in the model group. The rats treated with FFBJRGP and colchicine had less pronounced destruction of the liver architecture, with decreased collagen deposition (Table 2, Figure 2).

Table 2 Liver histopathological semiquantitative scores (mean ± SD)

Group	*n*	Scores					Staging scores
		0	I	II	III	IV	
Control	6	6					0.00 ± 0.00[b]
Model	13				2	11	26.08 ± 5.85
FFBJRGP-treated	9				4	5	20.33 ± 6.12[b]
Colchicine-treated	9		1	3	5	2	19.00 ± 6.38[b]

[b]$P < 0.01$ *vs* model group. FFBJRGP: Fufang Biejia Ruangan Pill.

Effect of FFBJRGP on TGF-β1 and Smad3 expression in liver

The expression of TGF-β1 and Smad3 in the rat liver was quantified. Expression of TGF-β1 was twofold higher in the model group than in the normal control group. FFBJRGP and colchicine therapy significantly decreased TGF-β1 expression (Table 3). Compared with the normal control group, the expression of Smad3 in the model group was increased ($P < 0.01$). Compared with the model group, expression of Smad3 was decreased in the FFBJRGP and colchicine groups (Table 3, Figure 3).

FFBJRGP significantly suppresses HSC-LX-2 cell proliferation

The antiproliferative activity in HSC-LX-2 cells was determined by cell viability using the MTT assay. HSC-LX-2 cell proliferation was inhibited by FFBJRGP. Compared with the blank group (100%), FFBJRGP at a dose of 0.55 g/kg inhibited HSC-LX-2 cell proliferation by 31%, and colchicine at a dose of 0.1 g/kg inhibited proliferation by 28%. The antiproliferative effects were not related to the nonspecific cytotoxic effects of FFBJRGP because cells showed normal morphology.

FFBJRGP significantly reduces hydroxyproline content

To assess the effect of FFBJRGP on ECM production in HSC-LX-2 cells, hydroxyproline content was examined. Hydroxyproline content was decreased in the FFBJRGP group (1.78 ± 0.06 μg/mL, $P < 0.01$) compared with the blank group (2.35 ± 0.12 μg/mL), and it was also decreased in the colchicine group (1.91 ±0.14 μg/mL, $P < 0.01$).

Effect of FFBJRGP on cell cycle

Flow-cytometric assays were carried out to evaluate the effect of FFBJRGP on the cell cycle of activated HSC-LX-2 cells. Compared with the blank control, FFBJRGP altered the percentage of cells in the G_0/G_1 and S phases. The percentage of cells in the G_0/G_1 phase was increased in the FFBJRGP group (52.6% ± 1.2%, $P < 0.01$) compared with the blank group (46.7% ± 0.0%), and the percentage of cells in S phase was decreased in the FFBJRGP group (34.9% ± 7.9%) compared with the blank group (42.1% ± 0.5%). However, the change in the percentage of cells in the G_0/G_1 and S phases was not obvious in the colchicine group.

DISCUSSION

Hepatic fibrosis is thought to be a reversible disease, however, at present there is no satisfactory method in clinical practice to reverse the pathological process. Several drugs, including antisense TGF-β receptor, cytokines[9], antioxidants, chemical drugs, soluble type Ⅱ receptor of TGF-β1, and TGF-β1 antibody have been used to block experimental hepatic fibrosis, but their effects are not as promising as we expected. Besides, some traditional Chinese drugs are effective in preventing fibrogenesis and other causes of chronic liver injury[10], and this offers more hope for the future control of liver fibrosis and cirrhosis[11]. These drugs have the advantages of being cheap, safe and easy to acquire, but most of them are limited in animal experiments and clinical observation, and systematic study at molecular level is lacking.

The activation of HSCs by cytokines is considered to be of importance during the long duration of liver fibrosis. These activated HSCs then become the main source of most cytokines and collagen. Among the cytokine-mediating factors, TGF-β1 is an essential profibrogenic factor[12-17]. In addition, the TGF-β-Smad signaling pathway is the main pathway of TGF-β1[18-20], which transfers the stimulating signal from outside into the affected cells. The Smad proteins consist of a large family of transcription factors, which are also found in vertebrates, insects and nematodes. To date, Smads are the only TGF-β receptor substrates with the ability to propagate signals. Two different transmembrane protein serine/threonine kinases, named as TGF-β receptor type Ⅰ and Ⅱ, are brought together by the ligand, which acts as a receptor assembly factor[21]. Before this occurs, receptor Ⅰ is inactive because a wedge-shaped GS region is inserted into the kinase domain, dislocating the catalytic center. During TGF-β signal transduction, receptor Ⅱ is activated firstly. TGF-β and its receptor then form an activated complex. In the ligand-induced complex, activated receptor Ⅱ phosphorylates the GS region of receptor type Ⅰ, resulting in the activation of the receptor Ⅰ kinase. The type Ⅰ receptors specifically recognize the Smad subgroup known as receptor-activated Smads (R-Smads), which are Smad 2 and Smad 3[22]. R-Smads are activated and form a complex consisting of R-Smads and Smad 4, which belongs to Co-Smad. The Smads complex accumulates in the nucleus. This procedure leads to the formation of the functional transcriptional complexes. The R-Smads and Co-Smads in this complex may participate in DNA binding and recruitment of transcriptional cofactors[23]. CREB binding protein is the main downstream molecule and the general transcriptional coactivator. After transfer into the nucleus, the transcriptional complex binds to the certain domain of the target gene and causes gene expression, such as collagen production. Excess collagen production

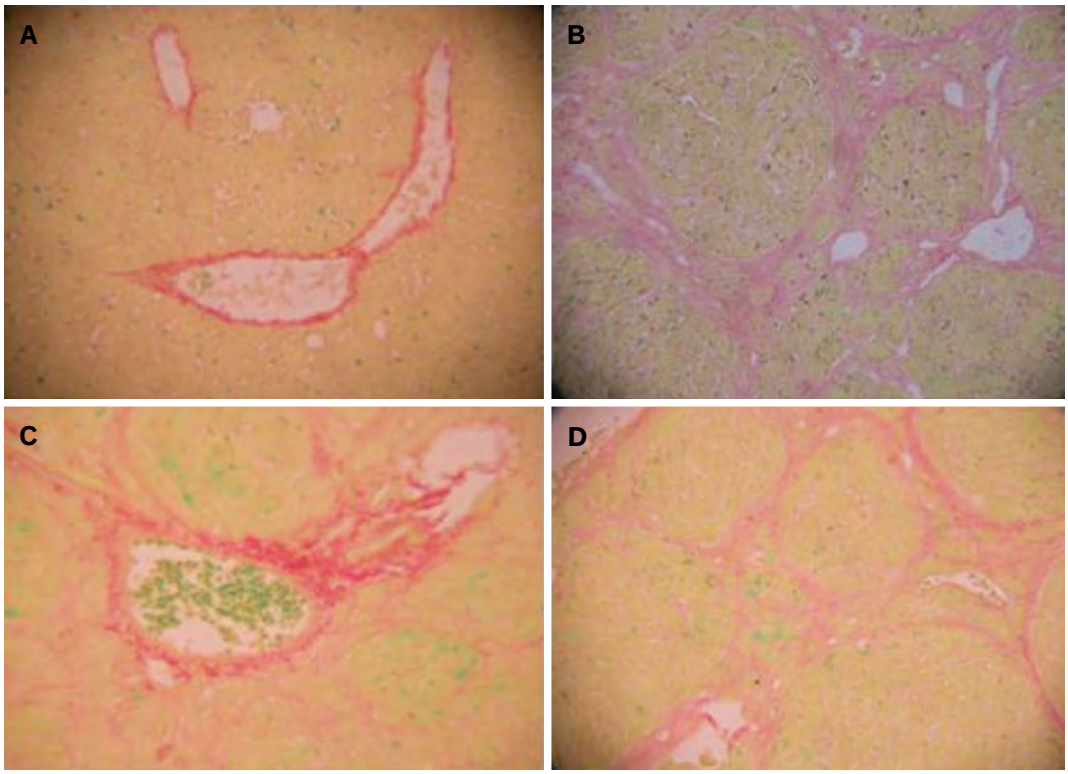

Figure 2 Profiles of liver tissues in rats. A: Normal rats; B: Rats with hepatic fibrosis; C: Fufang Biejia Ruangan Pill-treated rats; D: Colchicine-treated rats (stained with Ponceau S, × 100).

Table 3 Expression of transforming growth factor-β1 and Smad3 (mean ± SD)

Groups	TGF-β1/β-actin	Smad3/β-actin
Control	1.00 ± 0.00[b]	0.62 ± 0.08[b]
Model	3.29 ± 2.08	1.33 ± 0.10
FFBJRGD-treated	2.08 ± 0.57	0.95 ± 0.12[b]
Colchicine-treated	2.25 ± 0.82	1.15 ± 0.06[b]

[b]$P < 0.01$ *vs* model group. TGF-β: Transforming growth factor-β; FFBJRGP: Fufang Biejia Ruangan Pill.

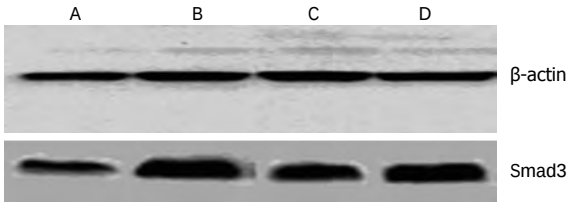

Figure 3 **Western blotting for Smad3 expression in rats.** A: Normal rats; B: Rats with hepatic fibrosis; C: Fufang Biejia Ruangan Pill-treated rats; D: Colchicine-treated rats.

leads to collagen deposition in liver tissues and eventually hepatic fibrosis or cirrhosis. The TGF-β-Smad signaling pathway is important in the formation of hepatic fibrosis, therefore, blocking its transduction may inhibit hepatic fibrosis. Inhibition of the TGF-β-Smad signaling pathway or modulating the gene expression of certain Smads can interfere with hepatic fibrosis[24].

Hepatic fibrosis is characterized by abnormal accumulation of ECM proteins, particularly collagen. The main collagen-producing cells in the liver are HSCs, which proliferate and undergo a process of activation during the development of fibrosis, resulting in increased capacity for collagen synthesis. A simple and reproducible tool is necessary to assess accurately the degree of hepatic fibrosis in clinical practice.

According to the theory of traditional Chinese medicine, hepatic fibrosis is characterized by internal damp (Shi), heat (Re), poison (Du), blood stasis (Yu), and both Qi and Yin asthenia[25,26]. In the present study, not only CCl_4, but also cholesterol, lard and alcohol were used to establish a model of hepatic fibrosis. CCl_4 is poison, and cholesterol, lard and alcohol produce damp and heat, which cause healthy energy asthenia, blood stasis exacerbation, unrelievable damp and heat, and induce hepatic fibrosis. This model well simulates these symptoms. The serum markers of ECM have been used for the assessment of hepatic fibrosis because they are neither invasive nor unavailable. Serum levels of CⅣ, PCⅢ, HA and LN are positively correlated with the inflammatory activity and degree of hepatic fibrosis. Hydroxyproline content in the liver is considered another index of collagen metabolism and provides valuable information about the biochemical and pathological states of liver fibrosis. The present study demonstrated that consumption of FFBJRGP prevented the development of hepatic fibrosis in a rat model of CCl_4-induced liver fibrosis. The results were confirmed by both liver histology and quantitative measurement of serum levels of CⅣ, PCⅢ, HA and LN.

Accordingly, inhibition of proliferation, and reduced collagen content, were also observed in activated HSC-LX-2 cells following FFBJRGP treatment. We also found that FFBJRGP downregulated the expression of TGF-β1 and Smad3, and altered the percentage of cells in the G_0/G_1 and S phases.

In conclusion, the traditional Chinese medicine FFBJRGP shows significant antifibrotic effects. Inhibiting activation of TGF-β/Smad signaling may be an underlying mechanism by which FFBJRGP protects against chronic liver disease associated with fibrosis.

COMMENTS

Background

In China, the incidence of hepatic cirrhosis is still high. Hepatic cirrhosis develops from fibrosis. If treated properly at the fibrosis stage, cirrhosis can be prevented. Fufang Biejia Ruangan Pill (FFBJRGP), a Chinese medical product, is used extensively for the treatment of hepatic fibrosis. FFBJRGP has better antifibrotic efficacy due to its effects of "softening and resolving hard masses, dissolving blood stasis and detoxication, replenishing Qi and Blood" in the philosophy of traditional Chinese medicine. However, the underlying therapeutic mechanisms of FFBJRGP in hepatic fibrosis are still unclear, even though it has become the best-selling traditional Chinese medicine. Thus, in the present study, the authors investigated the antifibrotic effect and potential mechanisms of action of FFBJRGP in hepatic fibrosis, in order to establish the clinical efficacy and make better application of FFBJRGP.

Research frontiers

Recent research shows that hepatic fibrosis can be reversed by regulating collagen metabolism, inhibiting the activation of hepatic stellate cells (HSC), or by promoting HSC apoptosis. Hepatic extracellular matrix mainly results from HSCs, which can be activated by the fibrogenesis signaling pathway.

Innovations and breakthroughs

This study confirmed that FFBJRGP can inhibit hepatic fibrosis *in vivo* and *in vitro*. FFBJRGP can improve liver function, inhibit collagen deposition, alleviate hepatic injury, inhibit HSC-LX-2 cell proliferation, and redistribute the cell cycle, which is probably associated with its downregulation of the fibrogenic transforming growth factor (TGF)-β-Smad signaling pathway.

Applications

FFBJRGP can inhibit hepatic fibrosis *in vivo* and *in vitro*, which implies it is a good drug for patients with hepatic fibrosis. This study provides scientific data for its better application.

Terminology

FFBJRGP is a Chinese medicine that can inhibit hepatic fibrosis. HSCs are key cells that can produce a considerable amount of extracellular matrix and promote collagen deposition. TGF-β1-Smads is a fibrogenic signal transduction pathway that can activate HSCs and promote collagen synthesis.

Peer review

This study describes the antifibrogenic effects of the Chinese herbal medicine FFBJRGP. The data strongly suggests that FFBJRGP may be therapeutically useful in patients with hepatic fibrosis.

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BRIEF ARTICLE

Prevalence and features of fatty liver detected by physical examination in Guangzhou

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Abstract

AIM: To investigate the prevalence of fatty liver discovered upon physical examination of Chinese patients and determine the associated clinical characteristics.

METHODS: A total of 3433 consecutive patients who received physical examinations at the Huangpu Division of the First Affiliated Hospital at Sun Yat-sen University in Guangzhou, China from June 2010 to December 2010 were retrospectively enrolled in the study. Results of biochemical tests, abdominal ultrasound, electrocardiography, and chest X-ray were collected. The diagnosis of fatty liver was made if a patient met any two of the three following ultrasonic criteria: (1) liver and kidney echo discrepancy and presence of an increased liver echogenicity (bright); (2) unclear intrahepatic duct structure; and (3) liver far field echo decay.

RESULTS: The study population consisted of 2201 males and 1232 females, with a mean age of 37.4 ± 12.8 years. When all 3433 patients were considered, the overall prevalence of hyperlipidemia was 38.1%, of fatty liver was 26.0%, of increased alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) levels was 11.9%, of gallstone was 11.4%, of hyperglycemia was 7.3%, of hypertension was 7.1%, and of hyperuricemia was 6.2%. Of the 2605 patients who completed the abdominal ultrasonography exam, 677 (26.0%) were diagnosed with fatty liver and the prevalence was higher in males (32.5% *vs* females: 15.3%, $P < 0.001$). The overall prevalence of fatty liver increased with age, with the peak prevalence (39.5%) found in the 60 to 70-year-old age group. Among patients between the ages of 18 to 50-year-old, the prevalence of fatty liver was significantly higher in males (20.2% *vs* females: 8.7%, $P < 0.001$); the difference in prevalence between the two sexes in patients > 50-year-old did not reach statistical significance. Only 430 of the patients diagnosed with fatty liver had complete information; among those, increased ALT and/or AST levels were detected in only 30%, with all disturbances being mild or moderate. In these 430 patients, the overall prevalence of hypertriglyceridemia was 31.4%, of mixed type hyperlipidemia was 20.9%, of hypercholesterolemia was 12.3%, of hyperglycemia was 17.6%, of hypertension was 16.0%, of hyperuricemia was 15.3%, and of gallstone was 14.4%. Again, the prevalences of hypertriglyceridemia and hyperuricemia were higher in males (hypertriglyceridemia, 36.0% *vs* females: 12.0%, $P < 0.05$; hyperuricemia, 17.3% *vs* females: 7.2%, $P < 0.05$); in contrast, however, the prevalences of mixed type hyperlipidemia and hypercholesterolemia was higher in females (mixed type hyperlipidemia, 18.7%

vs females: 30.1%, $P < 0.05$, hypercholesterolemia, 9.5% *vs* females: 24.1%, $P < 0.05$). Finally, comparison of the fatty liver group to the non-fatty liver group showed that prevalences of hyperlipidemia, hyperglycemia, hypertension, and hyperuricemia were higher in the former (all $P < 0.01$).

CONCLUSION: A high prevalence of fatty liver is detected upon physical examination in Guangzhou, and the primary associated clinical findings are hyperlipidemia, hyperglycemia, hypertension, and hyperuricemia.

Key words: Fatty liver; Nonalcoholic; Prevalence; Hyperlipidemia; Hyperglycemia; Hypertension

Core tip: This study represents the first published investigation of fatty liver prevalence detected by routine physical examinations of individuals residing in the Huangpu District of Guangzhou, China. A high prevalence of fatty liver (26.0%) was detected among the total physical examinees and was characterized by an age-related increasing trend, with the highest prevalence (39.5%) found among individuals between 60 and 70-year-old. The individuals diagnosed with fatty liver also showed significantly higher prevalences of hyperlipidemia, hyperglycemia, hypertension, and hyperuricemia than their non-fatty liver counterparts (all $P < 0.01$), suggesting a close association between fatty liver and dysmetabolic factors.

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INTRODUCTION

Prevalence of fatty liver in China has risen consistently over recent years, accompanying improvements in people's living conditions and adoption of a more Westernized diet. In Western countries, estimates of fatty liver prevalence in the adult population have ranged from 20% to 33%[1], and the most recent prevalence estimate reported for Shanghai, China is 20.82%[2]. In addition to being the most frequently diagnosed liver disease in Chinese clinics, fatty liver represents a particularly alarming threat to human health and public healthcare systems as it can readily progress to steatohepatitis, cirrhosis, or liver cancer.

To gain further understanding about the prevalence and presenting features of fatty liver in China, the current study was designed as a single-site retrospective analysis of adult patients who underwent physical examinations in Guangzhou and were diagnosed with fatty liver.

MATERIALS AND METHODS

Study participants

A total of 3433 consecutive adult patients who underwent routine physical examinations at the Huangpu Division of the First Affiliated Hospital of Sun Yat-sen University from June 2010 to December 2010 were retrospectively enrolled in the study.

Physical examination

Patients presented to the hospital for blood sampling after 10 h of fasting; all serological measurements were carried out on-site at the certified laboratory. Automated techniques (Architect C8000 automatic biochemistry analyzer; Abbott Laboratories, Abbott Park, IL, United States) were used to measure plasma concentrations of glucose, total cholesterol (CHOL), triglyceride (TG), serum uric acid, serum creatinine, blood urea nitrogen, alanine aminotransferase (ALT), and aspartate aminotransferase (AST). Direct sandwich enzyme-linked immunosorbent assay was used to measure hepatitis B virus markers.

Abdominal ultrasonography was performed to detect the presence of fatty infiltration in the liver, using standard imaging criteria to assess hepatic fat[3]. Electrocardiography and chest X-ray were performed to rule out serious heart and lung diseases.

Diagnostic criteria and definitions

Hypertension was diagnosed by a systolic pressure of $\geqslant$ 140 mmHg and/or a diastolic pressure of $\geqslant$ 90 mmHg, according to the 2010 Chinese guidelines for the management of hypertension[4]. Hyperglycemia was diagnosed by fasting plasma glucose level of $\geqslant$ 110 mg/dL[5]. Hyperuricemia was diagnosed by blood uric acid level of $\geqslant$ 7 mg/dL in men and $\geqslant$ 6 mg/dL in women[6]. Abnormal serum creatinine level was defined as $\geqslant$ 1.6 mg/dL. The various types of hyperlipidemia were diagnosed by CHOL $\geqslant$ 200 mg/dL and TG $\geqslant$ 150 mg/dL for mixed type hyperlipidemia, CHOL $\geqslant$ 200 mg/dL and TG < 150 mg/dL for hypercholesterolemia, and CHOL < 200 mg/dL and TG $\geqslant$ 150 mg/dL for hypertriglyceridemia[7]. Fatty liver was diagnosed when a patient met any two of the three following ultrasonic criteria: liver and kidney echo discrepancy and presence of increased liver echogenicity (bright); unclear intrahepatic duct structure; liver far field echo decay[3].

Statistical analysis

All statistical analyses were performed by the SPSS statistical software suite, version 13.0 (Chicago, IL, United States). All reported *P*-values were two-sided, and $P < 0.05$ was considered statistically significant. Descriptive data are expressed as mean ± SD. Comparisons between quantitative data were carried out by the Student's *t*-test, and comparisons between categorical variables were carried out by the χ^2 test.

Table 1 Prevalence of dysmetabolic diseases and biochemical abnormalities in the total study population *n* (%)

	Patients examined	Positive patients
Hyperlipidemia	2715	1035 (38.1)
Fatty liver	2605	677 (26.0)
Increased ALT and/or AST levels	3393	405 (11.9)
Gallstone	2605	296 (11.4)
HBsAg	2244	198 (8.8)
Hyperglycemia	2767	203 (7.3)
Hypertension	2938	210 (7.3)
Hyperuricemia	2700	167 (6.2)
Increased Scr levels	2150	10 (6.2)

HBsAg: Hepatitis B surface antigen; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Scr: Serum creatinine.

RESULTS

Demographic and clinical characteristics of physical examinees

The study population of physical examinees consisted of 2201 males and 1232 females. Three-thousand-two-hundred-and-five of the patients described themselves as employed, with the majority being mental laborers and a small percentage being physical laborers (84.8% and 15.2%, respectively). The mean age of the overall study population was 37.4 ± 12.8-year-old (range: 18-87 years). The overall prevalences of dysmetabolic diseases and perturbed biochemical findings are listed in Table 1.

Characteristics of the 677 patients diagnosed with fatty liver

Of the 2605 subjects who underwent abdominal ultrasonography, 677 subjects (26.0%) showed imaging signs of fatty liver. The prevalence of fatty liver was significantly higher in males than in females (32.5% *vs* 15.3%, $P < 0.001$). The overall prevalence of fatty liver increased with age, with the 60 to 70-year-old age group representing the peak prevalence (39.5%) and without age bias. However, when the larger age group of 18 to 50-year-old was considered, a significantly higher prevalence was found for males (20.2% *vs* females: 8.7%, $P < 0.001$); this trend did not exist for the > 50-year-old age group (34.6% *vs* females: 38.9%, $P = 0.301$) (Table 2). The overall prevalences of dysmetabolic diseases and perturbed biochemical findings for the 677 patients with fatty liver are listed in Table 3.

Characteristics of the 430 patients with fatty liver and complete physical examination data

In total, 430 of the patients diagnosed with fatty liver also had complete data accounting for all of the physical examination components; this group was comprised of 347 males (80.7%) and 83 females (19.3%), with a mean age of 43.8 ± 12.4-year-old (range: 19-78 years). One-hundred-and-twenty-nine patients (30.0%) showed mildly or moderately increased ALT and/or AST levels (40-200 U/L); however, the amount of patients with increased ALT was significantly higher than of patients with increased AST (29.8% *vs* 7.9%, $\chi^2 = 67.2$, $P < 0.001$). The majority of patients with abnormal ALT and/or AST levels showed a mild increase, and included 102 patients (70.1%) with ALT and/or AST levels < 2-times the upper normal limit and 27 patients (20.9%) with ALT and/or AST levels 2 to 5-times the upper normal limit. Males were more likely to have increased ALT and/or AST levels (32.6% *vs* 19.3%, $P < 0.05$).

The overall prevalences of dysmetabolic diseases and perturbed biochemical findings for the 430 patients with fatty liver and complete data are listed in Table 4. The prevalences of hypertriglyceridemia and hyperuricemia were higher in males than in females, but the prevalences of mixed type hyperlipidemia and hypercholesterolemia were higher in females than in males ($P < 0.05$). The difference in the prevalence of hypertension or hyperglycemia between males and females did not reach statistical significance ($P > 0.05$).

Risk factors associated with fatty liver

Patients with no signs of fatty liver and hepatitis B surface antigen negativity were assigned to a non-fatty liver group, which included 382 males (77.2%) and 113 females (22.8%), with a mean age of 42.5 ± 10.9-year-old. Compared to the fatty liver group, the sex and age distributions were not significantly different ($P > 0.05$). As shown in Table 5, the prevalences of hyperlipidemia, hyperglycemia, hypertension, and hyperuricemia were significantly higher in the fatty liver group than in the non-fatty liver group ($P < 0.01$).

DISCUSSION

Fatty liver is a clinicopathologic syndrome that manifests from hepatic steatosis and excessive fat accumulation caused by a variety of factors. The syndrome spectrum includes simple fatty liver, steatohepatitis, fatty liver cirrhosis, and associated hepatocellular carcinoma. While liver biopsy is the gold standard for diagnosis of fatty liver, ultrasonography is generally used as a non-invasive screening method for the general population. The reported estimates of fatty liver cases diagnosed by ultrasonography have ranged from 17% to 46% in Europe, United States and other Asian countries[8-11]. In the current survey of physical examinees in Huangpu District, Guangzhou, the prevalence of fatty liver detected in physical examination was 26%; this rate is similar to previous estimates made in other Chinese cities[12,13].

It is generally recognized that the prevalence of fatty liver increases with age, with the highest rates found in the age group of 50 to 70-year-old[10,14]. In the present study, the highest prevalence of fatty liver occurred in the age group of 60 to 70-year-old. It is important to note that elderly people harbor significantly more of the known risk factors for fatty liver, such as obesity, hypertension, diabetes, and hyperlipidaemia. Furthermore, aging brings restrictions on physical mobility, which in turn supports or promotes the above risk factors and can eventually lead to a higher prevalence of fatty liver[15].

Table 2 Age and sex distribution of overall fatty liver prevalence

Age (yr)	All patients	Patients with fatty liver	Overall prevalence	Prevalence in males	Prevalence in females	χ^2	*P* value
18-19	17	1	5.80%	7.10%	0.00%	-	-
20-29	1055	55	5.20%	6.40%	2.30%	7.47	0.006
30-39	1003	206	20.50%	26.70%	8.70%	44.80	< 0.001
40-49	799	212	26.50%	34.50%	16.80%	31.78	< 0.001
50-59	331	123	37.20%	35.90%	39.00%	0.32	0.569
60-69	147	58	39.50%	37.80%	42.10%	0.27	0.601
⩾ 70	81	22	27.10%	24.50%	32.10%	0.54	0.464
Total	2605	677	26.00%	32.50%	15.30%	95.18	< 0.001

Table 3 Prevalence of dysmetabolic diseases and biochemical abnormalities in the 677 patients diagnosed with fatty liver *n* (%)

	Patients examined	Positive patients
Increased ALT and/or AST levels	676	199 (29.4)
Hypertriglyceridemia	635	197 (31.0)
Mixed type hyperlipidemia	635	135 (21.3)
Hypercholesterolemia	635	92 (14.5)
Hyperglycemia	640	116 (18.1)
Hypertension	636	102 (16.0)
Gallstone	677	97 (14.4)
Hyperuricemia	627	87 (13.9)
HBsAg	506	39 (7.7)
Increased Scr levels	507	3 (0.6)

HBsAg: Hepatitis B surface antigen; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Scr: Serum creatinine.

Table 4 Sex distribution of prevalence of dysmetabolic diseases and biochemical abnormalities in the 430 subjects with fatty liver *n* (%)

	All patients	Male patients	Female patients	χ^2	*P* value
Increased ALT and/or AST levels	129 (30.0)	113 (32.6)	16 (19.3)	5.63	0.018
Hypertriglyceridemia	135 (31.4)	125 (36.0)	10 (12.0)	17.87	< 0.001
Mixed type hyperlipidemia	90 (20.9)	65 (18.7)	25 (30.1)	5.25	0.022
Hypercholesterolemia	53 (12.3)	33 (9.5)	20 (24.1)	13.19	< 0.001
Hyperglycemia	76 (17.6)	59 (17.0)	17 (20.5)	0.56	0.455
Hypertension	69 (16.0)	53 (15.3)	16 (19.3)	0.80	0.372
Hyperuricemia	66 (15.3)	60 (17.3)	6 (7.2)	5.22	0.022
Gallstone	62 (14.4)	50 (14.4)	12 (14.4)	0.00	0.991
HBsAg	34 (7.9)	27 (7.9)	7 (7.9)	0.04	0.843
Increased Scr levels	3 (0.7)	3 (0.9)	0 (0.0)	-	-

HBsAg: Hepatitis B surface antigen; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Scr: Serum creatinine.

Most studies have shown that men are more likely to develop fatty liver than women before the age of 50 years, but both sexes face a similar likelihood of developing the condition after 50[16,17]. Similarly, a study involving 26527 Chinese subjects who underwent routine health check-ups showed that the prevalence of fatty liver was 31% in men and 16% in women[18].

The significant difference in the prevalence of fatty liver between men and women before the age of 50 is probably a result of the clear differences in the amount and distribution of body fat between the sexes. Men usually store fat in the abdomen whereas women tend to store fat in the subcutaneous tissue. While the reasons for this differential fat accumulation in men and women remain unclear, evidence from cell research have suggested that lipid metabolism pathways may play important roles. Moreover, molecular studies have uncovered distinctions between men and women in the activity and metabolism of lipids. A Japanese study showed that the triglyceride and cholesterol particles were larger in men than those in women, and both of these factors are associated with risk for fatty liver[15,19].

The fact that the significant difference in prevalence of fatty liver among men and women is lost after the age of 50 is intriguing. Women of this age have decreased adiponectin levels, as a result of the lower estrogen and higher androgen that occur after menopause[20,21]; the resetting of postmenopausal women's physiology to that which more closely resembles the male physiology may account for the similar prevalence of fatty liver between the sexes at this age. The Chinese study mentioned above also showed that the mean ALT levels in men were significantly higher than those in women before 50; yet, the peak levels of ALT were observed in women older than 50 years, which might be related to menopause changes and the decreased physical exercise that frequently accompanies this period of life[18]. Nonetheless, these previously published findings, along with ours presented herein, highlight the importance of prevention and screening of fatty liver in men and postmenopausal women.

Most patients with fatty liver are diagnosed without or with mild clinical symptoms. In a study of fatty liver clinical characteristics by Powell *et al*[22], 79% of diagnosed patients were shown to have normal serum transaminase levels. In the current study, 30% of the patients diagnosed with fatty liver presented with mildly increased ALT and/or AST levels, and most of those were accounted for by ALT increase. Thus, the most common type of fatty liver was nonalcoholic fatty liver disease (NAFLD). Moreover, the fatty liver group showed higher prevalences of hyperlipidemia, hyperglycemia, hypertension, and hyperuricemia as compared to patients without fatty liver, suggesting that fatty liver may be closely associated with these disorders.

Risk factors known to be associated with NAFLD include metabolic syndrome, diabetes, and obesity. Prevalence estimates of NAFLD have ranged from 40% to 70% in patients with type 2 diabetes mellitus (T2DM)[23],

Table 5 Distribution of prevalence of risk factors in patients with and without fatty liver

	Patients with fatty liver (n = 430)		Patients without fatty liver (n = 495)		χ^2	P value
	All patients	Prevalence	All patients	Prevalence		
Hypertriglyceridemia	135	31.40%	45	9.10%	73.04	< 0.001
Mixed type hyperlipidemia	90	20.90%	33	6.70%	40.61	< 0.001
Hypercholesterolemia	53	12.30%	95	19.20%	8.07	0.004
Hyperglycemia	76	17.70%	25	5.00%	37.70	< 0.001
Hypertension	69	16.00%	32	6.50%	21.72	< 0.001
Hyperuricemia	66	15.30%	14	2.80%	45.66	< 0.001

57%-74% in individuals with obesity, and 27%-92% in patients with hyperlipidemia[24,25]. Donati *et al*[26] showed that the prevalence of NAFLD in the patients with hypertension but without obesity or T2DM was 2 to 3-times higher than that in the general population. Assy *et al*[25] showed that up to 50% of the patients with fatty liver were dyslipidemic, and that this dysmetabolic condition was chiefly characterized by high serum TG levels, which itself is an important risk factor for cardiovascular disease.

NAFLD is closely related to incidence of cardiovascular disease. In fact, the most common causes of death in patients with NAFLD are atherosclerotic cardiovascular disease and hepatic cirrhosis[27]. Therefore, clinicians should not only consider central obesity, type 2 diabetes, dyslipidemia and hypertension as risk factors for NAFLD, but also pay more attention to them as high risk factors for cardiovascular, kidney and liver diseases. A key strategy for clinical treatment of NAFLD is to reduce the above risk factors, and this can be accomplished by applying the existing knowledge to generate effective public health policies for the prevention of this disease.

In summary, a high prevalence of fatty liver was discovered in physical examinees in Guangzhou. Some of the cases presented with mild or moderate increase in ALT or AST levels, but many had concomitant hyperlipidemia, hyperglycemia, hypertension, or hyperuricemia. Clinicians should pay attention to the intervention and modification of these risk factors. It is important to note, however, that the retrospective nature of this study limits the risk factors that were available for analysis; for example, data on waist circumference, body mass index, dietary habits, and alcohol consumption - all potential risk factors - were lacking. In addition, the single-site population may limit generalization of our results. More studies of larger Chinese populations are needed to gain more detailed information on fatty liver in the general population and to better guide clinical treatment.

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COMMENTS

Background

The incidence of fatty liver is relatively high and on the rise in urban populations of China; however, consultation rates are low due to a lack in adequate knowledge of fatty liver. To date, no study has examined the prevalence and clinical features of fatty liver in urban Chinese who receive routine physical examinations. Thus, this study was designed to retrospectively investigate the prevalence and presenting features of fatty liver in physical examinees of Guangzhou.

Research frontiers

Public health programs of screening, education, and treatment of fatty liver should start with employed urban Chinese, who are generally characterized as relatively well-educated, financially secure, and compliant. The results of this study may offer guidance for clinical treatment by analyzing the presenting features of fatty liver in this population.

Innovations and breakthroughs

This study is the first to assess the presenting features of fatty liver in urban Chinese who received routine physical examinations in Guangzhou. The disease was found to be closely associated with concomitant hyperlipidemia, hyperglycemia, hypertension, or hyperuricemia. The results from this study, which itself is part of a continuous clinical research effort for determining fatty liver diagnostic and prognostic factors, are applicable to the development of new programs for screening and education of fatty liver targeting urban Chinese.

Applications

This study was undertaken mainly for practical purposes, *i.e.*, to raise public awareness of fatty liver and support performance of screening in the general population, which are expected to improve consultation rates and timely initiation of treatment for fatty liver.

Peer review

This study represents the first published investigation of fatty liver prevalence detected by routine physical examinations of individuals residing in the Huangpu District of Guangzhou; the results suggest a close association between fatty liver and dysmetabolic factors. This study was undertaken mainly for practical purposes, such as to raise public awareness of fatty liver and the benefits of screening the general population for this disease so that cases may be diagnosed and treated in a timely manner.

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BRIEF ARTICLE

Clonality analysis of neuroendocrine cells in gastric adenocarcinoma

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Abstract

AIM: To achieve a better understanding of the origination of neuroendocrine (NE) cells in gastric adenocarcinoma.

METHODS: In this study, 120 cases of gastric adenocarcinoma were obtained. First, frozen section-immunohistochemistrical samples were selected from a large quantity of neuroendocrine cells. Second, laser capture microdissection was used to get target cells from gastric adenocarcinoma and whole genome amplification was applied to get a large quantity of DNA for further study. Third, genome-wide microsatellite abnormalities [microsatellite instability (MSI), loss of heterozygosity (LOH)] and *p53* mutation were detected by polymerase chain reaction (PCR)-single-strand conformation polymer- phism-silver staining and PCR-sequencing in order to identify the clonality of NE cells.

RESULTS: The total incidence rate of MSI was 27.4%, while LOH was 17.9%. Ten cases had a highest concordance for the two types of cells. The other samples had similar microsatellite changes, except for cases 7 and 10. Concordant *p53* mutations exhibited in sample 4, 14, 21 and 27, and there were different mutations between two kinds of cells in case 7. In case 17, mutation took place only in adenocarcinoma cells. *p53* mutation was closely related with degree of differentiation, tumor-node-metastasis stage, vessel invasion and lymph node metastasis. In brief, NE and adenocarcinoma cells showed the same MSI, LOH or *p53* mutation in most cases (27/30). In the other three cases, different MSI, LOH or *p53* mutation occurred.

CONCLUSION: NE and the gastric adenocarcinoma cells may mainly derive from the same stem cells, but the remaining cases showing different origin needs further investigation.

Key words: Neuroendocrine differentiation; Clonal analysis; Gastric adenocarcinoma; Neuroendocrine cells

Core tip: There have been only a few studies of neuroendocrine differentiation (NED) in gastric adenocarcinoma. Therefore, we studied the clonality of neuroendocrine (NE) cells in gastric adenocarcinoma using laser capture microdissection, microsatellite instability (MSI), loss of heterozygosity (LOH) and *p53* mutation to evaluate the clonality of NED. NE and adenocarcinoma cells showed the same MSI, LOH or *p53* mutation in most cases (27/30), they may originate from the same stem cells, but the remaining three cases showed different origins, which warrants further research.

Wang LL, Yao GY, Zhao ZS, Wei XL, Xu RJ. Clonality analysis of neuroendocrine cells in gastric adenocarcinoma. *World J Gastroenterol* 2013; 19(32): 5340-5346 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5340.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5340

INTRODUCTION

Although the worldwide incidence and mortality of gastric cancer have been declining steadily, it remains one of the most common cancers and the leading cause of cancer death worldwide[1]. Previous studies have reported that mixed glandular-neuroendocrine (NE) tumors that arise from the gastrointestinal tract, such as the stomach and colon, normally contain both glandular and endocrine cells[2,3]. These studies have suggested that mixed tumors occur as a consequence of multidirectional differentiation of glandular of endocrine stem cells that are derived from the endoderm. However, it remains unclear whether the glandular and endocrine cells expand from two distinct precursors, or arise from a single progenitor cell.

Microsatellite instability (MSI) is a form of genetic instability that is characterized by new alleles that are not present in the normal genotype. This type of mutation occurs in various human carcinomas[4], and is believed to be caused by altered DNA mismatch repair genes. Several genetic alterations have been shown to play a significant role in tumourigenesis. The most frequently observed molecular changes occur in the *p53* gene[5]. There is now enough evidence to suggest that the functional inactivation of the *p53* gene through allelic loss and point mutation plays an important role[6]. The *p53* gene encodes a protein that is involved in control of the cell cycle and acts as a negative regulator in the cell response to damaged DNA. The most widely used molecular approach is single-strand conformation polymorphism (SSCP) analysis of DNA fragments amplified by the polymerase chain reaction (PCR), with subsequent sequence analysis. Functional alteration of p53 protein can occur through several mechanisms: point mutations, deletions, rearrangements in the *p53* gene, binding with viral proteins, binding with cellular proteins, and oligomerization[7]. Wild-type p53 protein has a very short half-life, whereas mutated p53 is stable and can accumulate at high concentrations in the nuclei of tumor cells. As a consequence, immunohistochemical staining with specific antibodies can be used to detect mutant p53 protein.

To achieve a better understanding of the origination of NE cells in gastric adenocarcinoma, and provide a clear method of evaluation to clinicians, we performed a prospective study on neuroendocrine differentiation in gastric adenocarcinoma by analyzing MSI, loss of heterozygosity (LOH) and *p53* mutation.

MATERIALS AND METHODS

Frozen section immunohistochemistry

In this study, 120 cases of gastric adenocarcinomas and corresponding non-neoplastic gastric mucosal tissues were obtained from the People's Hospital of Zhejiang Province, China. The tumors were staged according to the tumor-node-metastasis (TNM) classification and were graded according to the World Health Organization classification. Immunohistochemistry was carried out using the primary antibody against NE marker (chromogranin A, polyclonal, 1:100; Maixin, China). In brief, the tissue sections were incubated in methanol for 5 min. After washing with phosphate-buffered saline (PBS), the sections were incubated in 7.5% hydrogen peroxide for 5 min, followed by further washing with PBS. The sections were then incubated with primary antibodies in the case of chromogranin A at 4 ℃ overnight. Then these sections were detected using Two-Step Immunohistochemical Detection Reagent (ZSGB-BIO, Beijing, China). Frozen section immunohistochemistry samples were selected from a large quantity of NE cells. The study was approved by the Ethics Committee for Human Study in our institution.

Laser-capture microdissection

Laser-capture microdissection (LCM) was performed with the use of an Arcturus PixCell II microscope (Arcturus Engineering, Mountain View, CA, United States) to obtain cells from gastric adenocarcinoma. The technology for melting heat of infrared rays was used to melt the polymeride under microscope, followed by molecular biology analysis. Open the instrument, put the complete slice on the objective table, the cell image was exhibited on the computer screen through the microscope. If the cellular morphology was normal, with satisfactory staining, under 10 × 20 lens according to the following conditions: power, 65 mV; duration, 15.5 s; and spots size, 7.5 μm, we attached the transparent Elvax® ethylene vinyl acetate hot plastic film hat by the driving arm to lay aside precisely above the tissue slice. The target cell or the cell group was obtained through the control handle to the slice migration located at the field of vision centre. Press the button according to the target region's size, and the focusing infrared laser beam carries on the capture. When the laser beam launch ended, move the mechanical arm from the slice to emigrate the cover and the thin film, move the hat into 0.5 mL Eppendorf centrifuge tube (add the Micro-kit extraction reagent box extraction buffer solution beforehand), and proceed with the DNA extraction. A 7.5-mm-diameter laser beam was used to procure NE cells and a 15- or 30-mm-diameter beam for adenocarcinoma cells. LCM cells were pooled from multiple caps, which were stored at -20 ℃ until dissection was complete. Approximately 15000 laser hits to each specimen gave the necessary cell yield after transfer. LCM was performed with capture of 500 NE cells and thousands of adenocarcinoma cells from each sample. NE and adenocarcinoma cell populations were stored separately. Cell samples were frozen immediately at -20 ℃, and were sent on the same day, on caps frozen on dry ice, for DNA extraction and subsequent genetic analysis.

DNA extraction and whole-genome amplification

DNA extraction from the captured cells and whole

Table 1 Primer sequences for the analysis of microsatellite instability and *p53* mutations at exons 5-8

Microsatellite	Sequences
D1S104	ATCCTGCCCTTATGGAGTGCCCCAC
	TCCTCTGTCATTGTA
D2S119	CTTGGGGAACAGAGGTCATTGAGA
	ATCCCTCAATTTCTTTGGA
D2S123	AAACAGGATGCCTGCCTTTAGGACT
	TTCCACCTATGGGAC
D3S1766	ACCACATGAGCCAATTCTGTACCCA
	ATTATGGTGTTGTTACC
D3S2427	CTCCTCGTCACTGCAGTCTTCTGCCT
	CATCTGTTCAGGAT
D4S174	AAGAACCATGCGATACGACTCATT
	CCTAGATGGGTAAAGC
D4S402	CTTACTGTGTTGCCCAAGGTAGCTC
	TATGATTCATTTCAAGTTTG
D5S107	GATCCACTTTAACCCAAATACGGC
	ATCAACTTGAACAGCAT
D5S346	ACTCACTCTAGTGATAAATCGGGA
	GCAGATAAGACAGTATTACTAGTT
D5S409	GGGATGAAGTGTGGATAAACTAGG
	ATGGCAGTGCTCTTAG
D7S1805	CCTGCTTTGGCTTACCTGTACCCAC
	TTCTCTGCTATTACATAT
D9S157	AGCAAGGCAAGCCACATTTCTGGG
	GATGCCCAGATAACTATATC
D10S469	CAACAAGTGTGAGAGTCCATATGTT
	CTGTCTCTCCACAGT
AFMA086WG9	ATGTACGGTTCATTGACTTGACTGA
	CTACAAATGGGCA
D11S861	CTGAAACCAAGTGAAAAGGAGAA
	AGCTCCATTGTCTTCTGGC
D12S1899	TTCTTCCTTTCTCTTTCTCTCTTCCGC
	ACAAGTGACACATGGTCC
D16S398	CTTGCTCTTTCTAAACTCCAGAAAC
	CAAGTGGGTTAGGTC
D16S496	GAAAGGCTACTTCATAGATGGCAA
	TATAAGCCACTGCGCCCAT
D16S534	CAACAAAGCAAGACCCTGTCCATC
	TGCGGTTCTTTCCTC
D16S265	AGCTCTCTGAGTCCTCTGTGCGGAA
	GCATGGTGTCTCTCG
D16S752	AATTGACGGTATATCTATCTGTCTG
	GATTGGAGGAGGGTGATTCT
D17S250	GGAAGAATCAAATAGACAATGCTG
	GCCATATATATATTTAAACC
D17S796	CAATGGAACCAAATGTGGTCAGTC
	CGATAATGCCAGGATG
D19S416	CCTGTCCCAGAGAGACCCTAAAGA
	GAGTGTGCCATTTGCT
BAT 25	GTTTCGCCTCCAAGAATGTAAGTGT
	TTCTGCATTTTAACTATGGCTC
BAT 26	TGACTACTTTTGACTTCAGCCAACC
	ATTCAACATTTTAACCC
Exon 5	GACTTTCAACTCTGTCTCCTCTGGG
	GACCCTGGGCAAC
Exon 6	GAGACGACAGGGCTGGGTCCACTG
	ACAACCACCCTT
Exon 7	GTGTTGTCTCCTAGGTTGGCAAGTG
	GCTCCTGACCTGGAG
Exon 8	CCTTACTGCCTCTTGCTTTGAATCTG
	AGGCATAACTGC

genome amplification (WGA) were performed using DNA Micro-kit and DNA Repli-g Midi kit (QIAGEN, Germany) to obtain a large quantity of DNA. The brief processes were as follows: 15 μL buffer ATL (provided in kit) was added to a 0.5-mL microcentrifuge tube that contained the laser-microdissected cells; 10 μL proteinase K was added and mixed by pulse-vortexing for 15 s; the 0.5-mL tube was then placed in a thermomixer or heated orbital incubator, and incubated at 56 ℃ for 3 h, with occasional agitation; 25 μL buffer ATL was added with 50 μL buffer AL, and mixed well by pulse-vortexing for 15 s; 50 μL ethanol (96%-100%) was added and mixed thoroughly by pulse-vortexing for 15 s, incubated for 5 min at room temperature. Then, the entire lysate was carefully transferred to the QIAamp MinElute Column, centrifuged at 8000 *g* for 1 min and placed in a clean 2-mL collection tube; 500 μL buffer AW1 and AW2 (provided in kit) were added, respectively, and centrifuged at 8000 *g* for 1 min, followed by a full speed centrifugation at 14000 *g* for 3 min to dry the membrane completely. The QIAamp MinElute Column was placed in a clean 1.5-mL microcentrifuge tube and 20-30 μL buffer AE was added to the centre of the membrane, incubated at room temperature for 1 min, and finally centrifuged at 14000 *g* for 1 min. The DNA was denatured by adding denaturation buffer and stopped by adding of neutralization buffer that contained DNA polymerase. The isothermal amplification reaction proceeded for at least 8 h at 30 ℃. The method was used based on a technology that carries out isothermal genome amplification utilising a unique processive DNA polymerase, which could replicate up to 100 kb without dissociating from the genomic DNA template. The DNA polymerase had a 3'-5' exonuclease proofreading activity to maintain a high fidelity during replication, and was used with exonuclease-resistant primers to achieve a high yield of DNA product. The final processes were: TE buffer and denaturation solution were added, mixed well and incubated at room temperature for 3 min; neutralization buffer was added, mixed, followed by adding REPLI-g master mix, and incubated for 8-16 h at 30 ℃; and REPLI-g Midi DNA polymerase was inactivated by heating the sample at 65 ℃ for 3 min.

Analysis of MSI, loss of heterozygosity and p53 mutation

We chose 26 microsatellite markers with genome-wide scope for MSI analysis, and chose p53 exons 5-8 for *p53* mutation analysis. The primers for these analyses are listed in Table 1.

Genome-wide microsatellite abnormalities (MSI and LOH) and *p53* mutation were detected by PCR-SSCP silver staining and PCR sequencing to identify the clonality of NE cells. To evaluate microsatellite alterations, extra shadow bands above and below each intense principal allelic band were often visualized in microsatellite studies, and the most intense bands were considered the real alleles.

Statistical analysis

Statistical analyses were performed using SPSS for Windows version 15.0 (SPSS, Chicago, IL, United States). Survival data were analysed using the χ^2 test, Spearman rank correlation analysis, and Kaplan-Meier analysis, and

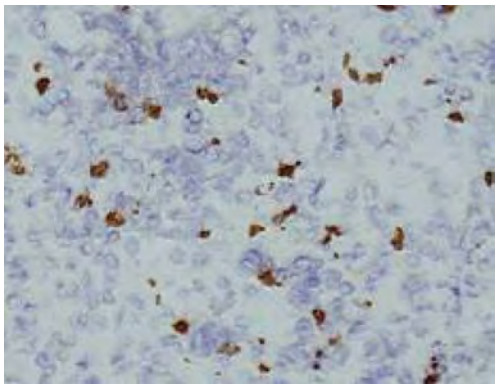

Figure 1 Chromogranin A expression in gastric cancer (× 100).

a survival curve was drawn. Differences were analysed using the log rank test and $P < 0.05$ was considered statistically significant.

RESULTS

Immunohistochemistry and LCM

Thirty samples from a total of 120 that contained a large number of NE cells were selected for LCM. About 500 NE cells were precisely captured from each sample (Figures 1 and 2).

Microsatellite analysis and p53 mutation

The total incidence rate of MSI was 27.4%, and LOH rate was 17.9%. The rates in gastric adenocarcinoma cells and NE cells were similar. There was no significant relationship between the MSI or LOH rate and clinicopathological characteristics. According to the coincidence of microsatellite changes, cases 2, 3, 5, 6, 11, 12, 18, 24, 27 and 30 had a highest concordance for the two types of cells. The other samples had similar microsatellite changes, except for cases 7 and 10 (Figure 3).

Most mutations of the *p53* gene were detected in exons 7 and 8. Concordant mutations were observed in cases 4, 14, 21 and 27, and there were different mutations in the two types of cells (*e.g.*, NE and gastric adenocarcinoma cells) in case 7. In case 17, the mutation was seen only in the adenocarcinoma cells not in the NE cells. *p53* mutation occurred six times in adenocarcinoma cells (20.0%) and five times in NE cells (16.7%). Clinicopathological analysis further showed that *p53* mutations were well associated with poor differentiation and TNM stages Ⅲ or Ⅳ tumors, the mutations were also linked to blood vessel invasion and lymph node metastasis (Table 2).

DISCUSSION

Our previous studies have demonstrated that NED occurred in 41.5% of colon cancers, 39.6% of gastric cancers, 38.1% of prostate cancers, 21% of breast cancers and 17.9% of pancreatic cancers, and NE in gastric adenocarcinoma was more frequently observed in poorly differentiated cancers than in well-differentiated tumors[8],

Figure 2 Images shown before and after laser-capture microdissection (× 200). A: Before laser-capture microdissection (LCM); B: After LCM.

which was different from other studies that showed that NE was associated with well-differentiated tumors[9,10]. However, it is not clear whether NE is derived from embryogenesis, histogenesis, or genetic changes that are associated with tumor etiology. It has been shown that NED occurs in adenocarcinoma of the prostate, gastrointestinal tract and lungs. These NE cells synthesize and excrete neuropeptides or amines hormones, leading to an increase of plasma hormone levels[11-13]. Hirano *et al*[14] found that the prognosis for gastric adenocarcinoma with choriocarcinoma and neuroendocrine cell carcinoma is exceedingly poor. Whereas, the biological functions of NED for the development or prognosis of gastric adenocarcinoma are largely unknown. We thus employed LCM to capture NE cells, distinguished from the gastric adenocarcinoma cells, and utilized molecular and genetic approaches to study the origin of NE cells and their association with gastric cancer biology. We found that the NE cells and gastric adenocarcinoma cells shared similar MSI, LOH and *p53* mutation, meaning both cell lines may be derived from same stem cells.

It has been well known that the NE cells are derived from multipotent stem cells. NED is initiated by hormonal change, microenvironmental change, and genomic instability. Some subdued genomic codes are randomly depressed and selectively activated by more than two regulatory genes during RNA translation, and as a result, multipotent stem cells generate differentiation or multidifferentiation[15]. Despite the apparently different morphological representation of NE cells in the tumor

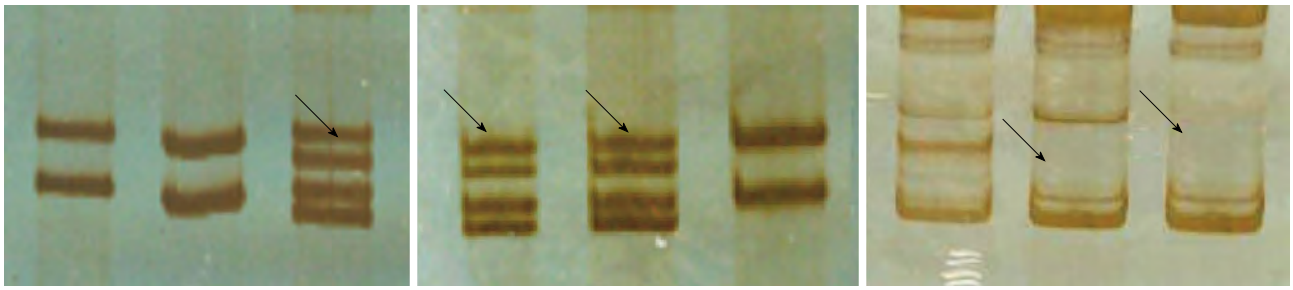

Figure 3 Examples of microsatellite instability and loss of heterozygosity in gastric cancer examined by single-strand conformation polymorphism analysis. NE: Neuroendocrine; T: Tumor samples; N: Normal tissue controls; MSI: Microsatellite instability; LOH: Loss of heterozygosity. Arrows show the increasing and missing of alleles.

Table 2 Concordance of *p53* mutation in gastric cancer and neuroendocrine cells

Case	Cell	Exon	Codon	Mutation	Amino acid	Differentiation	TNM	Metastasis
4	Cancer	8	273	GC→AT	Arg→Cys	Poor	Ⅳ	+
	NE	8	273	GC→AT	Arg→Cys			
7	Cancer	7	244	GC→AT	Gly→Ser	Poor	Ⅳ	+
	NE	8	287	GC→AT	Glu→Lys			
14	Cancer	8	287	GC→AT	Glu→Lys	Poor	Ⅰ B	+
	NE	8	287	GC→AT	Glu→Lys			
21	Cancer	7	244	GC→AT	Gly→Ser	Poor	Ⅳ	+
	NE	7	244	GC→AT	Gly→Ser			
27	Cancer	8	282	GC→AT	Arg→Arg	Moderate	Ⅲ	+
	NE	8	282	GC→AT	Arg→Arg			
17	Cancer	7	244	GC→AT	Gly→Ser	Poor	Ⅲ	+

NE: Neuroendocrine; TNM: Tumour-node-metastasis.

mass, it is largely unknown whether these cells have chromosomal or genetic alterations. Moreover, it is not clear whether NE cells are present as tumor or stromal components. NE cells from gastric adenocarcinoma were harvested by LCM, which ensured cell purity. Whole genome amplification (WGA) was then employed to compare genomic characteristics of NE cells with adenocarcinoma cells, for the identification of the clonality of the former. The development and prognosis of gastric cancer involves a number of genetic and epigenetic abnormalities[16]. MSI is thought to be an important molecular phenotype in gastric cancer[17]. In gastric cancer, the loss of genomic stability represents a key molecular step that occurs early in carcinogenesis, and creates a permissive environment for the accumulation of genetic and epigenetic alterations in tumor suppresser genes and oncogenes. It is widely accepted that gastric cancer can follow at least two major genomic instability pathways: MSI and chromosome instability[18]. LOH and MSI have strong sensitivity but poor specificity, whereas gene mutation has strong specificity but poor sensitivity. The appropriate combination of the two methods can give more precise results. Huang *et al*[19] have demonstrated whether different components of combined tumors contain the same or different genetic alterations, thus providing evidence for their clonality. As a result, he has suggested that, in the majority of combined tumors, cells with different phenotypes share similar genotypes and might arise from a single precursor cell. Only in a minority of these tumors are different areas derived from different precursor cells. Our study suggested that concordant microsatellite changes occurred in two types of cells in cases 2, 3, 5, 6, 11, 12, 18, 24, 27 and 30; different microsatellite changes in cases 7 and 10; and in the remaining 18 cases, there were no significant differences in microsatellite changes in the two types of cells. There was no correlation between MSI and degree of differentiation in gastric cancer. Semba *et al*[20] have suggested that MSI appears at a high frequency in well-differentiated adenocarcinoma, but others have come to the opposite conclusion[21].

Wild-type *p53* acts as an anti-oncogene in normal tissues, which is important in DNA repair and cell cycle regulation. Tumourigenesis is closely associated with *p53* mutation or loss of function[22]. Genetic changes (such as gain or loss of chromosomal segments, or gene mutation) in allelic genes are induced by unbalanced mitosis during stem cell differentiation. These genetic changes could be used for analysis of cell clonality. They can be detected by microsatellite changes (including LOH and MSI), gene mutation, and comparative genomic hybridisation. The functional inactivation of *p53* gene through allelic loss and point mutation plays an important part in the development of gastric cancer. We can detect mutant p53 protein by immunohistochemical staining with specific antibodies[23,24]. Nishikura *et al*[25] have suggested that NE carcinoma is composed of precursor NE cells that are

generated from adenocarcinoma, and p53 promotes this process. While studying gastrointestinal carcinomas, Eren has discovered that *p53* mutation might be associated with NED of adenocarcinoma[26]. The rate of p53 positivity in gastric carcinoma with NED was clearly higher than that in gastric carcinoma without NED. Our study showed that the rate of *p53* mutation in gastric adenocarcinoma cells was 20%, and it was 16.7% in NE cells. In cases 4, 14, 21 and 27, concordant mutations were seen in exons 7 and 8 in the two types of cells; in case 7, different *p53* mutations were observed; and in case 17, *p53* mutation was only seen in adenocarcinoma cells and not in the NE cells. The concordance rate of *p53* mutation in the two types of cells was 66.7%. Based on the similar microsatellite changes and *p53* mutations in both NE cells and adenocarcinoma cells in the 27 of 30 cases, we claimed that the NE and adenocarcinoma cells probably were derived from the same stem cells. Our results provided more evidence to support that the multipotent stem cells could differentiate to NE and adenocarcinoma cells. Whether NE cells can act as parenchyma of carcinoma and secrete hormones to promote carcinoma needs further investigation. We also found that 3 cases showed different MSI, LOH and *p53* mutation pattern, suggesting that the NE and gastric adenocarcinoma cells were derived from different stem cells. Further study on the underlying mechanisms is needed.

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COMMENTS

Background

Neuroendocrine differentiation (NED) is a common phenomenon in adenocarcinomas, but there have been only a few studies of NED in gastric adenocarcinoma. It remains unclear whether the glandular and endocrine cells expand from two distinct precursors, or arise from a single progenitor cell.

Research frontiers

Authors used laser capture microdissection, microsatellite instability (MSI), loss of heterozygosity (LOH) and *p53* mutation to evaluate the clonality of NED.

Innovations and breakthroughs

Authors studied the clonality of neuroendocrine (NE) cells in gastric adenocarcinoma using laser capture microdissection, MSI, LOH and *p53* mutation to evaluate the clonality of NED. NE and adenocarcinoma cells showed the same MSI, LOH or *p53* mutation in most cases (27/30), which may originate from the same stem cells. In the other three cases, different MSI, LOH or *p53* mutation occurred.

Applications

The article helps to achieve a better understanding of the origination of NE cells in gastric adenocarcinoma, and provide a clear method of evaluation to clinicians.

Terminology

Laser-capture microdissection (LCM): LCM was performed to obtain cells from gastric adenocarcinoma. The technology makes use of the melting heat of infrared rays to melt the polymeride under microscope, followed by molecular biology analysis.

Peer review

The authors discuss the available information on LOH and MSI in view of their findings and published reports. They presented from their and other groups the findings on p53 in gastric cancer and argued that NE and adenocarcinoma cell likely derive from the same stem cell in the majority of the tested tumors. In brief, this is an interesting study that is thoroughly performed and interpreted.

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BRIEF ARTICLE

Efficacy profiles for different concentrations of *Lactobacillus acidophilus* in experimental colitis

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Abstract

AIM: To determine the efficacy profiles of different concentrations of *Lactobacillus acidophilus* (*L. acidophilus*) for treating colitis using an experimental murine model.

METHODS: Colitis was established in 64 BALB/c mice by adding 5% dextran sodium sulfate (DSS) to the drinking water and allowing *ad libitum* access for 7 d. The mice were then randomly divided into the following control and experimental model groups (n = 8 each; day 0): untreated model control; negative-treatment model control (administered gavage of 1 mL/10 g normal saline); experimental-treatment models C4-C8 (administered gavage of 10^4, 10^5, 10^6, 10^7, or 10^8 CFU/10 g *L. acidophilus*, respectively); positive-treatment model control (administration of the anti-inflammatory agent prednisone acetate at 45 μg/10 g). Eight mice given regular water (no DSS) and no subsequent treatments served as the normal control group. Body weight, fecal traits, and presence of fecal occult blood were assessed daily. All animals were sacrificed on post-treatment day 7 to measure colonic length, perform histological scoring, and quantify the major bacteria in the proximal and distal colon. Intergroup differences were determined by one-way ANOVA and post-hoc Student-Newman-Keuls comparison.

RESULTS: All treatments (*L. acidophilus* and prednisone acetate) protected against colitis-induced weight loss ($P < 0.05$ *vs* model and normal control groups). The extent of colitis-induced colonic shortening was significantly reduced by all treatments (prednisone acetate > C4 > C5 > C7 > C8 > C6; $P < 0.05$ *vs* untreated model group), and the C6 group showed colonic length similar to that of the normal control group ($P > 0.05$). The C6 group also had the lowest disease activity index scores among the model groups. The bacterial profiles in the proximal colon were similar between all of the experimental-treatment model groups (all $P > 0.05$). In contrast, the bacterial profile in the distal colon of the C6 group showed the distinctive features ($P < 0.05$ *vs* all other experimental-treatment model groups) of *Lactobacillus* sp. and *Bifidobacterium* sp. being the most abundant bacteria and *Staphylococcus aureus* being the least abundant bacteria.

CONCLUSION: The most therapeutically efficacious concentration of *L. acidophilus* (10^6 CFU/10 g) may exert its effects by modulating the bacterial profile in the distal colon.

Key words: *Lactobacillus acidophilus*; Bifidobacterium; Colonic flora; Therapeutic dose; Experimental colitis; Efficacy profile

Core tip: Efficacies of the current treatments for ulcerative colitis (UC) are limited by procedure-related complications, poor patient compliance, and high re-

lapse rates. Administration of supplemental probiotics represents a promising new therapy of UC. Since UC pathogenic sites mainly involve the rectum and colon and UC patients show differential composition profiles of intestinal bacteria, this study was designed to evaluate the therapeutic efficacies of various concentrations for the standard probiotic, *Lactobacillus acidophilus*, using a well-known murine model of experimental colitis to examine the changes in colitis symptoms and the corresponding effects on the bacterial flora in the distal and proximal colon.

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INTRODUCTION

Ulcerative colitis (UC), a non-specific chronic inflammatory bowel disease, has emerged as a significant human health burden in Western countries and its prevalence is rising worldwide. As such, extensive research efforts have focused on determining its pathogenic mechanisms and developing efficacious therapies. While these studies have helped to define the clinical course of UC, identification of a safe and effective treatment has remained elusive. The primary drug therapies currently in use, including the anti-infective salazosulfamide, anti-inflammatory glucocorticoids and immunosuppressant probiotics, are limited by considerable side-effects, which lead to poor patient compliance and may contribute to the high relapse rates of UC[1].

Four fundamental components underlying UC pathogenesis have been identified, which represent likely sources for the yet undefined etiological factors: environment, microbiota, immune system, and genome[2,3]. A large number of experimental studies using animal models and clinical studies of human UC subjects have demonstrated that the intestinal microbiota, in particular, plays an important role in both UC onset and progression[4-7]. Moreover, sterile conditions (*i.e.*, germfree environments) have been shown to induce UC in mice and differential distributions of specific bacteria (*i.e.*, *Campylobacter* sp.) have been correlated with UC in adult humans[8,9]. Interactions between the microbiota and the immune system are well-described and recognized for their critical roles in normal physiological processes; accordingly, aberrant development and response of the immune system related to the microorganism environment in the gut, have been associated with UC[2,10].

The profile of normal human intestinal flora consists of about 30 genera of bacteria, representing hundreds of species and unknown thousands of strains. The most abundant species are anaerobic (including Bifidobacteria, Lactobacilli and Bacteroides), among which the *Lactobacillus* sp. appear to be the predominant flora, especially in the colon. Furthermore, quantitative analysis has indicated that many of these anaerobes are present at concentrations between 10^9-10^{12} CFU/mL. Many of these anaerobes, such as *Lactobacillus* sp. and *Bifidobacterium* sp., are characterized as probiotics, exerting beneficial effects on the human body. Indeed, detection of latent pathogenic species, such as *Clostridium* and *Staphylococcus*, is rare under normal physiological conditions[4].

Probiotics are considered a promising alternative therapy for UC. To date, Lactobacilli, Bifidobacteria, VSL#3 (a compound probiotic preparation composed of four Lactobacilli strains, three Bifidobacteria strains, and one *Streptococcus salivarius* strain), and *Escherichia coli* (*E. coli*) Nissle 1917 have been applied to UC subjects (both animal models and human cases) and shown to effectively resolve disease symptoms[11-14]. In addition, our laboratory showed that administration of *Lactobacillus acidophilus* (*L. acidophilus*) at the early stage is an effective therapy for UC but that administration of different probiotics does not provide the same efficacies[15,16]. These results suggest that the distinctive features of different bacteria, including their secretory functions and interactions with other bacteria and the host system, may have significant functional implications for their therapeutic efficacies in specific host tissues. Thus, while it is possible that introducing a large amount of a probiotic (or a mixture of probiotics) may beneficially impact the overall profile of intestinal bacteria, such an effort may also provide no benefit or be detrimental, possibly promoting latent pathogenicity, in specific intestinal regions.

Since UC pathogenic sites mainly involve the rectum and colon[3] and UC patients show differential composition profiles of intestinal bacteria[17,18], we hypothesize that development of probiotic therapy as an effective UC treatment modality will depend upon the particular probiotic's effects at a specific tissue site, dosage concentration, and relationship to other flora. Thus, the current study was designed to evaluate the efficacy profiles of different concentrations (10^4-10^8/10 g body weight) of the probiotic *L. acidophilus* in the proximal and distal colon of a well-established murine model of experimental colitis.

MATERIALS AND METHODS

Bacterial strain

L. acidophilus was isolated from a normal human intestinal tract SMC-S095 sample and sequence-identified by our laboratory. After culturing under anaerobic conditions with MRS medium for 24 h, the bacteria was collected, quantified by a spectrophotometer, and diluted with normal saline (NS) to 10^{10} CFU/mL.

Study design

Seventy-two female BALB/c mice (6-8-wk-old, 20.0 ± 2.0 g mean body weight) were purchased from Hunan Agricultural University and housed under standard con-

ditions (50% ± 10% humidity, 12 h light/dark cycle, *ad libitum* access to standard mouse chow). Colitis was established in 64 of the mice by adding 5% dextran sodium sulfate (DSS, MW 50000; Sigma Corp, St. Louis, MO) to the drinking water and allowing *ad libitum* access for 7 d. The mice were then randomly divided into the following control and experimental model groups (n = 8 each; day 0): untreated model control; negative-treatment model control (administered gavage of 1 mL/10 g normal saline); experimental-treatment models C4-C8 (administered gavage of 10^4, 10^5, 10^6, 10^7, or 10^8 CFU/10 g *L. acidophilus*, respectively); positive-treatment model control (administration of the anti-inflammatory agent prednisone acetate at 45 μg/10 g). Eight mice given regular water (no DSS) and no subsequent treatments served as the normal control group.

Body weight, fecal traits, presence of fecal occult blood, and disease activity index (DAI) scores were assessed daily, as previously described[19]. On post-treatment day 7, all mice were sacrificed by ether anesthesia overdose. The resected colon was measured (length-wise). Colonic segments (0.5 cm) were obtained starting from 1 cm to the ileocecus to 1 cm to the anus and used for bacterial analysis (described below). The remaining colonic segments approximately 0.5 cm to the anus were fixed in neutral formalin, prepared as paraffin-embedded sections, stained with hematoxylin and eosin (HE), and subjected to histological analysis by light microscopy and the damage scoring procedure described by Dieleman *et al*[20].

Bacterial analysis of intestinal flora

Colonic segments were weighed, homogenized, serially diluted in NS, and used to inoculate nonselective and selective culture mediums. For each animal, 10 L of intestinal fluid (10^0-10^{-5} *L. acidophilus* concentration) was collected and also inoculated in corresponding media. After culturing, three isolates each of anaerobic bacteria (Lactobacilli, Bifidobacteria, and Bacteroides) and aerobic bacteria [*Staphylococcus aureus* (*S. aureus*), *E. coli*, and Enterococci], as well as a sample of total aerobic bacteria, were selected for further analysis.

The anaerobic bacteria were incubated in the BAC. III-IE anaerobic workstation (Shel Lab, Cornelius, OR) at 37 ℃ for 48-72 h using the appropriate medium (Lactobacilli, LBS medium; Bifidobacteria, BS medium; Bacteroides, BDS medium; total aerobic bacteria, ordinary nutrition agar medium). The aerobic bacteria were incubated in the BSG-4 biochemical incubator (WanTong Precision Instruments Co., Ltd, Wuhan, China) at 37 ℃ for 24-48 h using the appropriate medium (*S. aureus*, high-salt mannitol medium; *E. coli*, eosin-methylene blue medium; Enterococci, TTC sodium azide medium). The resultant bacterial colonies were counted and converted to CFU/g by the following formula: number of bacterial colonies × [(diluted liquid volume + sample weight)/ sample weight] × dilution multiple.

Bacteria identification

Preliminary identification was performed on colonies of different bacteria according to morphological and Gram staining characteristics detected by light microscopy.

Identification of anaerobic bacteria (Lactobacilli and Bifidobacteria): Using Bifidobacteria as an example, the bacterial colonies of various forms were obtained from the BS medium, cultured respectively in MRS liquid culture medium. Resultant colonies were analyzed by light microscopy to confirm normal homogeneous morphology of Bifidobacteria, and isolates were inoculated onto MRS solid culture medium and cultured in the anaerobic incubator at 37 ℃ for 24 h. The resultant colonies were rescreened. After several generations' of serial cloning, the bacterial colonies with normal homogeneous appearance and morphology were selected for bacterial identification *via* the API20 A test (bioMérieux Vitek, Inc., Hazelwood, MO), according to the manufacturer's instructions. Results were within the API20 Analytical Profile Index. The protocol for Lactobacillus identification was similar.

Identification of Enterococci: The serially purified bacteria isolated from TTC culture were verified as Enterococci according to results of catalase test and verified by Gram staining. A single colony was resuspended in 0.3 mL sterile water, inoculated on a sheep blood agar plate, and incubated in the biochemical incubator at 37 ℃ for 24 h. One of the resultant colonies was selected for testing with the API20 Strep test (bioMérieux Vitek, Inc.), according to the manufacturer's instructions. Results (at 4 and 24 h of culture) were within the API20 Analytical Profile Index.

Statistical analysis

All statistical analyses were carried out with the SPSS statistical software suite (version 16.0; SPSS Inc, Chicago, IL). Results are expressed as mean ± SD. Quantitative data were converted to logarithm values and tested for homogeneity of variance. If the variance was homogeneous, single-factor analysis of variance (one-way ANOVA) was used to analyze the differences between groups. Mean values with significant difference ($P < 0.05$) were subjected to post-hoc pairwise comparison by the Student-Newman-Keuls test.

RESULTS

Effects of L. acidophilus treatments on colitis-induced changes in the general condition, body weight, and colonic length of mice

General condition: Mice in the normal control group were alert and had sleek, healthy coats. Mice in the untreated model control and the negative-treatment model control groups were apathetic, inert, had horripilated, dry coats with blood staining around the perianal area, and showed an obviously leaner body configuration than the normal controls. All experimental-treatment model groups and the positive-treatment model group showed similar disruptions in alertness, motor function, and coat condition as their model control counterparts, but to a much lesser extent.

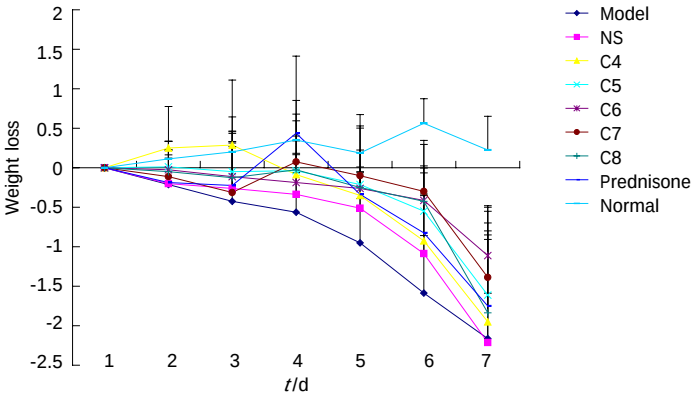

Figure 1 Effects of *Lactobacillus acidophilus* treatments on colitis-induced body weight loss. The *x*-axis shows the mean weight (mean ± SD) recorded for each group on each day of the 7-d study course. NS: Negative-treatment.

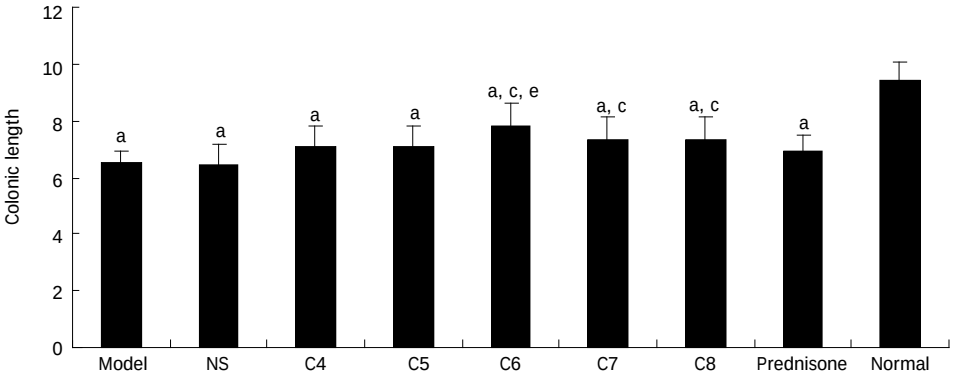

Figure 2 Effects of *Lactobacillus acidophilus* treatments on colitis-induced changes in colonic length. [a]$P < 0.05$ *vs* normal control group; [c]$P < 0.05$ *vs* untreated model control group; [e]$P < 0.05$ *vs* negative-treatment (NS) model control group.

Body weight: Mice in the normal control group experienced an increase in body weight over the duration of the study course; all mice were within normal weight range at sacrifice. In contrast, all other mice experienced a decrease in body weight over the study course. The greatest weight loss occurred in the untreated model control group, and the negative-treatment model control group showed only slightly less (and statistically similar; $P > 0.05$) weight loss. The body weight loss experienced by all experimental-treatment model groups and the positive-treatment model group showed trends of a more gradual decline over time than that of the untreated model control group. On the day of sacrifice, the extent of body weight loss among the model groups (Figure 1) showed the following hierarchical pattern: untreated model control = negative-treatment (NS) model control > C4 > C8 > positive-treatment (prednisone acetate) model control > C5 > C7 > C6.

Colonic length: All model groups had significantly shorter colonic lengths than the normal control group ($P < 0.05$), with the greatest extent of shortening observed in the untreated model control and negative-treatment model control groups. As shown in Figure 2, the following hierarchical pattern was observed for shortening degree among the treatment model groups: positive-treatment (prednisone acetate) model control > C4 > C5 > C7 > C8 > C6. Compared to the untreated model control group, the mean colonic lengths of C7, C8, and C6 were significantly longer ($P < 0.05$). Among those groups, the C6 group experienced the smallest degree of colonic shortening, and its mean colonic length was similar to that of the normal control group ($P > 0.05$).

Effects of L. acidophilus treatments on colitis-induced changes in DAI scores

When the mean DAI score of the normal control group was set to zero, the mean DAI score of all model groups showed a trend of significantly increasing values (indicating increasing detrimental pathology) over the study course. On the day of sacrifice, the untreated model control and the negative-treatment model control groups showed the highest (and similar) DAI scores. During the first four days after treatment, no significant differences were observed between the model groups (controls and experimentals). However, starting at day 5 post-treatment, the scores of the untreated model control and the negative-treatment model control groups markedly increased, indicating that the UC in these mice was aggravated. In addition, at this time, therapeutic effects begin to appear among the -treatment model groups. As shown in Figure 3A, while all model groups showed a sharp increase in mean DAI scores at day 7, the scores for the C4 and prednisone acetate treatment groups became significantly higher than the other experimental-treatment groups and statistically similar to those of the model control groups (both $P < 0.05$). As shown in Figure 3B, the experimental-treatment groups showed the following hierarchical

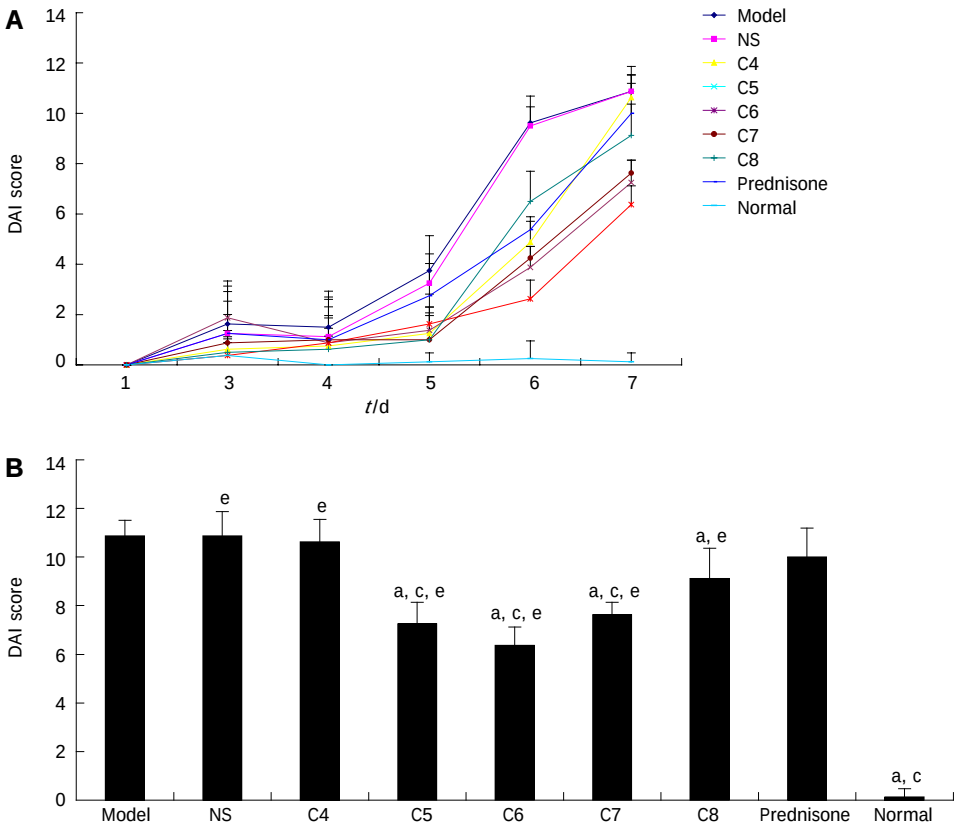

Figure 3 Effects of *Lactobacillus acidophilus* treatments on colitis-induced changes in disease activity index scores. Comprehensive evaluation of body weight decrease, fecal properties, and fecal occult blood was carried out by the disease activity index (DAI) scoring system throughout the study course. A: The daily mean ± SD DAI score is plotted for each group; B: Mean ± SD DAI scores on day 7 post-treatment. [a]$P < 0.05$ *vs* untreated model control group; [c]$P < 0.05$ *vs* positive-treatment (prednisone acetate) model control group; [e]$P < 0.05$ *vs* normal control group. NS: Negative-treatment.

pattern of decreasing DAI scores on day 7: C8 > C7 > C5 > C6.

Effects of L. acidophilus treatments on colitis-induced changes in histopathological features of the intestinal tissues

Mice in the normal control group showed normal orderly arrangement of the cellular structure of rectal and colonic tissue glands, with no obvious perturbations in goblet cell number or mucosal integrity. In contrast, mice in the untreated model control and the negative-treatment model control groups showed multifocal and deep ulcers distributed throughout the entire colon; moreover, the extent of ulceration increased along with disease severity, as evidenced by recess depth, histological perturbations, mucosal erosion, bleeding, necrosis, partially or completely damaged epithelial cell structure, and inflammation extending towards the submucosa and serosa. Mice in the experimental-treatment model groups and the positive-treatment model group also showed mucosal defects similar to but less extensive than those in the model control groups; moreover, the extent of relief of the mucosal defects varied among the different treatment models. The C6 group, in particular had the most apparent relief of colitis-induced mucosal defects, showing partially incomplete glands, rare occurrences of mucosal erosion, bleeding and necrosis, and only a small quantity of inflammatory cell infiltration (Figure 4A, panels 1-9).

The histological scores of colon damage observed in the various groups at day 7 are shown in Figure 4B. When the mean damage score of the normal control group was set to zero, the mean damage scores of all model groups showed a trend of significantly increasing values over the study course. The untreated model control and the negative-treatment model control groups had the highest mean damage scores. Compared to the untreated model control group, the mean damage scores of all experimental-treatment groups and the positive-treatment control model group were significantly lower ($P < 0.05$). Comparisons among the treatment model groups revealed that the mean damage scores of C6 and C7 groups were significantly lower than that of the prednisone acetate group ($P < 0.05$), with the C6 group having the lowest score.

Effects of L. acidophilus treatments on colitis-induced changes in the proximal and distal colonic bacterial profiles

The bacterial profiles detected in the proximal colons of the control, model, and treatment model groups were similar, and none of the between-group differences reached statistical significance (Figure 5A). In contrast, the bacterial profiles detected in the distal colons showed distinct features between the various groups (Figure 5B).

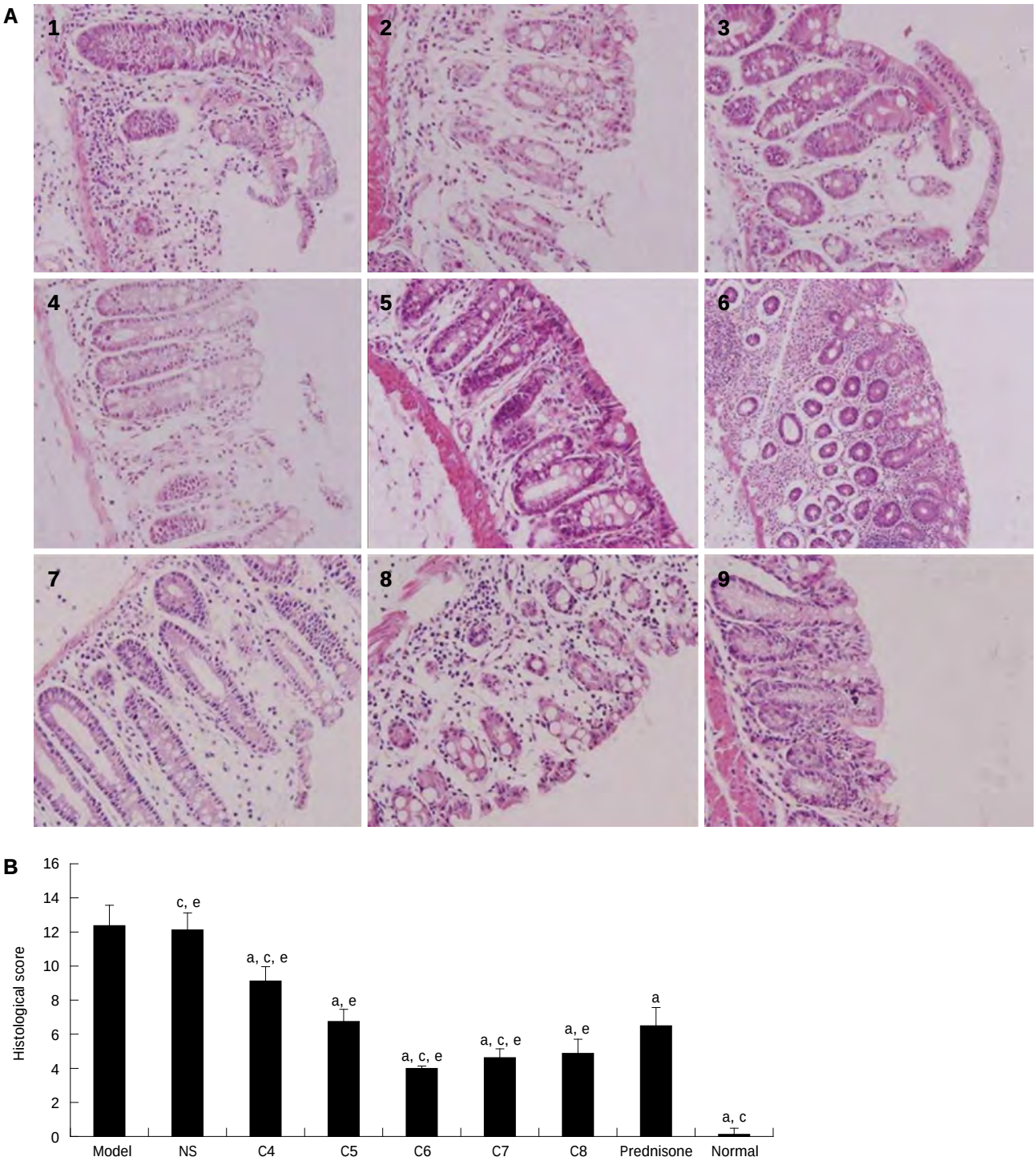

Figure 4 Effects of *Lactobacillus acidophilus* treatments on colitis-induced changes in intestinal histopathological features. A: Representative intestinal sections (HE, under light microscope, × 200) are shown. Panel 1: untreated model group, 2: negative-treatment (NS) model control group, 3-7: C4-C8 experimental treatment model groups respectively, 8: positive-treatment (prednisone acetate) model control group, and 9: normal control group; B: Average histopathological scores. $^{a}P < 0.05$ *vs* untreated model control group; $^{c}P < 0.05$ *vs* positive-treatment (prednisone acetate) model control group; $^{e}P < 0.05$ *vs* normal control group.

While the most abundant bacteria in all of the profiles were Lactobacilli, Bifidobacteria and Staphylococcus, the levels of each were different. For example, the levels of Lactobacilli among the untreated, negative-treatment, and positive-treatment model control groups were significantly higher than those in the normal control group ($P < 0.05$) but similar to one another ($P > 0.05$), while the experimental-treatment groups showed the highest levels ($P < 0.05$ *vs* the untreated model control group and the positive-treatment model control group), The levels of Bifidobacteria showed the same trends as the Lactobacilli levels. The levels of *S. aureus*, however, were similar between the untreated model control group, the negative-treatment model control group, the positive-treatment

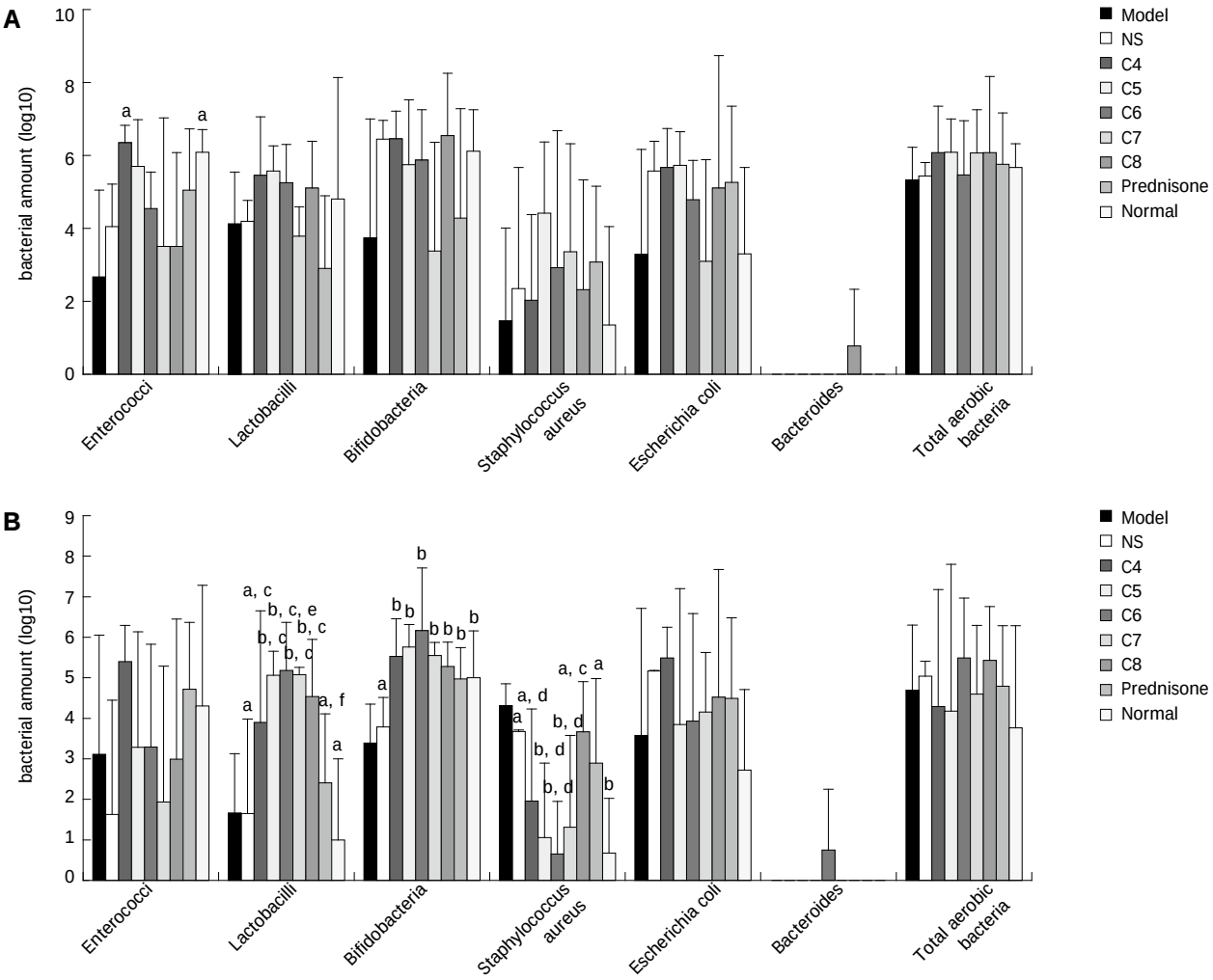

Figure 5 Bacterial profiles of the proximal (A) and distal (B) colon. Bacteria amount is expressed as the logarithm. [a]$P < 0.05$, [b]$P < 0.01$ *vs* untreated model control group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* positive-treatment (prednisone acetate) model control group; [e]$P < 0.05$, [f]$P < 0.01$ *vs* normal control group. NS: Negative-treatment.

model control group, the C4 group, and the C8 group ($P > 0.05$), but all five were significantly higher than that in the normal control group ($P < 0.05$). The C6 group showed the lowest level of *S. aureus* among all the model groups ($P < 0.05$), and this amount was not significantly different from that in the normal control group ($P > 0.05$). No other bacteria detected showed significantly different levels between any of the groups.

DISCUSSION

To date, investigations of the potential correlations between bacterial profiles and UC have been carried out from the perspective of disease cause (etiological factors) and resolution (therapeutic modalities). Despite the focused efforts of many experimental and clinical studies, no particular bacterial genus or species, or combined panel of such, has been identified as a causative agent of UC onset. Profiling of the organic acids metabolized by bacteria that are present in stool samples has indirectly provided some insights into this issue, suggesting that UC is likely to be related to a panel of multiple bacteria, rather than a single species or phenotype[21]. Profiling of human intestinal flora has indicated that individuals with UC have significantly higher numbers of intestinal *Bacteroides* sp., *Streptococcus* sp., and facultative anaerobes, but significantly lower numbers of *Lactobacillius* sp. and *Bifidobacterium* sp. than their healthy counterparts[21-23]. These results are considered to have clinical implications in that they suggest administration of corresponding probiotics may restore the profile of enteric microorganisms to match that of a non-UC status.

Indeed, several studies to date have evaluated the therapeutic efficacy of probiotic administration using Lactobacilli[24,25], Bifidobacteria[11,26,27], *E. coli* Nissle 1917[16,28], or VSL#3[15,29-31]. The degrees to which these individual supplements successfully resolved the UC varied, which led to the hypothesis that administration of a combination of probiotics may provide more benefit to the patients. In order to determine the most efficacious composition, the overall profile of bacteria present at the mucus barrier in UC needs to first be determined[32]. Another important issue that needs to be elucidated is the activities and effects of the various probiotics on the normal intestinal flora; otherwise, a suboptimal dose of any particular probiotic may negatively impact the overall

efficacy of the treatment.

Gionchetti *et al*[30] demonstrated that high-dose VSL#3 helps to maintain the remission status achieved by surgery to treat mild pouchitis of UC; lower doses were not evaluated. An informal review of the research studies of probiotic treatment for UC published in the publicly accessible science and medical literature databases suggested that most common concentrations of probiotics used range between 10^6-10^9 CFU/mL; the lack of a standardized concentration precludes direct comparison of the results from these studies. In addition, we have found no published reports of comparative analyses to evaluate the differential effects of probiotics at varying concentrations.

L. acidophilus was chosen as the focus of the current study based upon previous results showing its therapeutic benefit for early-stage experimental colitis and its abundance in the normal human intestine[33]. The current results indicated that the therapeutic effect of *L. acidophilus* did not increase in a concentration-dependent manner, but revealed that a moderate-dose concentration (10^6 CFU/10 g) provided the most alleviation of UC symptoms, as evidenced by the significant reductions in DAI and tissue damage scores. Most importantly, this moderate-dose restored UC-related parameters to the levels in non-UC healthy control mice.

Our detailed analyses of the proximal and distal colonic intestinal flora provided further insights into the relationship between the therapeutic effects observed and the concentration of Lactobacillus in the lesion. The symptoms' resolution mediated by the moderate-dose of *L. acidophilus* was accompanied by distinct and significant changes in the distal colon bacterial profile (*i.e.*, increases in Lactobacilli and Bifidobacteria, and decreases in *S. aureus*). Since UC lesions frequently involve the proximal colon and rectum, and the *L. acidophilus* intervention used in this study mainly affected the distal colonic bacterial flora, it is possible that the different therapeutic efficacies observed for the various probiotics in previous studies may result from effects in specific tissues.

In the current study, the number of Lactobacilli detected in specific lesions did not increase in conjunction with increased concentrations of the administered *L. acidophilus*. Since the Lactobacilli population is composed of many species, such as *L. acidophilus*, *L. casei*, *L. plantarum* and *L. bulgaricus*, researchers have started to investigate the therapeutic effects on UC related to the individual species[12,34]. Similarly, efficacy studies on the Lactobacilli compound bacteria VSL#3 have been carried out, and their results confirmed that the combined panel of *Lactobacillus* sp. in this compound is beneficial for treating inflammatory bowel diseases[15,35]. We hypothesize that, in the intestine, interactions between *L. acidophilus* and other local Lactobacilli (at low concentrations) may serve to promote each other mutually. It is possible then that increasing the concentration of *L. acidophilus* (*via* administration of the probiotic supplement) may serve to intensify this mutual promotion. However, the dose of the *L. acidophilus* supplement is important; too high of a dose may instead create an imbalance between the different *Lactobacillus* sp. and disrupt the mutual promotion, thereby leading to a decrease in the total number of the beneficial Lactobacilli. Gaining a detailed understanding of the mechanisms of the interactions between *Lactobacillus* sp. will provide important insights into the related roles in UC pathogenesis.

The optimal dose of *L. acidophilus* will promote the growth of endogenous probiotics, such as Bifidobacteria, and inhibit the growth of pathogenic bacteria, such as *S. aureus*. The moderate-dose of *L. acidophilus* in the current study increased the amount of other probiotics and reduced the amounts of the pathogenic species. However, it appears that simply modifying the dose of *L. acidophilus* will not be sufficient for designing a supplemental regimen with optimal efficacy as we also found that the concentration of endogenous Lactobacilli in the lesion is relatively positively correlated with the efficacy.

In conclusion, administration of *L. acidophilus* supplement at a dose of 10^6 CFU/10 g body weight provides optimal therapeutic effect on experimental colitis in a mouse model. The treatment-induced relief of UC symptoms was correlated with changes in the concentrations of endogenous Lactobacilli and other probiotic and pathogenic bacteria in the distal colon. Future studies should aim to determine the mechanisms underlying the interactions between *L. acidophilus* and other endogenous bacteria, as well as molecular effects on the host immune system, both of which may identify novel manipulable targets to further increase the therapeutic efficacy of this approach.

COMMENTS

Background

Ulcerative colitis (UC) is a nonspecific chronic inflammatory bowel disease with a high rate of recurrence. The etiological factors of UC remain largely unknown, but a large number of studies have demonstrated that the intestinal microorganism environment plays an important role in the development of UC.

Research frontiers

Despite the increased incidence of UC, there remains a distinct lack of efficacious and non-invasive methods of treatment. Besides surgical intervention, which is performed in the later stages of UC, the primary therapies are drug-based, with the most frequently administered agents being salazosulfamide, glucocorticoids and immunosuppressant probiotics. Yet all of these drugs produce significant side-effects that limit patient compliance, which may actually promote the high relapse rates. Because probiotics are beneficial (and predominant) components of the normal intestinal flora, they represent promising therapeutic agents for UC; yet, to date, no study has reported the comparative efficacies of different kinds of probiotics or different doses in UC.

Innovations and breakthroughs

Although probiotics are beneficial to the human body, the supplement preparation of these living bacteria is not static and their dynamic activities, such as proliferation and secreted signaling mechanisms, may influence the therapeutic effects at different sites within the host system. It is necessary to identify the most suitable probiotic type for use a supplemental therapy for UC, as well as the optimal dose that will replenish the endogenous probiotics and inhibit any latent pathogenic species.

Applications

By determining the most suitable concentration of *Lactobacillus acidophilus* (*L. acidophilus*) for use as a supplemental probiotic treatment of UC, and providing novel insights into the relationship between *L. acidophilus* and the other endogenous flora, this study not only promotes the clinical potential for probiotic treatment but also expands the base of knowledge about UC pathogenesis.

Terminology

Inflammatory bowel diseases, such as UC and Crohn's disease, are nonspecific chronic inflammatory conditions with highly complex etiologies. The dextran sodium sulfate-induced mouse model of experimental colitis is similar to human UC.

Peer review

Grammatical errors should be corrected but this manuscript will provide a good addition to the medical literature.

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BRIEF ARTICLE

Gastrointestinal symptoms and associated factors in Chinese patients with functional dyspepsia

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Abstract

AIM: To study the evolution of gastrointestinal symptoms and associated factors in Chinese patients with functional dyspepsia (FD).

METHODS: From June 2008 to November 2009, a total of 1049 patients with FD (65.3% female, mean age 42.80 ± 11.64 years) who visited the departments of gastroenterology in Wuhan, Beijing, Shanghai, Guangzhou, and Xi'an, China were referred for this study. All of the patients fulfilled the Rome Ⅲ criteria for FD. Baseline demographic data, dyspepsia symptoms, anxiety, depression, sleep disorder, and drug treatment were assessed using self-report questionnaires. Patients completed questionnaires at baseline and after 1, 3, 6 and 12 mo follow-up. Comparison of dyspepsia symptoms between baseline and after follow-up was explored using multivariate analysis of variance of repeated measuring. Multiple linear regression was done to examine factors associated with outcome, both longitudinally and horizontally.

RESULTS: Nine hundred and forty-three patients (89.9% of the original population) completed all four follow-ups. The average duration of follow-up was 12.24 ± 0.59 mo. During 1-year follow-up, the mean dyspeptic symptom score (DSS) in FD patients showed a significant gradually reduced trend ($P < 0.001$), and similar differences were found for all individual symptoms ($P < 0.001$). Multiple linear regression analysis showed that sex ($P < 0.001$), anxiety ($P = 0.018$), sleep disorder at 1-year follow-up ($P = 0.019$), weight loss ($P < 0.001$), consulting a physician ($P < 0.001$), and prokinetic use during 1-year follow-up ($P = 0.035$) were horizontally associated with DSS at 1-year follow-up. No relationship was found longitudinally between DSS at 1-year follow-up and patient characteristics at baseline.

CONCLUSION: Female sex, anxiety, and sleep disorder, weight loss, consulting a physician and prokinetic use during 1-year follow-up were associated with outcome of FD.

Key words: Functional dyspepsia; Gastrointestinal symptoms; Dyspeptic symptom score; *Helicobacter py-*

lori infection; Postprandial distress syndrome; Epigastric pain syndrome; Rome Ⅲ criteria

Core tip: This is a prospective study with Chinese patients to explore the clinical course of functional dyspepsia (FD), and evaluate the potential risk factors associated with it, using the Rome Ⅲ criteria both longitudinally and horizontally. The sample size in this study was large and there was a good response rate. The mean dyspepsia symptom score for both total and individual symptoms showed a significant gradually reduced trend. Female sex, anxiety, and sleep disorder, weight loss, consulting a physician and prokinetic use during 1-year follow-up were associated with the outcome of FD.

Yu J, Liu S, Fang XC, Zhang J, Gao J, Xiao YL, Zhu LM, Chen FR, Li ZS, Hu PJ, Ke MY, Hou XH. Gastrointestinal symptoms and associated factors in Chinese patients with functional dyspepsia. *World J Gastroenterol* 2013; 19(32): 5357-5364 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5357.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5357

INTRODUCTION

Functional dyspepsia (FD) is a highly prevalent gastrointestinal disorder that is defined by the presence of symptoms thought to originate in the gastroduodenal region, without identifiable cause and diagnosed by routine tests[1,2]. According to the Rome Ⅲ criteria, patients are classified as postprandial distress syndrome (PDS) or epigastric pain syndrome (EPS) based upon the predominant symptom (*i.e.*, postprandial fullness, early satiation, or epigastric pain, and burning)[1]. The reported prevalence of FD symptoms varies between 19% and 41%[3]. FD has a significant impact on quality of life and imposes a substantial economic burden on society due to costs of physician visits, medication, and absenteeism[3,4].

The course of FD is always chronic, with a relapsing-remitting pattern, and has been poorly studied[5]. In spite of the prevalence of FD being stable over time, the reverse in symptom status is high[6]. Many studies have reported that a significant number of patients with FD improve or become asymptomatic over time, suggesting that a proportion of patients go into symptom remission, but the rates of symptom disappearance varies widely[5,7].

The pathophysiology of FD also remains poorly understood and is likely to be multifactorial[8]. Many pathogenic factors have been proposed for FD including genetic, environmental, pathological, and psychological factors[9]. Psychosocial factors such as depression, anxiety and stressful life events (*e.g.*, history of abuse) are considered to play a role in the development of FD[10,11]. A relationship between *Helicobacter pylori* (*H. pylori*) infection and FD has also been reported[12]. Similarly, several studies have demonstrated that sleep disorder is associated with functional gastrointestinal disorders such as irritable bowel syndrome, gastroesophageal reflux disease, and FD[13-15]. Nevertheless, it is not clear whether such pathogenic factors affect the clinical course of FD.

Accordingly, this longitudinal study followed up a group of Chinese patients with FD over 1 year. We aimed to explore the evolution of FD symptoms, and evaluate the potential risk factors both longitudinally and horizontally.

MATERIALS AND METHODS

Patient selection

A total of 1049 patients with FD (364 male and 685 female, aged 20-79 years, mean 42.80 ± 11.64 years) who fulfilled the Rome Ⅲ criteria were enrolled. These were outpatients who visited departments of gastroenterology in five cities in China (Wuhan, Beijing, Shanghai, Guangzhou, and Xi'an) from June 2008 to November 2009.

The patients had one or more dyspeptic symptoms, including troublesome postprandial fullness, early satiation, epigastric pain, or epigastric burning for the past 3 mo, with symptom onset at least 6 mo before diagnosis. All of the FD patients had undergone upper gastrointestinal endoscopy, abdominal ultrasound, and/or barium meal X-ray examination in a tertiary hospital. In all cases, there was no evidence of organic, systemic, or metabolic disease that was likely to explain the symptoms.

Patients were excluded if they: (1) had upper gastrointestinal organic diseases such as esophagitis, peptic ulcer, or peptic neoplasm that were found by gastroscopy or barium meal X-ray examination and abdomen ultrasonography; (2) had chronic diseases such as diabetes mellitus, hyperthyroidism, scleroderma, chronic renal failure, or congestive heart failure; (3) had a history of abdominal surgery; or (4) were pregnant or preparing to conceive a child, or lactating during the study period.

Data collection and synthesis

Baseline data: All 1049 FD patients were asked to finish a self-report questionnaire face to face. To ensure content validity and usability, physicians were trained initially to give instructions to patients and did not intervene with the patients' medical management.

The baseline self-reported questionnaire included several clinical variables involving demographics (age, sex, height, weight, and marital status), tobacco and alcohol use, educational level, economic situation, life satisfaction, physical labor, *H. pylori* status, severity and frequency of each dyspepsia symptom, bowel symptom comorbidity, psychosocial factors (anxiety and depression), sleep disorder, major mental stimulation, history of abuse and drug treatment (prokinetics, gastric mucosa protectants, antacids, anti-*H. pylori* therapy, and traditional Chinese medicine). The data for dyspepsia and bowel symptoms were collected using a Chinese version of the

validated Rome Ⅲ diagnostic questionnaire for adult functional gastrointestinal disease[16]. This questionnaire has been repeatedly tested and carefully validated[17].

Follow-up data: FD patients were asked to visit the department of gastroenterology to finish a follow-up questionnaire at 1, 3, 6 and 12 mo after the first visit. The follow-up questionnaire was the same as the baseline questionnaire, but did not include some details such as sex, educational level, economic situation, life satisfaction, physical labor, major mental stimulation, and history of abuse.

Diagnosis of H. pylori infection

At upper gastrointestinal endoscopy, biopsies were acquired and processed for rapid urease test. A $^{13}C/^{14}C$-urea breath test was also used to assess *H. pylori* status.

Definition of body mass index

Body mass index (BMI) was calculated and categorized as weight (kg)/height (m^2) according to World Health Organization recommendations.

Economic situation

Economic situation was classified as rich, sufficient, well-off and poor according to the expending percentage for food in whole income as < 1/5, < 1/3, 1/2, and > 1/2.

Educational level

Educational level was divided into seven: illiteracy, elementary school, junior high school, high school, junior college, university, and graduate and above. If the patients were illiterate or had finished elementary school education, they were judged as having a low level of education. If the patients had completed junior high school or high school education, they were regarded as having a medium level of education. Patients who had completed junior college education or above were considered to have a high level of education.

Tobacco and alcohol use

Current smokers were defined as individuals smoking cigarettes and having no other former tobacco use. Alcohol use was defined as consumption of > 100 g/wk alcohol.

Assessment of dyspeptic symptoms

Dyspeptic symptoms that were recorded and assessed included postprandial fullness, early satiation, epigastric pain, epigastric burning, belching, nausea, vomiting, and bloating. Each symptom was graded and scored on a Likert scale according to its severity as follows: 0, absent; 1, mild (not influencing daily activities); 2, relevant (diverting from but not urging modifications in daily activities); and 3, severe (influencing daily activities markedly enough to urge modifications). Frequency of each symptom was also graded as follows: 1, occurring < 1 d/mo; 2, occurring 1 d/mo; 3, occurring 2-3 d/mo; 4, occurring 1 d/wk; 5, occurring > 1 d/wk; and 6, occurring every day. The score for a single dyspeptic symptom was an aggregate of frequency and severity ratings, ranging from 0 to 9. Dyspeptic symptoms score (DSS) was assessed by summing the score of eight dyspepsia symptoms.

Psychosocial factors (anxiety and depression) and sleep disorder

The questionnaires used for assessment of psychological factors and sleep disorder were established according to a Chinese version of the Validated Rome Ⅲ Psychosocial Alarm Questionnaire for functional gastrointestinal disease[16,18]. In previous studies these questionnaires have been used to assess the psychological factors and sleep status of Chinese patients[19].

For problems related to anxiety and depression in the past 3 mo, the patients answered the question: Did you feel nervous irritable or depressed (yes/no)? If patients chose yes, they had to answer the next question about how often they felt nervous irritable or depressed: 1, occasionally; 2, sometimes; 3, frequently; 4, most of the time. Patients felt nervous irritable or depressed frequently or most of the time, indicating that anxiety or depression was present[16]. In the present study, we judged "nervous irritability" or "depression" occurring frequently or most of the time as "anxiety state" or "depression state".

Subjective sleep disorder in patients was measured with one question (yes/no). Symptoms of sleep disorder included trouble falling asleep, shallow sleep/dreaminess, sleep time < 6 h, early morning awakening, and daytime sleepiness.

History of abuse

A history of abuse in patients was measured with a question as follows: Have you ever been abused (yes/no)? If patients chose yes, they stated whether the abuse was physical or mental.

Statistical analysis

All statistical analyses were assessed using SPSS for Windows version 13. A two-sided P value < 0.05 was regarded as statistically significant. Data are presented as mean ± SD. To assess whether those who completed all four follow-ups were representative of the original study population, we compared the baseline characteristics between the follow-up population and those who were lost to follow-up, using Pearson's χ^2 test (categorical variables), Mann-Whitney U test (ordinal variables, such as BMI) and t test (continuous variables). Comparison between all individual dyspepsia symptoms at initial visit and at the four follow-ups was explored using multivariate analysis of variance (MANOVA) of repeated measuring. Univariate association measures between patient characteristics (baseline as well as 1-year follow-up) and DSS at 1-year follow-up were calculated using Pearson'

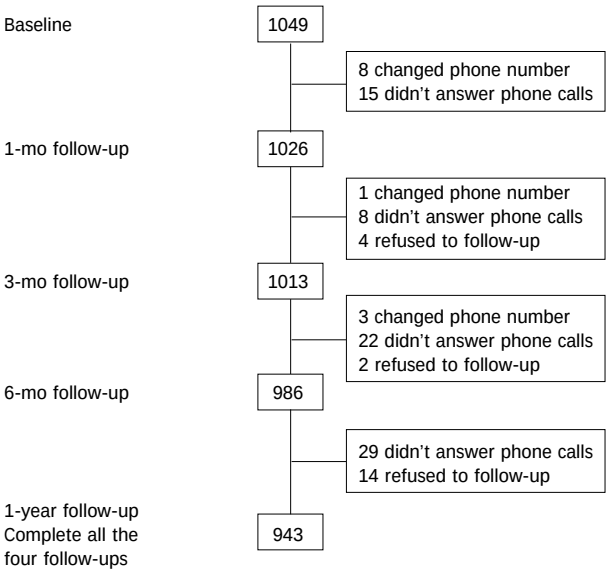

Figure 1 Flowchart of study participants.

s correlation and non-parametric one-way ANOVA. Risk factors associated with DSS at final follow-up, both longitudinally and horizontally, were determined by performing multiple linear regressions.

RESULTS

Patient characteristics and response rate

Of the 1049 FD patients originally enrolled, 1026 patients (97.8% of the baseline sample) completed the 1-mo study; 1013 patients (96.6% of the baseline sample) completed the 3-mo study; 986 patients (94.0% of the baseline sample) completed the 6-mo study; and 943 patients (89.9% of the baseline sample) completed the 1-year study (Figure 1).

The 943 patients who completed the baseline and all four follow-up questionnaires were included in this study. The average duration of follow-up was 12.24 ± 0.59 mo. The mean age of the follow-up population was 42.99 ± 11.74 years, and 603 (63.9%) were female; 176 (18.7%) had bowel symptom comorbidity, and 230 (24.4%) were positive for *H. pylori*. Men, alcohol users, those with higher educational level and better economic situation, and those who had consulted a physician were significantly more likely to be successfully followed up ($P < 0.05$ for all analyses) (Table 1).

Comparison of dyspepsia symptoms between baseline and at follow-up

The mean DSS in FD patients at baseline, and 1, 3 and 6 mo and 1 year follow-up was 22.05 ± 9.89, 14.04 ± 9.38, 12.05 ± 9.09, 10.08 ± 8.89 and 8.97 ± 8.62, respectively. This means that during 1 year follow-up, the mean DSS in FD patients showed a significant gradually reduced trend and all pairwise comparisons were statistically significant (all $P < 0.001$).

Table 1 Demographics and baseline characteristics of patients who completed all four follow-ups compared with those who were lost to follow-up

Characteristic	Complete all four follow-ups (n = 943)	Lost to follow-up (n = 106)	P value
Sex (female)	603 (63.9)	82 (77.4)	0.006
Smoker	202 (21.4)	16 (15.1)	0.128
Alcohol user	278 (29.5)	15 (14.2)	0.001
Marital status (married)	815 (86.4)	95 (89.6)	0.357
Physical labor			0.537
High	34 (3.6)	3 (2.8)	
Medium	238 (25.2)	31 (29.2)	
Low	671 (71.2)	72 (67.9)	
Life satisfaction			
High	171 (18.1)	13 (12.3)	0.292
Medium	732 (77.6)	90 (84.9)	
Low	40 (4.2)	3 (2.8)	
Educational level			0.011
High	336 (35.6)	28 (26.4)	
Medium	455 (48.3)	51 (48.1)	
Low	152 (16.1)	27 (25.5)	
Economic situation			0.03
Rich	40 (4.2)	3 (2.8)	
Sufficient	405 (42.9)	34 (32.1)	
Well-off	469 (49.7)	67 (63.2)	
Poor	29 (3.1)	2 (1.9)	
BMI (kg/m^2)			0.071
Obesity	138 (14.6)	10 (9.4)	
Normal	709 (75.2)	81 (76.4)	
Thin	96 (10.2)	15 (14.2)	
Bowel symptom	176 (18.7)	23 (21.7)	0.45
H. pylori status (positive)	230 (24.4)	22 (20.8)	0.14
Consulting a physician	908 (96.3)	91 (85.8)	< 0.001
Mean age (yr)	42.99 ± 11.74	41.11 ± 10.65	0.116
DSS	22.05 ± 9.89	20.25 ± 10.77	0.079

Data are expressed as absolute numbers (percentage) or mean ± SD. Significant variables in italic/bold ($P < 0.05$); BMI: Body mass index calculated; *H.pylori*: *Helicobacter pylori*; DSS: Dyspeptic symptom score.

Similar differences were observed for all individual symptoms (Figure 2). The mean symptom scores for both postprandial fullness and belching during 1 year follow-up showed a significant reduced trend and all pairwise comparisons were statistically significant (all $P < 0.001$).

The mean symptom scores for early satiation, nausea and bloating during 1 year follow-up decreased significantly and all pairwise comparisons were statistically different (all $P < 0.001$, except for the difference between 3 and 6 mo follow-up, $P = 0.037$, $P = 0.035$, $P = 0.102$, and the difference between 6 mo and 1 year follow-up, $P = 0.333$, $P = 0.034$, $P = 0.213$).

There was a marked decreased trend in mean symptom scores for both epigastric pain and epigastric burning during 1-year follow-up, and there were significant differences in all pairwise comparisons (all $P < 0.001$, except for the difference between 6 mo and 1 year follow-up, $P = 0.401$, $P = 0.028$).

The mean symptom scores for vomiting was reduced markedly, with all pairwise comparisons showing a significant difference (all $P < 0.001$, except for the difference

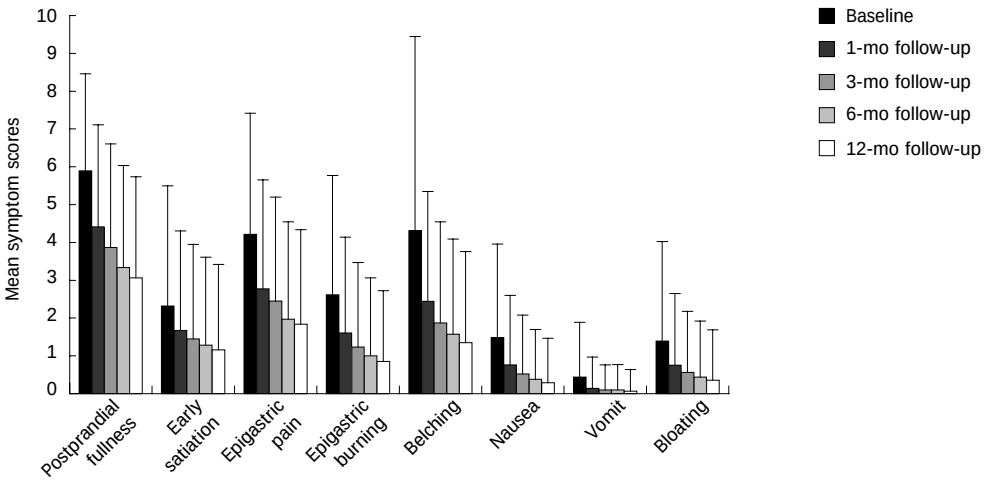

Figure 2 Comparison of dyspeptic symptoms between initial visit and at four follow-ups of repeated measures.

between 1 mo and 3 mo follow-up, $P = 0.330$; the difference between 3 mo and 6 mo follow-up, $P = 0.959$; the difference between 6 mo and 1 year follow-up, $P = 1.0$).

Factors associated with DSS at final follow-up (1-year follow-up)

Longitudinal associations: For patient characteristics at baseline, univariate correlates analysis revealed that history of abuse was associated with DSS at 1-year follow-up ($P = 0.025$), while no association was found for other variables such as sex, age, BMI, anxiety, depression, sleep disorder, *H. pylori* status, DSS at baseline, and drug treatment before baseline (Table 2). Multiple linear regression analysis showed no relationship between DSS at 1-year follow-up and patient characteristics at baseline (Table 2).

Horizontal associations: For patient characteristics at 1-year follow-up, univariate correlates analysis found that age ($P < 0.001$), alcohol consumption ($P = 0.024$), anxiety ($P < 0.001$), depression ($P < 0.001$), sleep disorder ($P < 0.001$), bowel symptoms ($P < 0.001$), weight loss ($P < 0.001$), consulting a physician ($P < 0.001$), prokinetic use ($P < 0.001$), gastric mucosa protectant use ($P < 0.001$), antacid use ($P < 0.001$), and traditional Chinese medicine use ($P < 0.001$) were significantly associated with DSS (Table 3).

Multiple linear regression analysis showed that sex ($P < 0.001$), anxiety ($P = 0.018$), sleep disorder ($P = 0.019$), weight loss ($P < 0.001$), consulting a physician ($P < 0.001$) and prokinetic use ($P = 0.035$) were significantly associated with DSS, while age, depression, alcohol consumption, bowel symptoms, and use of gastric mucosa protectants, antacids and traditional Chinese medicine were not associated with it (Table 3).

DISCUSSION

To the best of our knowledge, this is the first published prospective study with Chinese patients to explore the clinical course of FD, and evaluate potential risk factors associated with FD, using the Rome Ⅲ criteria, both longitudinally and horizontally. We selected a large group of FD patients from five cities in China. After their initial visit, patients were followed up at 1, 3 and 6 mo and 1 year.

The sample size was large and we had a good response rate to all parts of the study. We compared the baseline characteristics between the follow-up population and those who were lost to follow-up. We found that men, alcohol users, those with higher educational level and better economic situation, and those who had consulted a physician were significantly more likely to be successfully followed up. This is consistent with previous reports[20,21]. There were some demographic differences between responders and non-responders, but the magnitude of these differences was small and the individuals included in our follow-up study were broadly representative of the original enrolled FD patients, suggesting that the results of our study are persuasive.

The novel finding of our study was that the total DSS in FD patients showed a significant gradually reduced trend during 1 year follow-up, and similar differences were found for all individual symptoms. It seems that patients feel much better at the final follow-up and complain of less discomfort. Several previous studies reported improved symptoms during a period of follow-up, which is in line with our findings[5]. Kindt *et al*[22], in a 5-year follow-up study, found that about half of FD patients reported disappeared or improved symptoms. Pajala *et al*[23] observed a marked reduction in DSS in FD patients in Finland after 1 year follow-up. Heikkinen *et al*[24], in a long-term perspective study, also concluded that the stability of the symptom-based subgroups over time was poor. However, all of these studies only compared two time points, while our study compared five points.

Furthermore, we identified risk factors that influenced the clinical course of FD. Over the past decade, the correlation between psychological factors and func-

Table 2 Longitudinal associations between functional dyspepsia patients' baseline characteristics and dyspeptic symptom score at 1-year follow-up

Variable (at baseline)	Longitudinal associations							
	Univariate correlates			Multiple linear regression				
	r[1]	F[2]	P	B	β	t	P	R^2_{Model}
Sex[3]		3.489	0.062	0.161	0.009	0.208	0.835	0.019
Age	0.002		0.959	-0.007	-0.009	-0.224	0.823	
BMI	0.007		0.835	-0.024	-0.009	-0.248	0.804	
Smoking		1.894	0.169	1.228	0.058	1.368	0.172	
Alcohol consumption		0.02	0.887	-0.665	-0.035	-0.876	0.381	
Major mental stimulation		1.288	0.257	-0.421	-0.013	-0.365	0.715	
History of abuse		5.022	0.025[4]	-3.774	-0.062	-1.762	0.078	
Anxiety		0.106	0.745	0.897	0.030	0.777	0.437	
Depression		0.937	0.333	-1.549	-0.044	-1.157	0.248	
Sleep disorder		1.831	0.176	-0.683	-0.040	-1.131	0.259	
Bowel symptom		0.17	0.680	0.442	0.020	0.580	0.562	
H. pylori status		0.461	0.631	-0.269	-0.027	-0.782	0.434	
DSS	-0.014		0.667	-0.015	-0.018	-0.502	0.616	
Treatments in the previous months before baseline								
Consulting a physician		0.753	0.386	0.468	0.027	0.804	0.421	
Prokinetic use		0.077	0.782	-0.598	-0.034	-0.933	0.351	
Gastric mucosa protectant use		1.407	0.236	0.611	0.034	0.971	0.332	
Antacid use		1.381	0.24	0.589	0.034	0.906	0.365	
Anti-*H. pylori* therapy		1.014	0.314	0.431	0.018	0.539	0.590	
Traditional Chinese medicine use		0.005	0.945	-0.025	-0.001	-0.042	0.966	

[1]Pearson's correlation; [2]Non-parametric one-way ANOVA; [3]Male = 0, female = 1 reference category: female; [4]Significant variables ($P < 0.05$). BMI: Body mass index; *H.pylori*: *Helicobacter pylori*; DSS: Dyspeptic symptom score.

Table 3 Horizontal associations between functional dyspepsia patients' baseline characteristics and dyspeptic symptom score at 1-year follow-up

Variable (at 1-yr follow-up)	Horizontal associations							
	Univariate correlates			Multiple linear regression				
	r[1]	F[2]	P	B	β	t	P	R^2_{Model}
Sex[3]		3.489	0.062	0.585	0.033	6.356	< 0.001[4]	0.98
Age	0.139		< 0.001[4]	-0.002	-0.002	-0.456	0.649	
Time of follow-up (mo)	-0.035		0.288	-0.121	-0.008	-1.837	0.067	
Smoking		1.109	0.293	-0.242	-0.009	-1.533	0.126	
Alcohol consumption		5.132	0.024[4]	0.204	0.008	1.346	0.179	
Anxiety		13.257	< 0.001[4]	0.292	0.015	2.373	0.018[4]	
Depression		27.452	< 0.001[4]	-0.052	-0.002	-0.392	0.695	
Sleep disorder		69.219	< 0.001[4]	0.216	0.012	2.346	0.019[4]	
Bowel symptom		51.053	< 0.001[4]	0.185	0.007	1.377	0.169	
H. pylori status		1.563	0.210	-0.035	-0.003	-0.759	0.448	
Treatments during 1-yr follow-up period								
Consulting a physician		224.718	< 0.001[4]	0.893	0.051	9.168	< 0.001[4]	
Prokinetic use		59.340	< 0.001[4]	0.200	0.012	2.113	0.035[4]	
Gastric mucosa protectant use		22.857	< 0.001[4]	-0.014	-0.001	-0.147	0.883	
Antacid use		19.313	< 0.001[4]	-0.023	-0.001	-0.266	0.790	
Anti-*H. pylori* therapy		1.825	0.177	-0.075	-0.003	-0.635	0.526	
Traditional Chinese medicine use		50.799	< 0.001[4]	-0.062	-0.004	-0.755	0.450	
Weight loss during 1-yr follow-up period	0.988		< 0.001[4]	1.040	0.961	186.775	< 0.001[4]	

[1]Pearson's correlation; [2]Non-parametric one-way ANOVA; [3]Male = 0, female = 1 reference category: female; [4]Significant variables ($P < 0.05$). BMI: Body mass index; *H.pylori*: *Helicobacter pylori*; DSS: Dyspeptic symptom score.

tional gastrointestinal disorders has been confirmed in several clinical case-control studies[25,26]. Koloski *et al*[27] found that anxiety was an evident independent predictor for FD. Aro *et al*[28], in a Swedish population-based study, showed that anxiety but not depression was linked to FD and PDS but not to EPS. In the present study, anxiety at 1-year follow-up was also found to be horizontally associated with DSS, which is in keeping with previous studies.

Sleep disorder is a common phenomenon in all FD patients. Dyspeptic symptoms can interfere with sleep, and disrupted sleep may also potentially exacerbate FD

symptoms due to the hyperalgesic effect of sleep loss. Cremonini *et al*[14], in a study involving 3228 respondents, found that sleep disturbances were linked to both upper and lower gastrointestinal symptoms in the general population. Lacy *et al*[15] revealed that there was a relationship between FD and sleep disorder, and sleep disorder in FD patients appeared to be associated with symptom severity and higher levels of anxiety. We also discovered an association between sleep disorder at 1-year follow-up and FD outcome.

Recent cross-sectional population studies discovered that weight loss correlated most strongly with early satiety, followed by nausea and vomiting[29]. A longitudinal study in Belgium found that weight loss was independently associated with FD-specific quality of life at follow-up, and there was a trend association between weight loss and DSS at follow-up[22]. In this study, we did not have information about weight difference between dyspepsia symptom onset and initial visit. However, we collected data on patients' weight at baseline and at final follow-up, and observed that weight loss during 1 year follow-up was independently associated with DSS.

We showed an association between sex and FD outcome, indicating that women may have higher DSS at 1-year follow-up than men have, which is consistent with a cross-sectional study in Taiwan[30]. There was no association between *H. pylori* status and DSS at 1-year follow-up, which is similar to another prospective 2-year follow-up study from Taiwan[31].

An important finding in our study was that many individuals reported persistent symptoms despite consultation and prokinetic use during 1 year follow-up. Similarly, two recent studies have also reported persistence of symptoms in drug-treated patients[32,33]. It is probable that patients consulting a physician have the most severe symptoms, and they often take prescribed drugs on an on-demand basis. In addition, most individuals (n = 511, 54% of follow-up patients) in our study had a prescription of prokinetic during 1 year follow-up. These may be the reasons why patients consulting a physician and taking prokinetic still have continuous symptoms or even more severe symptoms.

In bivariate analysis, we also found a correlation between history of abuse and DSS at 1-year follow-up, which was similar to several other characteristics (*i.e.*, age, alcohol consumption, depression, bowel symptoms, and use of gastric mucosa protectants, antacids and traditional Chinese medicine during 1 year follow-up period). However, this correlation was not found in multiple linear regression analysis, indicating that it was weak.

In conclusion, in this large sample of individuals with FD, 89.9% of patients completed all four follow-ups, and the average duration of follow-up was 12.24 ± 0.59 mo. During 1 year follow-up, the total DSS in FD patients showed a significant gradually reduced trend, and similar differences were found for all individual symptoms. Female sex, anxiety, sleep disorder, weight loss, consulting a physician, and prokinetic use during 1 year follow-up were associated with outcome. Our study described the fluctuations in symptoms and found that several associated factors affected outcome. We believe that these findings provide evidence for the role of psychosocial factors in determining long-term clinical course in patients with FD. In the future, more research is needed to confirm and extend our study.

COMMENTS

Background

Functional dyspepsia (FD) is a chronic functional gastrointestinal disorder, and it has a significant impact on quality of life and imposes a substantial economic burden on society. However, the clinical course and risk factors for FD remain poorly studied.

Research frontiers

In the present study, the authors selected a large group of FD patients from five cities in China and explored the clinical course and risk factors for FD both longitudinally and horizontally.

Innovations and breakthroughs

Some cross-sectional population studies have discovered several risk factors associated with FD. This is believed to be the first follow-up study showing that the total dyspeptic symptoms score and single symptom score in FD patients present a significant gradually reduced trend, and female sex, anxiety, sleep disorder, weight loss, consulting a physician, and prokinetic use during 1-year follow-up were associated with outcome.

Applications

The study described the fluctuations in dyspeptic symptoms and found several factors were associated with the outcome. This may provide evidence for the role of psychosocial factors in determining the long-term clinical course of patients with FD.

Terminology

FD is a highly prevalent gastrointestinal disorder that is defined by the presence of symptoms thought to originate in the gastroduodenal region, without identifiable cause by routine diagnostic methods.

Peer review

This is a follow-up study evaluating the clinical course and potential risk factors for FD. This is an interesting article discussing an important area in functional gastrointestinal disorders.

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META-ANALYSIS

Systematic review and meta-analysis of laparoscopy-assisted and open total gastrectomy for gastric cancer

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Abstract

AIM: To evaluate the safety and efficacy of laparoscopy-assisted total gastrectomy (LATG) and open total gastrectomy (OTG) for gastric cancer.

METHODS: A comprehensive search of PubMed, Cochrane Library, Web of Science and BIOSIS Previews was performed to identify studies that compared LATG and OTG. The following factors were checked: operating time, blood loss, harvested lymph nodes, flatus time, hospital stay, mortality and morbidity. Data synthesis and statistical analysis were carried out using RevMan 5.1 software.

RESULTS: Nine studies with 1221 participants were included (436 LATG and 785 OTG). Compared to OTG, LATG involved a longer operating time [weighted mean difference (WMD) = 57.68 min, 95%CI: 30.48-84.88; $P < 0.001$]; less blood loss [standard mean difference (SMD) = -1.71; 95%CI: -2.48 - -0.49; $P < 0.001$]; earlier time to flatus (WMD= -0.76 d; 95%CI: -1.22 - -0.30; $P < 0.001$); shorter hospital stay (WMD = -2.67 d; 95%CI: -3.96 - -1.38, $P < 0.001$); and a decrease in medical complications (RR = 0.41, 95%CI: 0.19-0.90, $P = 0.03$). The number of harvested lymph nodes, mortality, surgical complications, cancer recurrence rate and long-term survival rate of patients undergoing LATG were similar to those in patients undergoing OTG.

CONCLUSION: Despite a longer operation, LATG can be performed safely in experienced surgical centers with a shorter hospital stay and fewer complications than open surgery.

Key words: Laparoscopy; Total gastrectomy; Gastric cancer; Complications; Meta-analysis

Core tip: This study evaluated the safety and efficacy of laparoscopy-assisted total gastrectomy (LATG) and open total gastrectomy (OTG) for gastric cancer through systematic review and meta-analysis. The existing research shows that LATG is safe and feasible, which can achieve similar lymph node dissection effects as OTG, characterized by such advantages as less pain, fewer postoperative complications, and rapid recovery, and which is expected to achieve the same effect in oncological treatment as OTG.

Chen K, Xu XW, Zhang RC, Pan Y, Wu D, Mou YP. Systematic review and meta-analysis of laparoscopy-assisted and open total gastrectomy for gastric cancer. *World J Gastroenterol* 2013; 19(32): 5365-5376 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5365.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5365

INTRODUCTION

Since it was first reported in 1994[1], laparoscopy-assisted distal gastrectomy (LADG) for gastric cancer has undergone rapid development and gained popularity in the past 20 years. Compared to traditional open gastrectomy, most studies have reported that LADG can achieve better cosmesis, shorter hospital stay, faster postoperative recovery, and better postoperative quality of life[2-6]. However, laparoscopy-assisted total gastrectomy (LATG) is technically demanding and the incidence of upper gastric carcinoma is relatively low in East Asia[7,8]. Therefore, although LADG has been accepted worldwide for tumors located in the lower stomach, LATG for upper and middle gastric cancer has not been generalized. In fact, there are only a few reports on the technical feasibility and safety of LATG and its long-term oncologic outcomes[9-12]. Although several meta-analyses and systematic reviews have been published for LADG[13-19], such studies have not been conducted for the potential benefits and disadvantages of LATG.

In order to assess accurately the current status of LATG, we strictly limited inclusion criteria by focusing exclusively on LATG and carried out a comprehensive meta-analysis. We believe that such research will contribute to a more systematic and objective evaluation of the safety of the LATG in cancer treatment.

MATERIALS AND METHODS

Search strategy

We searched PubMed, Cochrane Library, Web of Science and BIOSIS Previews for literature comparing LATG and open total gastrectomy (OTG) published between January 1995 and March 2013, and broadened the search range by browsing the related summary, methods, and references of retrieved articles. The following keywords were used: "laparoscopy", "laparoscopic", "gastric cancer", "gastric carcinoma", and "gastrectomy". The language of the publications was confined to English. Two investigators reviewed the titles and abstracts, and assessed the full text to establish eligibility.

Inclusion and exclusion criteria

All clinical studies should meet the following criteria for the meta-analysis: (1) published in English with data comparing LATG and OTG; (2) clear case selection criteria, containing at least the following information: the number of cases, surgical methods and perioperative data; and (3) if there was overlap between authors or centers, the higher quality or more recent literature were selected. However, articles from the same authors or centers but with different patient cohorts were included. The papers containing any of the following were excluded: (1) totally laparoscopic, laparoscopic hand-assisted, or robot-assisted gastrectomy; (2) non-gastric carcinoma cases; (3) palliative resection cases; and (4) extent of lymphadenectomy was not required for grouping in this study, but the articles with significant differences between the two groups in the extent of lymphadenectomy were excluded.

Data extraction and quality assessment

Two authors independently extracted the data using a unified datasheet, and decided upon the controversial issues through discussion. Extracted data included: author, study period, geographical region, number of patients, operating time, blood loss, number of retrieved lymph nodes, proximal and distal margin distance, time to flatus, time to oral intake, length of hospital stay, morbidity and mortality. Postoperative complications were classified as medical (cardiovascular, respiratory, or metabolic events; nonsurgical infections; deep venous thrombosis; and pulmonary embolism) or surgical (any anastomotic leakage or fistula, any complication that required reoperation, intra-abdominal collections, wound complications, bleeding events, pancreatitis, ileus, delayed gastric emptying, and anastomotic stricture). This classification system is based on the Memorial Sloan-Kettering Cancer Center complication reporting system[20]. If necessary, the first authors were contacted to retrieve further information. Selected documents were rated according to the grading of the Centre of Evidence-Based Medicine (CEBM, Oxford, United Kingdom; http://www.cebm.net), which, in brief, assigns level 1 to randomised controlled trials (RCTs), level 2 to cohort studies, level 3 to case-control studies, level 4 to case series or poor quality observational study and level 5 to expert opinion.

Statistical analysis

The meta-analysis was performed in line with recommendations from the Cochrane Collaboration and the Quality of Reporting of Meta-Analyses guidelines[21,22]. Continuous variables, when both means and standard deviations were presented, were assessed using weighted mean difference (WMD) or standard mean difference (SMD), the postoperative morbidity and mortality were analyzed using the risk ratio (RR), and the risk difference (RD) was used to evaluate cancer recurrence because there may be no recurrence events in either groups during follow-up. When heterogeneity test showed no significant differences ($P > 0.05$), we used a fixed-effects model to calculate the summary statistics. When the heterogeneity test showed statistically significant differences ($P < 0.05$), we used a random effects model based on the DerSimonian and Laird method. Subgroup analysis of intraoperative outcomes, such as operating time, blood loss, and number of retrieved lymph nodes, was conducted for the number of LATG cases performed (40 cases were used as a cut-point), because the learning curve may have an impact on the operative outcomes. Potential publication bias was determined by conducting informal visual inspection of funnel plots based on the complications. Data analyses were performed using Review Manage Version 5.1 (RevMan 5.1) software downloaded from Cochrane Library. $P < 0.05$ was considered statistically significant.

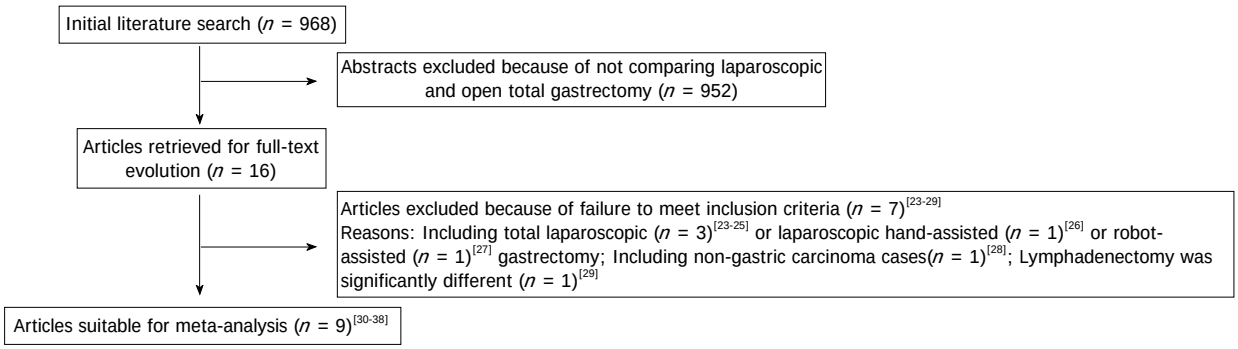


Figure 1 Flow chart of literature search strategies.

Table 1 Characteristics of included studies

Ref.	Nation	Study type	Study period	Sample size		Stage	Level of lymphadenectomy	Follow-up (mo)		Level of evidence
				LATG	OTG			LATG	OTG	
Kim *et al*[30]	South Korea	Retro	2004-2006	27	33	EC + AC	D1 + α/β, D2	NR	NR	2b
Mochiki *et al*[31]	Japan	Retro	1999-2007	20	18	EC + AC	D1 + β	31 (3-60)	46 (13-60)	2b
Sakuramoto *et al*[32]	Japan	Retro	2003-2007	30	44	EC + AC	D1 + β, D2	30		4
Kawamura *et al*[33]	Japan	Retro	2003-2008	46	35	EC	D2	NR	NR	4
Du *et al*[34]	China	Retro	2005-2009	82	94	AC	D2	25 (2-44)		2b
Kim *et al*[35]	South Korea	Pros	2009-2010	63	127	EC + AC	D2	NR	NR	2b
Kunisaki *et al*[36]	Japan	Pros	2002-2008	27	30	EC + AC	D1 + β	NR	NR	3b
Eom *et al*[37]	Korea	Retro	2003-2008	100	348	EC + AC	D2	52.6 (0.3-95.7)		4
Guan *et al*[38]	China	Pros	2007-2010	41	56	EC + AC	D2	NR	NR	3b

Retro: Retrospective observational study; Pros: Prospective observational study; EC: Early gastric cancer; AC: Advanced gastric cancer; NR: Not reported; LATG: Laparoscopy-assisted total gastrectomy; OTG: Open total gastrectomy.

RESULTS

Studies selected

The initial search strategy retrieved 968 publications in English. After the titles and abstracts were reviewed, papers without comparison of LATG and OTG were excluded, which left 16 comparative studies, seven[23-29] of which did not meet the inclusion criteria and were excluded. This left a total of nine comparative observational studies[30-38]. A flow chart of the search strategies is illustrated in Figure 1.

Study characteristics and quality

A total of 1221 patients were included in the analysis with 436 undergoing LATG (35.7%) and 785 undergoing OTG (64.3%). Only one study reported a case converted to open surgery because of extensive abdominal adhesions[38]. Regarding the tumor stage, only one study was limited to early stage cancer[33]. In another study, only patients with advanced gastric cancer were described[34]. The other seven studies included both populations. All studies had Asian data from Japan, South Korea and China. In the included studies, four studies was considered as level of evidence 2b, two studies as level of evidence 3b, and the remaining three as level of evidence 4 (according to the grading of the CEBM). The characteristics and methodological quality assessment scores of the included studies are shown in Table 1.

Intraoperative effects

Most of the studies considered suitable for the meta-analysis reported a longer operating time for LATG than for OTG. The mean operating time of LATG was 57.68 min longer than for OTG (WMD = 57.68 min; 95%CI: 30.48-84.88, $P < 0.001$) (Figure 2A). Two studies[31,32] used grams but the others[33-36,38] used milliliters as the unit of measurement for intraoperative blood loss, therefore, SMD was used to synthesize the data. The intraoperative blood loss was lower in LATG than OTG (SMD = -1.71; 95%CI: -2.48 - -0.94, $P < 0.001$) (Figure 2B). All studies contained the number of retrieved lymph nodes. The difference in the mean number of retrieved lymph nodes between LATG and OTG was not significant in the pooled data (WMD = -1.41; 95%CI: -3.15 - 0.32, $P = 0.11$) (Figure 2C). Two studies described the proximal and distal margin distances[35,38]. Meta-analysis of the distal margin distance showed no significant difference between the two groups (WMD = 0.46 cm; 95%CI: -0.40 - 1.32, $P = 0.29$). However, the proximal margin distance of OTG was longer than that of LATG with a marginal difference (WMD = -0.40 cm; 95%CI: -0.82 - 0.02, $P = 0.06$). All intraoperative effect outcomes are summarized in Table 2.

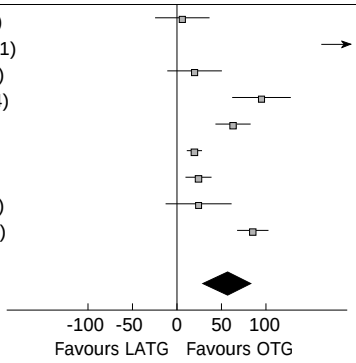
Subgroup analysis for learning curve

The overall effects of operating time and blood loss remained unchanged in subgroups, although performing

A

Study or subgroup	LATG Mean	LATG SD	LATG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, random, 95%CI
Mochiki *et al*[31]	254	44.7	20	248	50.9	18	10.8%	6.00 (-24.61, 36.61)
Kim *et al*[30]	527.5	95.7	27	320.9	75.8	33	9.4%	206.60 (162.19, 251.01)
Kawamura *et al*[33]	291.9	59.4	46	272.1	76.8	35	10.8%	19.80 (-10.89, 50.49)
Sakuramoto *et al*[32]	313	81	30	218	53	44	10.6%	95.00 (62.06, 127.94)
Du *et al*[34]	275	78	82	212	51	94	11.8%	63.00 (43.22, 82.78)
Kim *et al*[35]	150.8	31.2	63	131.2	21.6	127	12.4%	19.60 (11.03, 28.17)
Guan *et al*[38]	235.7	38.5	41	211.5	33.2	56	12.1%	24.20 (9.55, 38.85)
Kunisaki *et al*[36]	286.4	68	27	262.1	74.9	30	10.2%	24.30 (-12.80, 61.40)
Eom *et al*[37]	283.7	84.1	100	198.5	59.7	348	11.9%	85.20 (67.56, 102.84)
Total (95%CI)			436			785	100.0%	57.68 (30.48, 84.88)

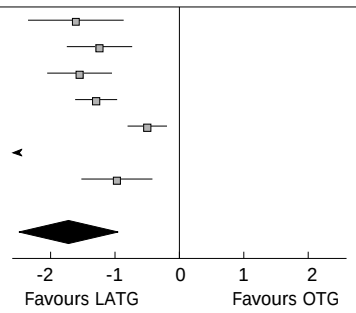
Heterogeneity: Tau² = 1535.02; χ^2 = 127.63, *df* = 8 (*P* < 0.00001); I^2 = 94%
Test for overall effect: *Z* = 4.16 (*P* < 0.0001)



B

Study or subgroup	LATG Mean	LATG SD	LATG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, random, 95%CI
Mochiki *et al*[31]	299	223.6	20	758	330.9	18	13.5%	-1.61 (-2.35, -0.87)
Sakuramoto *et al*[32]	134	98	30	407	270	44	14.5%	-1.24 (-1.75, -0.73)
Kawamura *et al*[33]	54.9	45.3	46	304.3	237.3	35	14.5%	-1.55 (-2.05, -1.05)
Du *et al*[34]	156	112	82	339	162	94	15.1%	-1.29 (-1.62, -0.97)
Kim *et al*[35]	179.7	123.8	63	272.7	209.6	127	15.1%	-0.50 (-0.80, -0.19)
Guan *et al*[38]	104.2	42.9	41	355.6	51.3	56	13.0%	-5.20 (-6.05, -4.35)
Kunisaki *et al*[36]	155	138.8	27	422.4	350.4	30	14.3%	-0.97 (-1.52, -0.42)
Total (95%CI)			309			404	100.0%	-1.71 (-2.48, -0.94)

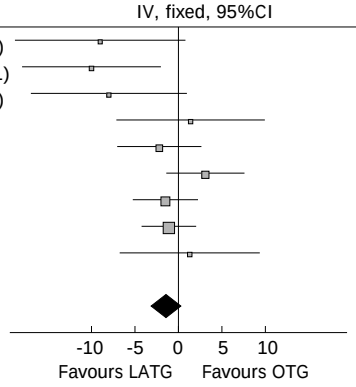
Heterogeneity: Tau² = 0.99; χ^2 = 109.49, *df* = 6 (*P* < 0.00001); I^2 = 95%
Test for overall effect: *Z* = 4.36 (*P* < 0.0001)



C

Study or subgroup	LATG Mean	LATG SD	LATG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, fixed, 95%CI
Mochiki *et al*[31]	26	13.4	20	35	17	18	3.1%	-9.00 (-18.81, 0.81)
Kim *et al*[30]	27.2	15.7	27	37.2	15.7	33	4.7%	-10.00 (-17.99, -2.01)
Sakuramoto *et al*[32]	43.2	17.2	30	51.2	22.1	44	3.7%	-8.00 (-16.97, 0.97)
Kawamura *et al*[33]	48.5	16.3	46	47.1	21.5	35	4.1%	1.40 (-7.14, 9.94)
Du *et al*[34]	34.2	13.5	82	36.4	19.1	94	12.8%	-2.20 (-7.04, 2.64)
Kim *et al*[35]	38.7	15.7	63	35.6	13.1	127	14.9%	3.10 (-1.40, 7.60)
Eom *et al*[37]	48.3	16.4	100	49.8	18.4	348	21.4%	-1.50 (-5.25, 2.25)
Guan *et al*[38]	23.1	8	41	24.2	7.5	56	30.5%	-1.10 (-4.24, 2.04)
Kunisaki *et al*[36]	38.1	13.9	27	36.8	17.1	30	4.6%	1.30 (-6.76, 9.36)
Total (95%CI)			436			785	100.0%	-1.41 (-3.15, 0.32)

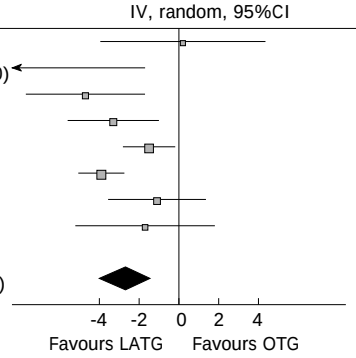
Heterogeneity: χ^2 = 13.67, *df* = 8 (*P* = 0.09); I^2 = 41%
Test for overall effect: *Z* = 1.60 (*P* = 0.11)



D

Study or subgroup	LATG Mean	LATG SD	LATG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, random, 95%CI
Kim *et al*[30]	16.2	7.1	27	16	9.3	33	7.1%	0.20 (-3.95, 4.35)
Mochiki *et al*[31]	19	13.4	20	29	12.7	18	2.2%	-10.00 (-18.30, -1.70)
Sakuramoto *et al*[32]	13.5	2.7	30	18.2	9.6	44	10.9%	-4.70 (-7.70, -1.70)
Kawamura *et al*[33]	15.5	3.3	46	18.8	6.3	35	14.5%	-3.30 (-5.59, -1.01)
Kim *et al*[35]	8.1	3.8	63	9.6	5.3	127	20.9%	-1.50 (-2.82, -0.18)
Guan *et al*[38]	9.7	2.2	41	13.6	3.6	56	21.9%	-3.90 (-5.06, -2.74)
Kunisaki *et al*[36]	14.5	3.5	27	15.6	5.8	30	13.5%	-1.10 (-3.56, 1.36)
Eom *et al*[37]	12.6	15.5	100	14.3	16.7	348	9.0%	-1.70 (-5.21, 1.81)
Total (95%CI)			354			691	100.0%	-2.67 (-3.96, -1.38)

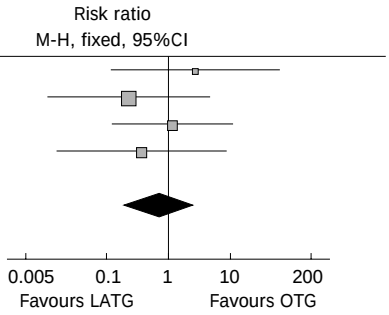
Heterogeneity: Tau² = 1.63; χ^2 = 16.04, *df* = 7 (*P* = 0.02); I^2 = 56%
Test for overall effect: *Z* = 4.05 (*P* < 0.0001)



E

Study or subgroup	LATG Events	LATG Total	OTG Events	OTG Total	Weight	Risk ratio M-H, fixed, 95%CI
Mochiki *et al*[31]	1	20	0	18	9.3%	2.71 (0.12, 62.70)
Du *et al*[34]	0	82	2	94	41.5%	0.23 (0.01, 4.70)
Eom *et al*[37]	1	100	3	348	23.8%	1.16 (0.12, 11.03)
Kunisaki *et al*[36]	0	27	1	30	25.3%	0.37 (0.02, 8.69)
Total (95%CI)		229		490	100.0%	0.72 (0.20, 2.57)
Total events	2		6			

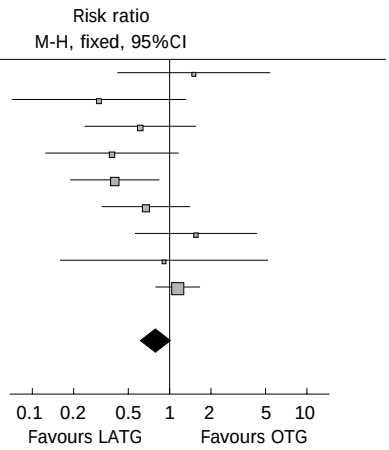
Heterogeneity: χ^2 = 1.58, *df* = 3 (*P* = 0.66); I^2 = 0%
Test for overall effect: *Z* = 0.51 (*P* = 0.61)



F

Study or subgroup	LATG Events	LATG Total	OTG Events	OTG Total	Weight	Risk ratio M-H, fixed, 95%CI
Mochiki *et al*[31]	5	20	3	18	2.9%	1.50 (0.42, 5.41)
Kim *et al*[30]	2	27	8	33	6.5%	0.31 (0.07, 1.32)
Sakuramoto *et al*[32]	5	30	12	44	8.8%	0.61 (0.24, 1.56)
Kawamura *et al*[33]	4	46	8	35	8.2%	0.38 (0.12, 1.16)
Du *et al*[34]	8	82	23	94	19.4%	0.40 (0.19, 0.84)
Kim *et al*[35]	8	63	24	127	14.4%	0.67 (0.32, 1.41)
Kunisaki *et al*[36]	7	27	5	30	4.3%	1.56 (0.56, 4.33)
Guan *et al*[38]	2	41	3	56	2.3%	0.91 (0.16, 5.20)
Eom *et al*[37]	27	100	82	348	33.2%	1.15 (0.79, 1.67)
Total (95%CI)		436		785	100.0%	0.79 (0.61, 1.02)
Total events	68		168			

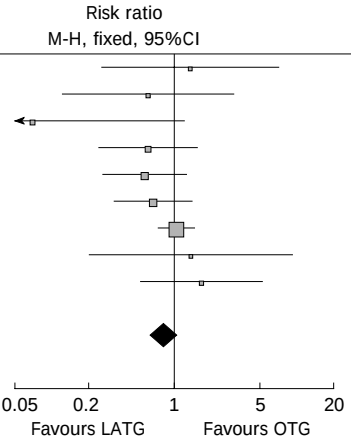
Heterogeneity: χ^2 = 13.42, *df* = 8 (*P* = 0.10); I^2 = 40%
Test for overall effect: *Z* = 1.83 (*P* = 0.07)



G

Study or subgroup	LATG Events	LATG Total	OTG Events	OTG Total	Weight	Risk ratio M-H, fixed, 95%CI
Mochiki *et al*[31]	3	20	2	18	2.1%	1.35 (0.25, 7.19)
Kim *et al*[30]	2	27	4	33	3.6%	0.61 (0.12, 3.09)
Kawamura *et al*[33]	0	46	5	35	6.2%	0.07 (0.00, 1.22)
Sakuramoto *et al*[32]	5	30	12	44	9.6%	0.61 (0.24, 1.56)
Du *et al*[34]	8	82	16	94	14.7%	0.57 (0.26, 1.27)
Kim *et al*[35]	8	63	24	127	15.7%	0.67 (0.32, 1.41)
Eom *et al*[37]	29	100	97	348	42.8%	1.04 (0.73, 1.48)
Guan *et al*[38]	2	41	2	56	1.7%	1.37 (0.20, 9.30)
Kunisaki *et al*[36]	6	27	4	30	3.7%	1.67 (0.53, 5.28)
Total (95%CI)		436		785	100.0%	0.83 (0.64, 1.08)
Total events	63		166			

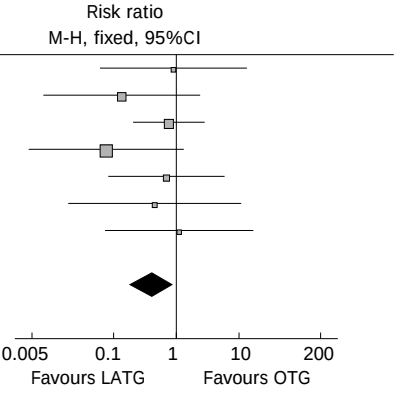
Heterogeneity: χ^2 = 8.14, *df* = 8 (*P* = 0.42); I^2 = 2%
Test for overall effect: *Z* = 1.39 (*P* = 0.16)



H

Study or subgroup	LATG Events	LATG Total	OTG Events	OTG Total	Weight	Risk ratio M-H, fixed, 95%CI
Mochiki *et al*[31]	1	20	1	18	5.0%	0.90 (0.06, 13.36)
Kim *et al*[30]	0	27	4	33	19.3%	0.13 (0.01, 2.40)
Kawamura *et al*[33]	4	46	4	35	21.5%	0.76 (0.20, 2.83)
Du *et al*[34]	0	82	7	94	33.1%	0.08 (0.00, 1.32)
Eom *et al*[37]	1	100	5	348	10.6%	0.70 (0.08, 5.89)
Guan *et al*[38]	0	41	1	56	6.0%	0.45 (0.02, 10.83)
Kunisaki *et al*[36]	1	27	1	30	4.5%	1.11 (0.07, 16.91)
Total (95%CI)		343		614	100.0%	0.41 (0.19, 0.90)
Total events	7		23			

Heterogeneity: χ^2 = 3.84, *df* = 6 (*P* = 0.70); I^2 = 0%
Test for overall effect: *Z* = 2.21 (*P* = 0.03)



I

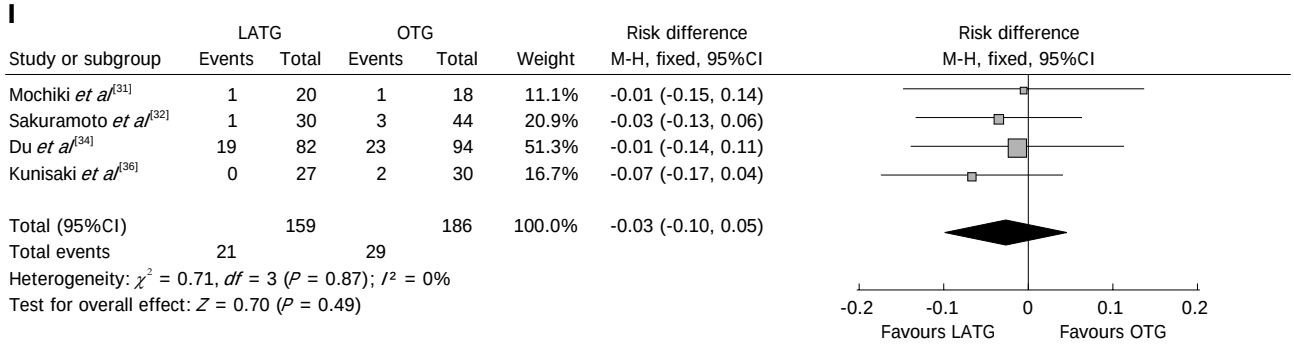


Figure 2 Meta-analysis. A: The pooled data: operating time; B: The pooled data: intraoperative blood loss; C The pooled data: number of retrieved lymph nodes; D: The pooled data: duration of hospital stay; E: The pooled data: mortality; F: The pooled data: overall postoperative complications; G: The pooled data: surgical complications; H: The pooled data: medical complications; I: The pooled data: recurrences.

Table 2 Results of meta-analysis

Outcome	No. of study	Sample size		Heterogeneity (P, I^2)	Overall effect size	95%CI of overall effect	P value
		LATG	OTG				
Operating time (min)	9	436	785	< 0.001, 94%	WMD = 57.68	30.48-84.88	< 0.001
Blood loss	7	309	404	< 0.001, 95%	SMD = -1.71	-2.48 - -0.94	< 0.001
Retrieved lymph nodes	9	436	785	0.09, 41%	WMD = -1.41	-3.15 - 0.32	0.11
Proximal margin (cm)	2	163	475	1.00, 0%	WMD = -0.40	-0.82 - 0.02	0.06
Distal margin (cm)	2	163	475	0.67, 0%	WMD = 0.46	-0.40 - 1.32	0.29
Analgesics given	4	221	300	< 0.001, 93%	SMD = -0.86	-1.62 - -0.11	0.02
Duration of fever (d)	2	112	138	0.47, 0%	WMD = -1.58	-1.80 - -1.37	< 0.001
Time to first flatus (d)	7	316	419	< 0.001, 91%	WMD = -0.76	-1.22 - -0.30	0.001
Time to oral intake (d)	4	161	257	0.04, 63%	WMD = -0.81	-1.26 - -0.35	< 0.001
Hospital stay (d)	8	354	691	0.02, 56%	WMD = -2.67	-3.96 - -1.38	< 0.001
Overall complications	9	436	785	0.10, 40%	RR = 0.79	0.61-1.02	0.07
Surgical complications	9	436	785	0.42, 2%	RR = 0.83	0.64-1.08	0.16
Medical complications	7	343	614	0.70, 0%	RR = 0.41	0.19-0.90	0.03
Mortality	4	229	490	0.66, 0%	RR = 0.72	0.20-2.57	0.61

WMD: Weighted mean difference; SMD: Standard mean difference; LATG: Laparoscopy-assisted total gastrectomy; OTG: Open total gastrectomy.

> 40 LATG cases demonstrated a moderate reduction in operating time and blood loss. Lymph node retrieval was lower in the subgroup with < 40 LATG cases performed (WMD = -6.12; 95%CI: -10.42 - -1.81, P = 0.005). However, there was no difference when > 40 LATG procedures were performed (WMD = -0.50; 95%CI: -2.4 - 1.39, P = 0.60). The outcomes of subgroup analysis are summarized in Table 3.

Postoperative outcome

Flatus is one of the outcome measures for evaluating postoperative recovery of gastrointestinal functions. The mean time to first flatus was shorter in LATG than in OTG (WMD= -0.76 d; 95%CI: -1.22 - -0.30, P = 0.001), as was the time to restart oral intake after surgery (WMD = -0.81 d; 95%CI: -1.26 - -0.35, P < 0.001). Postoperative analgesic consumption was less in LATG than in OTG (SMD = -0.86; 95%CI: -1.62 - -0.11, P = 0.02). A shorter hospital stay was also observed in the LATG group (WMD = -2.67 d; 95%CI: -3.96 - -1.38, P < 0.001) (Figure 2D). All postoperative outcomes are summarized in Table 2.

Two studies reported inflammatory response index such as white blood cell (WBC) count and C-reactive protein (CRP)[32,33]. A significantly lower WBC count for LATG compared with OTG was found on postoperative days 1, 3, 7[32,33] and 10[33], and lower CRP for LATG was found on postoperative day 1 in both studies[32,33].

Mortality was described in four studies, and there was no significant difference in postoperative mortality (RR = 0.72, 95%CI: 0.20-2.57, P = 0.61) (Figure 2E). Morbidity was addressed and specified in all studies with exception of Kunisaki's study[36]. We contacted the authors of this study to get information about the specific complications. The rate of overall postoperative complications was lower for LATG with a marginal difference (RR = 0.79, 95%CI: 0.61-1.02, P = 0.07) (Figure 2F). Visual inspection of the funnel plot revealed symmetry, indicating no serious publication bias (Figure 3). After further analysis, surgical complications were similar between the two groups (RR = 0.83, 95%CI: 0.64-1.08, P = 0.16) (Figure 2G), without the exception of any specific complications such as anastomotic leakage, intra-abdominal collections, bleeding, or anastomotic stricture. LATG was associated, however, with a significant reduction in medical complications (RR = 0.41, 95%CI: 0.19-0.90, P = 0.03) (Figure 2H) with a

Table 3 Subgroup analysis for learning curve using a cut-point of 40 laparoscopy-assisted total gastrectomy cases

Outcome	No. of study	Sample size		Heterogeneity (P, I^2)	Overall effect size	95%CI of overall effect	P value
		LATG	OTG				
Operating time (min)							
< 40 LATG cases	4	104	125	< 0.001, 95%	WMD = 81.99	1.47-162.5	0.05
> 40 LATG cases	5	332	660	< 0.001, 93%	WMD = 42.53	16.23-68.82	0.002
Blood loss							
< 40 LATG cases	3	77	92	0.40, 0%	SMD = -1.22	-1.55 - -0.88	< 0.001
> 40 LATG cases	4	232	312	< 0.001, 97%	SMD = -2.07	-3.35 - -0.79	0.002
Retrieved lymph nodes							
< 40 LATG cases	4	104	125	0.20, 36%	WMD = -6.12	-10.42 - -1.81	0.005
> 40 LATG cases	5	332	660	0.47, 0%	WMD = -0.50	-2.4 - 1.39	0.60

LATG: Laparoscopy-assisted total gastrectomy; OTG: Open total gastrectomy; WMD: Weighted mean difference; SMD: Standard mean difference.

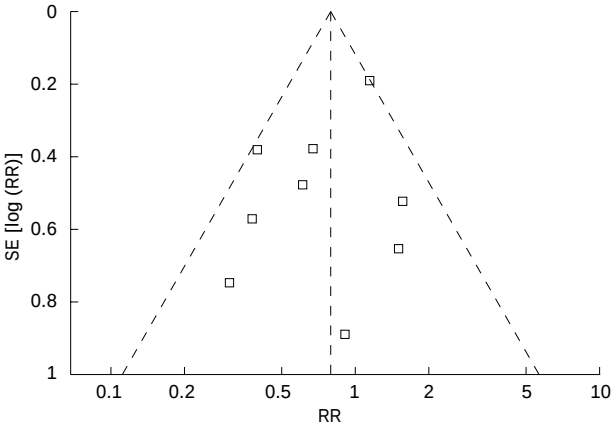


Figure 3 Funnel plot of the overall postoperative complications.

possible contribution from respiratory complications (RR = 0.34, 95%CI: 0.11-1.03, P = 0.06). The outcomes of mortality and morbidity are summarized in Table 2.

Recurrence and long-term survival rate

During the follow-up period, cancer recurrence was observed in four studies[31,32,34,36]. The recurrence risk in LATG was 13.2% (21/159) and 15.6% (29/186) in OTG, but the difference between LATG and OTG was not significant (RD = -0.03, 95%CI: -0.10-0.05, P = 0.49) (Figure 2I).

Three trials reported the long-term survival rate[31,36,37]. Mochiki *et al*[31] have reported that there was no significant difference in the cumulative or disease-specific 5-year survival rates between LATG and OTG (cumulative: 95% in LATG, 90.9% in OTG; disease-specific: 100% in LATG, 91.7% in OTG, P = 0.81). Eom *et al*[37] have reported that the survival rates were similar between groups; the hazard ratio of LATG *vs* OTG was 0.43 (95%CI: 0.15-1.20; P = 0.107) for overall survival and 0.47 (95%CI: 0.19-1.18; P = 0.106) for disease-free survival. Kunisaki *et al*[36] also have reported that there was no significant differences in overall and disease-specific survival between groups.

DISCUSSION

RCTs are the most ideal tools for meta-analysis. However, no RCTs on LATG have yet been conducted because the history and popularity of LATG are insufficient compared with LADG, due to the fact that it is difficult to dissect splenic hilar lymph nodes and mobilize the esophagus under a laparoscope, while it is demanding to perform Roux-en-Y esophagojejunostomy through mini-laparotomy. Thus, our meta-analysis synthesized the existing observational studies with strictly limiting inclusion and exclusion criteria. The included studies were primarily derived from the countries with the most widespread use of laparoscopic gastrectomy (four from Japan, three from Korea, and two from China), and all published in the past 5 years (2008-2012), and the total number of cases incorporated in the study was 1221. The meta-analysis conducted based on this point will contribute a more comprehensive and objective evaluation for the current LATG surgical status.

Similar to most reports comparing laparoscopic and open surgery in many different clinical situations, the intraoperative blooding in the LATG group was less than that in the OTG group, as is the need for transfusions. The reduced length of incision wound and the application of energy-dividing devices, such as the Harmonic Scalpel and Ligasure, contribute to the reduction in blood loss. Lack of blood is a common problem faced by many hospitals, especially in developing countries such as China. Therefore, less-invasive laparoscopic surgery can reduce the clinical requirement for blood and lower the rate of complications associated with blood transfusions such as virus infection and allergic reaction. In addition, some researchers have suggested that transfusions are associated with increased perioperative mortality and morbidity[39].

Regarding the operating time, LATG is more time-consuming than OTG. LATG combined with lymphadenectomy is a complex operation and needs a lot of technical expertise. Almost all of the studies included in this meta-analysis demonstrated prolonged operating time in LATG, despite significant heterogeneity. Learning curve which related to the surgeon's experience, familiarity with instruments, and assistant compliance could influence some outcomes studied, such as operating time or lymph node retrieval[40]. Because several of the researches included in this study reported on their initial experience, so

we performed a subgroup analysis using 40 LATG cases as a cut-point and demonstrated a moderate reduction in LATG operating time. Another reason for the prolonged operating time for LATG may be related to the reconstructive step, which is more difficult to complete through minilaparotomy than open surgery because of the narrow operating window for manual suture or anvil insertion and application of other instruments, especially in obese patients. To overcome these potential problems, various modified techniques have been reported, such as laparoscopic purse-string suture technique using Endo Stitch (Covidien, Mansfield, MA, United States)[41], Endo-PSI (Hope Electronics Co., Ltd, Shenzhen, China)[42], or a hemi-double stapling technique[43]. Another two intracorporeal reconstruction methods may be most representative; one using a transorally inserted anvil (OrVil; Covidien) to make an end-to-side esophagojejunostomy[44], the other using linear staplers to make a side-to-side anastomosis[45]. These methods not only avoid auxiliary incision, but also help to simplify the procedure of reconstruction and shorten the operating time[46,47].

The inflammatory stress reaction is an inevitable outcome of operative trauma and is an important index for measuring its extent. Some studies have compared inflammatory cytokines such as interleukin (IL)-6, IL-10 and CRP in plasma of patients who have undergone laparoscopic or laparotomic resection for gastroenteric cancer. The postoperative level of IL-6, IL-10 and CRP increased but the levels in the laparoscopic group are significantly lower than in the laparotomic group[48-50]. A meta-analysis of laparoscopic colectomy has also demonstrated that the postoperative IL-6 level of laparotomic group patients was significantly lower than that of laparotomic group[51]. The studies included in this research show that the WBC count and CRP of patients in the LATG group were lower than those in the OTG group, and serum protein was higher[32,33], indicating that LATG imposes few inflammatory stimuli on patients and consumes less protein. Kawamura *et al*[33] have also found that postoperative blood glucose in OTG patients is significantly higher than that in LATG patients when the same amount of calories was ingested, indicating that LATG has a lower effect on sugar metabolism.

The most striking finding was a reduced number of complications in the LATG *vs* OTG group, which may have resulted from a reduction in medical complications. It was conceivable that surgical complications were similar between groups because LATG results in the same organ and lymphatic resection as OTG. However, it is worth noting that some studies have found that there is a high risk of anastomotic stricture after LATG[10,52], whereas our study found morbidity associated with anastomotic stricture was similar between the two groups. Prevention of anastomotic stricture has long been one of the main tasks in total gastrectomy and also should not be ignored in LATG. Some researchers hold that side-to-side esophagojejunostomy could be used to reduce the risk of anastomotic stricture because a larger anastomotic stoma can be made from it[45,53]. Besides, the significantly decreased medical complications could be explained by the reduced invasiveness of the laparoscopic technique and less postoperative pain. We also found that respiratory complications occurred in LATG less often than in OTG, although the difference was not significant (P = 0.06). The pain caused by large incision as well as the use of tension sutures and abdominal bandages after laparotomy can make it difficult for patients to cough, expectorate and perform exercise breathing effectively, thus leading to such complications as pulmonary infection[54]. Pain after surgery was less serious in LATG than in OTG due to the shorter duration or the lower dosage of analgesic application[32-35]. The time to first flatus was also earlier in LATG than in OTG, which indicated a rapid recovery of gastrointestinal function after LATG. Reduced use of analgesic drugs, shortened time of abdominal cavity exposure, alleviated inflammatory reactions, and earlier postoperative activities are considered to be the main reasons for earlier gastrointestinal recovery from LATG; all of which may also contribute to shortening the duration of postoperative hospital stay.

The adequacy of the radical resection should be evaluated by the extent of lymph node dissection performed and the number of harvested lymph nodes, as well as the length of the resection margins. We found that fewer lymph nodes were obtained after LATG than in OTG, even though the difference was not significant. However, the subgroup analysis with 40 cases in LATG showed that the difference was shrinking. The number of laparoscopic lymph nodes dissected was closely related to the level of surgical technique. In recent years, with increasingly mature techniques, some researchers have reported not only a similar number of overall retrieved lymph nodes between LADG and open distal gastrectomy, but also a similar number of specific lymph nodes, such as group 7, 8a, 9, 11p, 12a and 14v, which used to be considered difficult for laparoscopic dissection[55,56]. Splenic hilar lymph node dissection is one of the difficulties in radical total gastrectomy, which is because the splenic vessels run circuitously, and the branches vary substantially and they are in a narrow space at a very deep location. It is easy to cause hemorrhage because of splenic vascular injury or cause spleen ischemia and further necrosis by accidental cutting of the splenic artery branches of when dissecting the lymph nodes in this area. Compared to laparotomy, laparoscopy allows the operator to complete the spleen hilum lymph node dissection under a clear field of view and helps to improve surgical safety[57].

With regard to the length of the resection margin, we found that the proximal margin in LATG was shorter than that of OTG. Such result may relate to the nature for LATG which should resect specimen and make reconstruction all through mini-laparotomy; and it is difficult to pull the proximal stomach using a narrow incision, which may influence the distance of proximal margin. Therefore, patients with smaller neoplasms are more likely to receive LATG instead of OTG, thus allowing

the surgeon to choose a smaller excision extension.

Cancer recurrence and long-term survival rate are two critical outcomes for evaluating surgical interventions in oncological therapy. LATG is not superior to LADG in both history and popularity, and only three studies have compared the long-term survival rate between the two groups[31,36,37], and another two have performed a descriptive analysis of cancer recurrence[32,34]. Based on these data, postoperative cancer recurrence and long-term survival rate in LATG were similar to those in OTG. However, as the cases in the studies included in our analysis were mostly concerned with early gastric cancer, the effect of LATG for early gastric cancer should be affirmed. Some RCTs and meta-analyses have demonstrated that long-term follow-up outcome of laparoscopic gastrectomy for advanced gastric cancer is similar to that of laparotomy[58,59]. Recently, Park *et al*[60] have analyzed the follow-up results of 239 cases of advanced gastric cancer treated with laparoscopic radical gastrectomy. Among these cases, 130 were T_2 stage, 63 were T_3, and 46 were T_4, and the 5-year survival rates were 86.6%, 77.4% and 58.7%, respectively. The result is similar to that for concurrent laparotomy and is encouraging. However, there should be an attitude of caution for laparoscopic resection of advanced gastric cancer because relevant studies and clinical evidence are still deficient.

During our research, a similar article by Haverkamp *et al*[61] was published, which had several limitations. The clinical heterogeneity could have been caused by the different underlying conditions and interventions. It is well known that gastric submucosal tumors (SMTs) such as lymphoma, leiomyosarcoma, and gastrointestinal stromal tumors are significantly different from adenocarcinoma in terms of biological characteristics, clinical diagnosis, and treatment. In our study, only patients who underwent gastrectomy for gastric adenocarcinoma were included, but Haverkamp *et al* included 8 patients undergoing total gastrectomy for *SMTs*; this may influence the reliability the results[28]. The difference in surgical methods is a major cause of clinical heterogeneity. In laparoscopy-assisted gastrectomy (LAG), an incision is almost always required for extracting a relatively large specimen and involves some complicated steps. However, totally laparoscopic gastrectomy (TLG) is considered to be incisionless, except for the trocar wounds, and it is a laparoscopic approach for intracorporeal anastomosis without auxiliary incision and touching the tumor. Hence, these are two different operative methods. Furthermore, some studies have shown that TLG may be less invasive than LAG, with the disadvantage of prolonged operating time[47,62-66]. Therefore, it is inappropriate to pool trials that differ in terms of these two methods in a meta-analysis. However, the existing meta-analysis included a study in which the TLG was performed using a totally laparoscopic method[23]. In addition, for the trials without the mean and standard deviation, Haverkamp *et al* used the median and range to estimate them based on the Hozo method[67]. However, this method may lead to deviation, especially when the sample size is small or the samples exhibit serious skewness. In the study of Topal for example[23], the median intraoperative blood in the laparoscopic group (n = 38) was 10 (5-400) mL, so the estimated mean blood loss was 10 mL. In fact, however, even the minimum mean blood loss could be 15.4 mL, which differed from the estimated value. Besides, since the study by Haverkamp *et al*[61] was published, several clinical observational studies have become available. The larger the number of patients in a meta-analysis, the greater its power to detect a possible treatment effect. Therefore, our comprehensive meta-analysis will contribute to a more systematic and objective evaluation for the safety and cancer treatment of LATG.

In conclusion, the existing research shows that LATG is safe and feasible, which can achieve similar lymph node dissection effects as OTG, characterized by such advantages as less pain, fewer postoperative complications, and rapid recovery, and which is expected to achieve the same effect in oncological treatment as OTG. However, most of the published studies were retrospective, the sample sizes were relatively small, most of the cases were early gastric cancer, the follow-up periods were not long enough, and the results exhibited substantial heterogeneity. Therefore, the results mentioned above should be subject to verification by strictly designed, large-sample, multicenter, RCTs.

COMMENTS

Background

Since it was first reported in 1994, laparoscopy-assisted distal gastrectomy (LADG) for gastric cancer has undergone rapid development and gained popularity in the past 20 years. Compared with traditional open gastrectomy, LADG can achieve better cosmesis, shorter hospital stay, faster postoperative recovery, and better postoperative quality of life. Although LADG has been accepted worldwide for tumors located in the lower stomach, laparoscopy-assisted total gastrectomy (LATG) for upper and middle gastric cancer has not been generalized. Although several meta-analyses and systematic reviews have been published for LADG, such studies have not been conducted for the potential benefits and disadvantages of LATG.

Research frontiers

In order to assess accurately the current status of LATG, the authors strictly limited inclusion criteria by focusing exclusively on LATG and carried out a comprehensive meta-analysis. This will contribute to a more systematic and objective evaluation of the safety of the LATG in cancer treatment.

Innovations and breakthroughs

LATG is safe and feasible, which can achieve similar lymph node dissection effects as open total gastrectomy (OTG), characterized by such advantages as less pain, fewer postoperative complications, and rapid recovery, and which is expected to achieve the same effect in oncological treatment as OTG.

Applications

Despite a longer operation, LATG can be performed safely in experienced surgical centers with a shorter hospital stay and fewer complications than open surgery.

Peer review

This is a well written paper which will add a great deal to the literature on the subject. One of the most significant conclusions from this work is the lack of randomised controlled trials surrounding the field. Future research should compare LADG and LATG to further verify the safety and feasibility of LATG.

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CASE REPORT

Noninfectious interstitial lung disease during infliximab therapy: Case report and literature review

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Abstract

Pulmonary abnormalities are not frequently encountered in patients with inflammatory bowel diseases. However, lung toxicity can be induced by conventional medications used to maintain remission, and similar evidence is also emerging for biologics. We present the case of a young woman affected by colonic Crohn's disease who was treated with oral mesalamine and became steroid-dependent and refractory to azathioprine and adalimumab. She was referred to our clinic with a severe relapse and was treated with infliximab, an anti-tumor necrosis factor α (TNF-α) antibody, to induce remission. After an initial benefit, with decreases in bowel movements, rectal bleeding and C-reactive protein levels, she experienced shortness of breath after the 5th infusion. Noninfectious interstitial lung disease was diagnosed. Both mesalamine and infliximab were discontinued, and steroids were introduced with slow but progressive improvement of symptoms, radiology and functional tests. This represents a rare case of interstitial lung disease associated with infliximab therapy and the effect of drug withdrawal on these lung alterations. Given the increasing use of anti-TNF-α therapies and the increasing reports of pulmonary abnormalities in patients with inflammatory bowel diseases, this case underlines the importance of a careful evaluation of respiratory symptoms in patients undergoing infliximab therapy.

Key words: Interstitial lung disease; Crohn's disease; Infliximab; Mesalamine; Drug-induced toxicity

Core tip: Safety during anti-tumor necrosis factor (TNF)-α therapy is a major concern. Paradoxical inflammatory and autoimmune phenomena can be induced by this treatment and should always be considered. Interstitial lung disease is an emerging complication often observed early after the beginning of treatment, particularly when combination immunosuppressive regimens are employed. This case demonstrates that interstitial lung disease can also occur later during anti-TNF-α treatment and during monotherapy. Thus, great vigilance is recommended when patients start complaining of any respiratory symptom.

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INTRODUCTION

The occurrence of pulmonary involvement in patients with inflammatory bowel disease (IBD) was first described in 1976 and has been explained either as a potential extra-intestinal manifestation of the disease itself or as a secondary effect of medications employed to control

inflammation[1-4]. The common embryological origin of both the gastrointestinal tract and the respiratory system could be responsible for the shared antigenicity leading to the pulmonary manifestations. However, noninfectious drug-induced lung disease has been described using sulfasalazine, mesalamine, methotrexate and azathioprine[2,4]. Anti-tumor necrosis factor (TNF)-α agents have also been implicated as a cause of drug-induced interstitial lung disease and account for most of the cases reported in the rheumatology literature[5,6].

We report the case of a noninfectious interstitial pneumonia that occurred during infliximab (IFX) treatment in a young woman with colonic Crohn's disease (CD).

CASE REPORT

A 25-year-old female was diagnosed with left-sided ulcerative colitis (UC) in 2004 (16-year-old) and treated with oral and rectal mesalamine. She required several courses of oral prednisone during the subsequent 4-year follow up. Azathioprine was introduced in 2008 because of steroid dependency; however, despite the optimization of the dosage up to 2.5 mg/kg, the patient never experienced a full clinical remission. Colonoscopy demonstrated a segmental distribution of the ulcerative lesions, and histology confirmed CD. According to these findings, in December 2010, the patient discontinued azathioprine and was screened for biologics. Adalimumab (ADA) was started with an induction regimen followed by maintenance. After 4 mo, the patient was referred for a new disease flare and did not respond to concomitant therapy with 25 mg of prednisone. Biochemical parameters demonstrated thrombocytosis (810 × 10^3/μL) and elevated C-reactive protein (25 mg/L) and fecal lactoferrin (538 μg/mL). The new endoscopic assessment showed moderate activity in the left colon and mild lesions in the cecum and terminal ileum (Simple Endoscopic Score for CD 13). The interval between ADA administrations was then reduced to every week for one month, without any significant clinical or biochemical improvement. ADA was stopped, and IFX was started (5 mg/kg) with concomitant steroid tapering. She improved clinically, and her C-reactive protein levels normalized. After the 5th infusion, the patient reported the onset of shortness of breath and fatigue, without concomitant cough or fever. The patient had no history of asthma, atopy or allergy to medications. Chest X-ray did not demonstrate any significant lesion, and thorax auscultation was normal. In accordance with the lung specialist who preliminarily suspected pulmonary sarcoidosis, the 6th dose of IFX was administered, and the patient was admitted to the Pneumology Unit for monitoring. High-resolution computed tomography (HRCT) of the thorax revealed bilateral shadowing nodules and adjacent interstitial thickening with a predominant distribution in the middle and basal regions and relative sparing of the apices (Figure 1). Pulmonary function tests were compatible with a moderately restrictive pattern, without any oximetric deficiency. Bronchoscopy did not demonstrate any endobronchial abnormality, and a bronchoalveolar lavage fluid analysis was negative for *Pneumocystis carinii*, fungi and alcohol-acid resistant bacilli. Cyto-immunological analysis revealed increased cellularity (213 × 10^6/L) with a decreased percentage of macrophages (66%) and a well-represented component of eosinophils (20%) (Table 1). Transbronchial biopsy showed a mild chronic, nonspecific, non-granulomatous infiltrate and thickening of the basal membrane. A QuantiFERON-TB Gold test, auto-antibodies, serum angiotensin-converting enzyme, serum precipitins and blood cultures were unremarkable. Mesalamine and infliximab were discontinued, and prednisone was started at a dose of 50 mg/d for 7 d and subsequently tapered to 25 mg/d in association with inhalations of budesonide and a long-acting beta2-agonist. Clinical improvement occurred over the following 6 wk, with mild symptoms still present at 8 wk. HRCT performed after 10 wk showed minimal peripheral irregularities in both apices. All the symptoms had subsided by week 14. The spirometric values during the follow up are reported in Table 2.

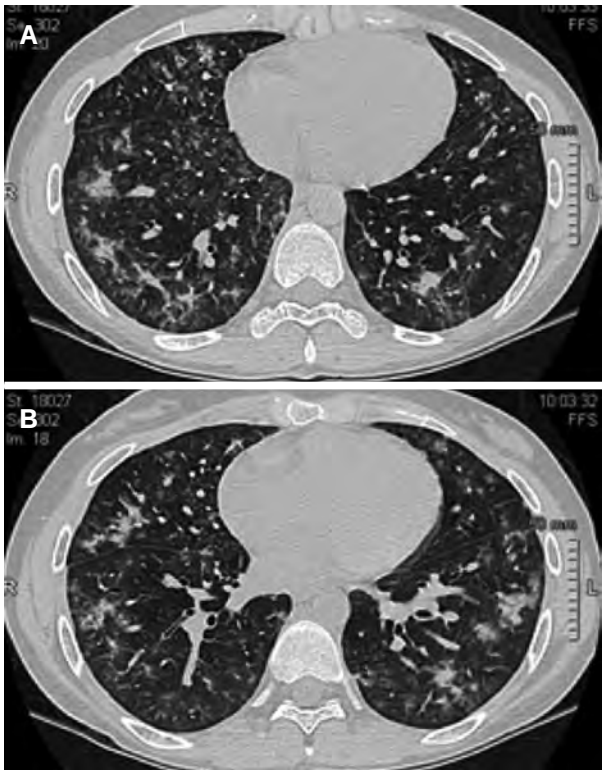

Figure 1 High-resolution computed tomography at hospital admission in the Pneumology Unit. High-resolution computed tomography of the thorax revealed bilateral shadowing nodules and adjacent interstitial (A) thickening with a predominant distribution in the middle and basal regions and relative sparing of the apices (B). R: Right; L: Left.

Table 1 Analysis of the bronchoalveolar lavage fluid

	Cells (10^6/L)	Macrophages	Lymphocytes	Neutrophils	Eosinophils
Patient	213	66%	13%	1%	20%
Ref. values	120-190	85%-93%[1]	5%-12%[1]	2%[1]	< 1%[1]

[1]According to Meyer[16].

Table 2 Spirometric values during the follow up

	4-wk follow up	8-wk follow up	12-wk follow up
FEV1, L	2.03 (58%)	2.24 (64%)	2.56 (73%)
FVC, L	2.08 (52%)	2.33 (58%)	2.62 (66%)
TLC, L	3.04 (56%)	3.63 (67%)	3.73 (69%)
RV, L	0.91 (62%)	1.30 (88%)	0.97 (66%)
FRC, L	1.62 (57%)	2.47 (87%)	2.44 (86%)
DLCO, mL/mmHg per minute	NA	17.3 (58%)	19.0 (64%)

FEV1: Forced expiratory volume in one second; FVC: Forced vital capacity; TLC: Total lung capacity; RV: Residual volume; FRC: Functional residual capacity; DLCO: Diffusion capacity of carbon monoxide; NA: Not available.

DISCUSSION

Although IBDs are pathologic conditions of the gastrointestinal tract, they should be considered as systemic diseases because almost all organs can be involved, although the most frequent extra-intestinal manifestations are articular, dermatologic, ophthalmologic and hepatobiliary[7,8].

Pulmonary involvement can manifest with different patterns[9]. A significant proportion of IBD patients show abnormal functional tests compared to healthy matched controls, suggesting the potential presence of subclinical pulmonary dysfunctions[2,10-13]. In addition and more importantly, HRCT scans performed in 2 series of consecutive IBD patients were pathological in 53.00% and 64.10% of patients, respectively[14,15]. Interestingly, these findings were unrelated to the presence of respiratory symptoms.

Our patient had never previously experienced respiratory symptoms; she did not smoke and did not suffer from asthma or atopy. The previous existence of subclinical respiratory defects cannot be excluded because the patient did not perform a functional respiratory test before the onset of pulmonary symptoms. Nonetheless, it is reasonable to predict the absence of abnormalities because the baseline chest-X ray performed before starting anti-TNF-α therapy was unremarkable.

In addition to IBD-associated pulmonary manifestations, the occurrence of drug-induced effects has to be considered, particularly according to the cyto-immunological analysis of bronchoalveolar lavage fluid, which in our case, was consistent with subacute respiratory illness compatible with either nonspecific interstitial pneumonia or cryptogenic pneumonia[16-18]. Several cases of pulmonary toxicity induced by sulfasalazine and mesalamine have been reported, particularly eosinophilic pneumonia, which is characterized by eosinophilic infiltration of the lungs with or without peripheral eosinophilia[2]. In our patient, we detected normal levels of peripheral eosinophils, and transbronchial biopsy did not reveal an abnormal number of these cells; however, the examination of specimens obtained by fiberoptic bronchoscopy is suboptimal for the diagnosis of interstitial lung diseases. Most of the reported reactions occurred between 2 and 6 mo after the introduction of the drug, with rare cases occurring later on (44 mo)[2,19,20]. Peripheral eosinophilia was often present, and the resolution of symptoms (dyspnea, fever, chest pain, cough) with the discontinuation of the drug was prompt[2]. When our patient first developed shortness of breath, she had been under mesalamine treatment for 8 years. The possibility of mesalamine-induced pneumonia seems unlikely. However, reports of lung toxicity associated with TNF-α antagonists have recently appeared, particularly in the rheumatology literature[5,6]. Our patient was exposed to two different biologics: ADA for 1 year and subsequently IFX for 6 mo. The pulmonary toxicity of ADA is controversial: there are reported cases of induced interstitial pneumonia[21,22] as well as reports of efficacy in the treatment of rheumatoid arthritis- and dermatomyositis-associated lung disease[21,23]. Our patient did not experience any respiratory symptoms during treatment with ADA, and she started complaining of dyspnea on exertion after the 5th dose of IFX. This timing is delayed compared with the previously reported experiences that occurred in the majority of rheumatology cases after the 2nd-3rd infusion[24-28]. Similarly, in the CD patient described by Weatherhead *et al*, symptoms appeared after the first infusion and were exacerbated after the 2nd[27]. In the most recently reported case of nonspecific interstitial pneumonia in a young female with UC, symptoms appeared after the 2nd infusion of IFX[30]. The timing of respiratory symptoms after the 5th infusion of IFX observed in our patient is similar to that reported by Wiener and colleagues in a 63-year-old woman affected by UC[31]. Most of the reported cases received anti-TNF α associated with other immunomodulators[6]; additionally, in the reports by Weatherhead and Wiener, the patients were taking other medications for IBD (azathioprine and balsalazide, respectively)[29,31]. Thus, it was hypothesized that TNF-α blocking agents might provide a favorable environment for the induction and/or the progression of iatrogenic lung disease through modulation of the immune system[32].

In the present case, it is reasonable to suspect drug-induced interstitial lung disease attributable to IFX for several reasons: (1) the onset of respiratory symptoms shortly after IFX introduction; (2) the 8-year treatment with mesalamine without any symptoms; and (3) the slow improvement after mesalamine discontinuation (despite its rapid wash-out period) and after IFX discontinuation (consistent with its long wash-out period). Given the seriousness of the adverse event, definite proof is unrealistic because a re-challenge with the drug would be unethical and dangerous; however, the lack of an anatomical diagnosis using open-lung biopsy limited the differential diagnosis.

In conclusion, there is emerging evidence that anti-TNF-α agents might induce lung toxicity even in the long term. High vigilance is recommended for the occurrence of respiratory symptoms in patients undergoing biological treatment.

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CASE REPORT

Hepatotoxicity associated with glucosamine and chondroitin sulfate in patients with chronic liver disease

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Abstract

Glucosamine and chondroitin sulfate are molecules involved in the formation of articular cartilage and are frequently used for symptom relief in patients with arthrosis. These molecules are well tolerated with scarce secondary effects. Very few cases of possible hepatotoxicity due to these substances have been described. The aim of this paper is to report the frequency of presumed glucosamine hepatotoxicity in patients with liver disease. A questionnaire was given to 151 consecutive patients with chronic liver disease of different etiology (mean age 59 years, 56.9% women) attended in an outpatient clinic with the aim of evaluating the frequency of consumption of these drugs and determine whether their use coincided with a worsening in liver function test results. Twenty-three patients (15.2%) recognized having taken products containing glucosamine or chondroitin sulfate previously or at the time of the questionnaire. Review of the clinical records and liver function tests identified 2 patients presenting an elevation in aminotransferase values temporarily associated with glucosamine treatment; one of the cases simultaneously presented a skin rash attributed to the drug. Review of these two patients and the cases described in the literature suggest toxicity of glucosamine and chondroitin sulfate. The clinical spectrum is variable, and the mechanism of toxicity is not clear but may involve reactions of hypersensitivity. The consumption of products containing glucosamine and/or chondroitin sulfate is frequent among patients with chronic liver diseases and should be taken into account on the appearance of alterations in liver function tests not explained by the underlying disease.

Key words: Toxic hepatitis; Hepatotoxicity; Glucosamine; Chondroitin sulphate; Osteoarthritis

Core tip: A questionnaire was given to 151 consecutive patients with chronic liver disease of different etiology (mean age 59 years, 56.9% women) attended in an outpatient clinic with the aim of evaluating the frequency of consumption of these drugs and determine whether their use coincided with a worsening in liver function test results. Twenty-three patients (15.2%) recognized having taken products containing glucosamine or chondroitin sulfate previously or at the time of the questionnaire.

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INTRODUCTION

Glucosamine and chondroitin sulfate are precursor molecules involved in the synthesis of glycosaminoglycans

which make up the connective tissue. Their integrity is important to maintain the strength and elasticity of articular cartilage which confers resistance to mechanical stress[1]. In addition to its role as a substrate in the synthesis of cartilage and connective tissue, anti-inflammatory properties through the inhibition of the synthesis of nitric oxide have been attributed to glucosamine[2]. In relation to these functions, glucosamine and chondroitin sulfate have been used in the treatment of articular hyaline cartilage degeneration with the aim of stimulating the production of cartilaginous matrix[3].

Adverse effects from the exogenous administration of glucosamine and/or chondroitin sulfate are observed in less than 5% of the patients, with the most frequent being: gastrointestinal disturbances (discomfort/epigastric pain, pyrosis, diarrhea, nausea, and dyspepsia), somnolence, cutaneous reactions and headache. Two patients receiving glucosamine who were attended in our unit presented an unexplained elevation in transaminase values with no associated symptoms which reverted on discontinuation of the medication and was interpreted as a possible toxic hepatitis by this drug. We therefore decided to prospectively investigate the frequency of use of glucosamine and the incidence of elevations in aminotransferase which might be related to this drug. We studied patients with chronic liver disease since we considered that these patients may have limitations for the use of non steroidal anti-inflammatory drugs due to the risk of gastrointestinal bleeding or renal failure and may, thus, be a group in which the use of glucosamine or chondroitin sulfate is frequent.

CASE REPORT

From May 2011 to July 2011, 151 consecutive patients attended in an outpatient clinic for liver diseases by one of the authors were evaluated. All were asked about previous or current intake of drug products composed of glucosamine or chondroitin sulfate using a questionnaire including the name of all the commercial products containing these compounds on sale in Spain as well as those which can be obtained by Internet. None of the patients refused to answer the questionnaire. Patients who replied affirmatively with respect to the use of these products were asked about the date of treatment initiation and the duration of drug use. The clinical charts were thereafter reviewed to determine if there were hepatic biochemical alterations coinciding with the use of the drug.

During the period mentioned, 151 patients, ranging from 19 to 85 years of age (mean 59.2 years), were interviewed; 56.9% being women. The liver diseases were chronic hepatitis/hepatic cirrhosis due to hepatitis C virus (38.2%), autoimmune hepatitis (12%), chronic hepatitis/cirrhosis by hepatitis B virus (7.1%), alcoholic cirrhosis (4.9%) and Wilson disease and primary biliary cirrhosis (3.5%).

Twenty-three patients (15.2% of the total) acknowledged having consumed products containing glucosamine (6 patients), chondroitin sulfate (16 patients) or both (1 patient). Ten were receiving the drug at the time of the interview. In 21 out of the 23 patients it could not be established whether the liver had sustained drug-induced damage since no elevation in aminotransferase above the usual values was observed in association with the administration of glucosamine or chondroitin sulfate. A relationship between an elevation in transaminases and product consumption was detected in 2 cases, both of which had taken glucosamine. The first was a 71-year-old woman with chronic hepatitis C who had taken glucosamine sulfate during one year and presented an elevation in aminotransferases of 5 to 7-fold greater than the normal values during this treatment. The clinical records did not mention the use of other drugs. Viral infection due to hepatitis A, hepatitis B, and cytomegalovirus was excluded by serological tests. Serum transaminases returned to the usual values after treatment discontinuation. The second case was a 77-year-old woman with chronic hepatitis C who had taken glucosamine for 3 mo in 1977 and had presented an allergic cutaneous reaction attributed to the drug. She had not been treated with any other drug. At that time the transaminase values rose 4-fold above normal. In the follow-up liver tests taken in June 2011, a minimum elevation of alanine aminotransferase similar to previous analyses was observed. Neither of these two cases presented hyperbilirubinemia or changes in the biochemical indices of cholestasis and did not present either symptoms or decompensation of their liver disease. The details of the analyses performed during the episode compared with previous and posterior registries of each case are shown in Table 1.

DISCUSSION

To our knowledge this is the first report on the consumption of products containing glucosamine and/or chondroitin sulfate in patients with chronic liver disease. The frequent intake observed in the population studied is substantial (23 out of 150 patients, that is 15%), with 2 cases of possible toxicity among 23 patients who acknowledged current or past intake. This represents hepatic toxicity of almost 9% in patients with chronic liver disease reporting consumption of these drugs. The two cases with liver damage coinciding with the treatment had chronic hepatitis C.

Review of the literature has shown several cases of alleged hepatotoxicity by glucosamine and chondroitin sulfate (Table 2). In 2007 one case of hepatitis with elevations in alanine aminotransferase and total bilirubin of 6- and 10-fold, respectively above normal values was reported in a patient who had taken glucosamine at the therapeutic doses for 4 wk prior to presenting jaundice and pruritus[4]. Other etiologies were ruled out with a complete study of the patient, and liver biopsy showed findings compatible with drug-induced hepatitis. Another report identified 3 cases of probable hepatotoxicity. The first was a patient with severe cholestatic hepatitis who developed fulminant liver failure resulting in death after having taken glucosamine for 4 wk. The second case was a woman who had consumed a glucosamine/

Table 1 Results of the liver function tests in cases of hypertransaminasemia related to glucosamine consumption

	1-yr prior to consumption	During consumption	1-yr after consumption
AST (UI/L) (normal < 40)			
Case 1	25	182	71
Case 2	36	161	36
ALT (UI/L) (normal < 40)			
Case 1	33	282	85
Case 2	53	162	37
GGT (UI/L) (normal < 40)			
Case 1	32	150	77
Case 2	18	31	15
AP (U/L) (normal < 290)		229	219
Case 1	240	183	213
Case 2	144		
Total bilirubin (mg/dL) (normal < 1.2)			
Case 1	0.3	0.8	0.4
Case 2	0.8	0.9	0.9

The results of the two cases are compared with analyses performed 1-yr prior to and after the episode. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma Glutamyl transpeptidase; AP: Alkaline phosphatase.

chondroitin compound and presented anorexia, jaundice and cutaneous rash with persistence of biochemical alterations 6 mo after the onset of the symptoms. During the follow up the patient developed signs of hepatic dysfunction, and liver biopsy showed chronic portal hepatitis. The third case was a patient presenting an asymptomatic elevation in transaminases after having consumed a compound containing glucosamine for 6 mo. Complete recovery was achieved on discontinuation of the drug[5]. Two cases of probable hepatotoxicity were described in 2010 in relation to the consumption of a dietetic supplement (move free advanced) which contained glucosamine and chondroitin sulfate. The first of these cases presented diarrhea with an elevation in aminotransferases and alkaline phosphatase and the second showed a slight increase in aminotransferases with no specific symptoms. Neither case presented jaundice nor features of hepatic failure and the two patients improved 7 and 12 wk after withdrawal of the product[6].

The two cases attended by one of us (AP) were patients with no previous liver disease in whom a relationship was observed between the consumption of glucosamine and alterations in liver function tests. One was a 28-year-old woman who presented features of acute hepatitis with jaundice and pruritus at one month of starting treatment with glucosamine because of rough discomfort in both knees after minor trauma. Blood tests improved slightly on discontinuation of treatment with glucosamine with the subsequent disappearance of the symptoms. Viral, alcoholic, metabolic and autoimmune etiologies of the disease were ruled out. A liver biopsy performed one year later due to the persistence of biochemical alterations showed signs of chronic hepatitis. The other patient was a 56 year-old woman who had persistent transaminase values 3-fold greater than normal. All the potential causes of liver diseased were discarded and transaminase values normalized on withdrawal of glucosamine. The treatment was prescribed to improve initial symptoms of osteoarthrosis.

Taking our cases and those reported in the literature into account several characteristics may be pointed out. Firstly, all the cases had consumed compounds with glucosamine or chondroitin sulfate at the recommended therapeutic doses with no warning on the possible influence of doses within the range of the risk of hepatotoxicity. Neither was any other possible cause of liver damage identified. Suspicion of a toxic etiology in our cases was based on the infrequency of episodes of important elevations in transaminase values in chronic hepatitis C with no concomitant cause as well as regression on drug discontinuation. Jaundice was the most frequent initial symptom of hepatic compromise in the published cases but some cases presented asymptomatic alterations in liver biochemistries, one being severe hepatic failure and another developed chronic liver disease.

The mechanisms involved in the drug-induced hepatotoxicity are not clear. It is of note that the raw material used in compounds containing glucosamine are obtained from biopolymers of shells from marine invertebrates (shrimps, crabs, lobsters) and chondroitin sulfate is taken from cow trachea cartilage and shark cartilage in Japan[7]. One of our cases and two of those reported in the literature simultaneously presented hypersensitivity reactions and thus, hypersensitivity may have been the contributory mechanism, at least in one of the cases. Responsibility of additives contained in the glucosamine preparations used for our patients seems unlikely, because neither aspartame, sorbitol, citric acid or polietilenglicol have been related to liver injury.

Glucosamine is a precursor to glycosaminoglycan, which is believed to play a role in the growth of cartilage and its repair. Chondroitin is part of a large proteoglycan molecule that gives cartilage flexibility and is thought to inhibit enzymes that break down cartilage. Glucosamine is used in the treatment of osteoarthritis, a disease resulting from the articular hyaline cartilage degeneration which leads to the loss of cartilage. Osteoarthritis is very prevalent in general population, particularly in elder subjects. It causes significant morbidity due to pain and functional disability of joints, and increased health care costs as well[8]. The reason for the use of glucosamine or chondroitin sulfate in these patients lies in the belief that osteoarthritis is associated with a deficiency in key natural substances and these products provide a substrate for the synthesis of cartilaginous matrix. In addition, they provide protection against enzymes which degrade the cartilage[7]. Some randomized placebo-controlled trials using glucosamine showed a decrease of the symptoms of osteoarthritis in the group receiving glucosamine in comparison with the control group, but this not found in others[8-11]. No side effects related to the liver were observed in these trials. In the 2005 Cochrane review it was reported that in studies older and of lesser quality the effect of placebo was greater, while pain relief was

Table 2 Summary of the cases reported in the literature on hepatotoxicity by glucosamine and/or chondroitin sulfate

Ref.	Age (yr) Sex (F/M)	Drug consumed	Length of consumption	Latency	Jaundice	Peak in AST/ ALT (IU/L)	Hypersensitivity	Hepatic failure	Follow-up
Ossendza *et al*[4]	52/M	Glucosamine	3 wk	4 wk	Yes	263/63	Pruritus, eosinophilia	No	Complete recovery
Smith *et al*[5]	64/M	Glucosamine/ chondroitin sulfate	4 wk	5 wk	Yes	-/1461	-	Yes	Death
	57/F	Glucosamine	4 wk	5 wk	Yes	-/1130	Pruriginous rash	No	Chronic hepatitis
	55/F	Glucosamine	6 mo	8 mo	No	-/175	-	No	Complete recovery
Linnebur *et al*[6]	71/F	Glucosamine/ chondroitin sulfate	7 wk	3 wk	No	600-700/ 00-500	-	No	Complete recovery
	85/F	Glucosamine/ chondroitin sulfate	3 wk	3 wk	No	54/37	-	No	Complete recovery
Authors' cases	71/F	Glucosamine	1 yr	NA	No	182/282	-	No	Liver tests return to pretreatment (basal) values
	77/F	Glucosamine	3 mo	NA	No	161/162	Pruriginous rash	No	Liver tests return to (basa) values

M: Male; F: Female; AST/ALT: Aspartate aminotransferase/ alanine aminotransferase; NA: Not available.

similar in patients receiving glucosamine or placebo in studies of better quality[12]. In Europe the different compounds containing glucosamine or chondroitin alone or in combination require a medical prescription, but in North America they may be purchased as a supplement without prescription, thus adding an extra risk of potential adverse effects because the drugs are taken without any medical judgment or are poorly purified.

Mild forms of hepatotoxicity may remain undiagnosed because of the absence of clinical expression with laboratory analyses not being performed in patients complaining of joint pain before and during treatment with glucosamine or chondroitin sulfate. Our observations suggest that these products should be suspected as a possible cause for the analytical changes in patients receiving treatment with these drugs who show an alteration in transaminase values. In these cases, drug discontinuation seems justified taking into account their low or questionable therapeutic efficacy and the possibility of developing more severe liver damage with continued use.

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CASE REPORT

A case of rapidly progressing leiomyosarcoma combined with squamous cell carcinoma in the esophagus

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Abstract

Esophageal leiomyosarcoma is a rare tumor that accounts for less than 1% of all malignant esophageal tumors. Esophageal leiomyosarcoma combined with squamous cell carcinoma is even rarer than solitary leiomyosarcoma. We experienced a case of leiomyosarcoma combined with squamous cell carcinoma that progressed very rapidly.

Key words: Leiomyosarcoma; Carcinoma; Squamous cell; Esophagus; Sarcoma

Core tip: We performed esophagectomy with esophagogastrostomy to resect the tumor. Pathological examination of the surgical specimen revealed that it was combined with squamous cell carcinoma. Combined carcinoma should therefore be considered when leiomyosarcoma shows rapid progression.

Jang SS, Kim WT, Ko BS, Kim EH, Kim JO, Park K, Lee SW. A case of rapidly progressing leiomyosarcoma combined with squamous cell carcinoma in the esophagus. *World J Gastroenterol* 2013; 19(32): 5385-5388 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5385.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5385

INTRODUCTION

Leiomyosarcoma of the esophagus is a rare malignant tumor, accounting for less than 1% of all malignant esophageal tumors[1-3]. Esophageal leiomyosarcoma combined with squamous cell carcinoma is even rarer than solitary leiomyosarcoma. Simultaneous esophageal leiomyosarcoma and squamous cell carcinoma were first described by Ovens *et al*[4] in 1951. Leiomyosarcomas are characterized by slow growth and late metastases, and hence have a better prognosis than squamous cell carcinomas of the esophagus[5,6]. However, we experienced a case of leiomyosarcoma combined with squamous cell carcinoma that progressed very rapidly. We report this case and review the literature.

CASE REPORT

A 72-year-old male visited to our hospital suffering from chest pain that had been present for 1 mo. The patient had been diagnosed with colon cancer and received laparoscopic surgery 1 year prior. The physical examination was unremarkable. He was admitted to the cardiology department, and received electrocardiogram and cardiac single photon emission computed tomography, but no cardiac problem was found. Endoscopic examination

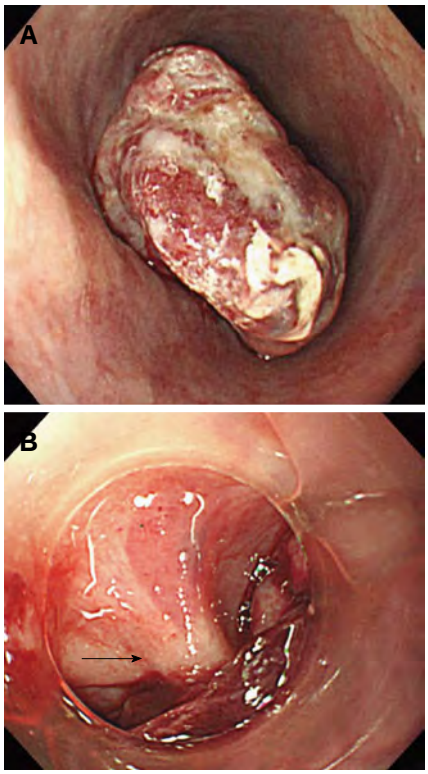

Figure 1 Endoscopic finding. A: Intraluminal polypoid mass; B: Stalk of the mass (arrow).

demonstrated a stalked intraluminal polypoid mass in the mid esophagus, 30 cm from the incisor (Figure 1). The tumor was large enough to fill the esophageal lumen, but allowed passage of a gastrofiberscope (Q260, Olympus, Tokyo, Japan) to the distal part of the esophagus. An endoscopic biopsy was performed, and the patient was suspected of leiomyoma and leiomyosarcoma. A computed tomography scan showed a large, well enhancing soft tissue mass in the mid esophagus (Figure 2), but no regional lymph node enlargement or liver metastasis. Positron emission tomography/computed tomography (PET-CT) showed intense segmental F-18 fluorodeoxyglucose (FDG) uptake [Standardized Uptake Value (SUV) max 17.3] at the mid-thoracic esophagus. Compared with the previous PET-CT for colon cancer follow-up from 3 mo prior, there was only physiologic FDG uptake at the esophagus (Figure 3). The patient underwent surgery; an esophagectomy with esophagogastrostomy. Macroscopically, the resected specimen was a polypoid tumor measuring 9.8 cm × 5.0 cm × 2.5 cm (Figure 4). Histopathologically, the tumor consisted of pleomorphic spindle cells with mitosis and cell necrosis compatible with leiomyosarcoma (Figure 5A). Tumor invasion involved the muscularis propria, submucosa, and mucosa. Nine regional lymph nodes were free of metastasis. An immunohistochemical examination stained positive for smooth muscle actin, but negative for cytokeratin and S-100 protein (Figure 5B). These were stained by an automated Ventana immunohistochemical/*in situ* hybridization staining platforms machine (BenchMark XT). Squamous

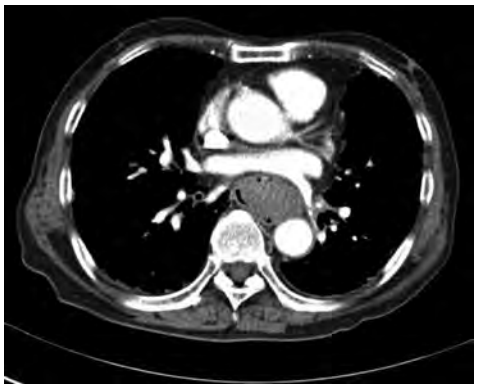

Figure 2 Computed tomography scan showed a large, homogeneously **enhancing soft tissue mass.**

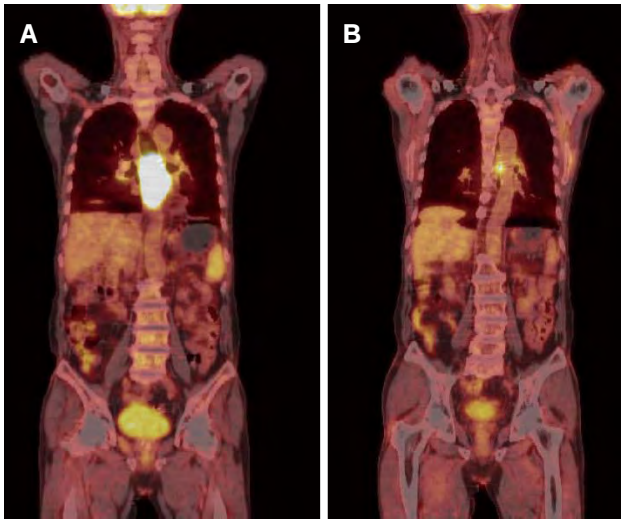

Figure 3 Positron emission tomography/computed tomography. A: Positron emission tomography/computed tomography (PET-CT) showed intense segmental fluorodeoxyglucose uptake (SUV max 17.3) at mid esophagus; B: PET-CT performed at 3 mo ago.

severe dysplasia and focal stratified squamous epithelial invasion into the lamina propria was also noted in the mucosa (Figure 5C). We diagnosed the patient with leiomyosarcoma combined with squamous cell carcinoma.

In the post-operative period, the patient recovered uneventfully and was discharged 18 d after operation. No adjuvant radiotherapy or chemotherapy was administered. At the last follow up visit to our hospital 2 mo after surgery, the patient was in good condition without any recurrence or distant metastasis.

DISCUSSION

Leiomyosarcoma is a high grade, smooth muscle soft tissue tumor that can occur in any tissue containing smooth muscle fibers. A leiomyosarcoma combined with squamous cell carcinoma is an extremely rare disease of the esophagus, with very few such cases described[4,5,7-9]. Leiomyosarcomas are most commonly located in the middle and lower thoracic esophagus because smooth muscle

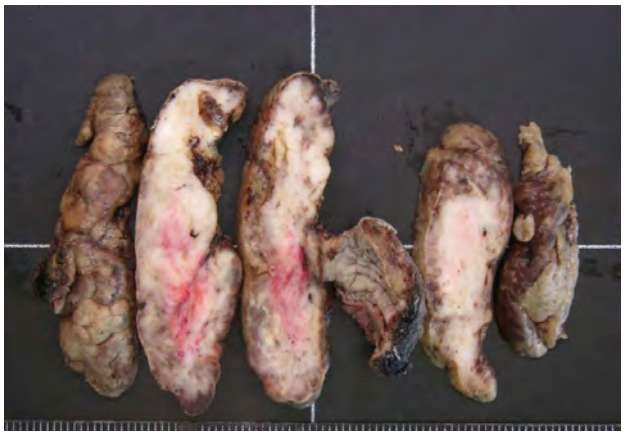

Figure 4 Resected specimen measured about 9.8 cm × 5.0 cm × 2.5 cm.

predominates in that area[10]. Esophageal leiomyosarcomas are typically divided into two types: the polypoid type in 60% of cases and the infiltrative type in 40% of cases[11,12]. Our case was the polypoid type. The prognosis of esophageal leiomyosarcoma is better than esophageal squamous cell carcinoma because of its characteristics of slow growth and late metastases[5,6]. Patients with polypoid and intramural tumors, tumors in an intrathoracic location, and well-differentiated tumors have a better prognosis than patients with infiltrating lesions, tumors in cervical locations, and poorly differentiated tumors[13,14]. Koga *et al*[13] reported a case of esophageal leiomyosarcoma that grew rapidly and had a poor prognosis. We think this case is unique because the tumor had good prognostic factors, such as the polypoid type and intrathoracic location, but it grew very rapidly and was combined with squamous cell carcinoma. Eroğlu *et al*[7] suggested that mutability or metaplasia between mesenchymal and epithelial tissues or multipotent stem cells with the ability to undergo biphasic differentiation toward mesenchymal and epithelial elements could be a mechanism of this combined malignancy. It is possible that these are separate entities that have arisen independently and combined squamous cell carcinoma may affect the growth of leiomyosarcoma by cytokines or growth factors.

The role of FDG-PET-CT in the diagnosis of leiomyosarcoma was reported recently[15-17]. Our case showed intense FDG uptake on PET-CT. The standard treatment is esophagectomy, but the role of adjuvant radiotherapy or chemotherapy is controversial with some authors[2,6,14,18]. In our case, the leiomyosarcoma grew exceptionally rapidly and was combined with squamous cell carcinoma, so further research will be needed to reveal the relationship between leiomyosarcoma and squamous cell carcinoma.

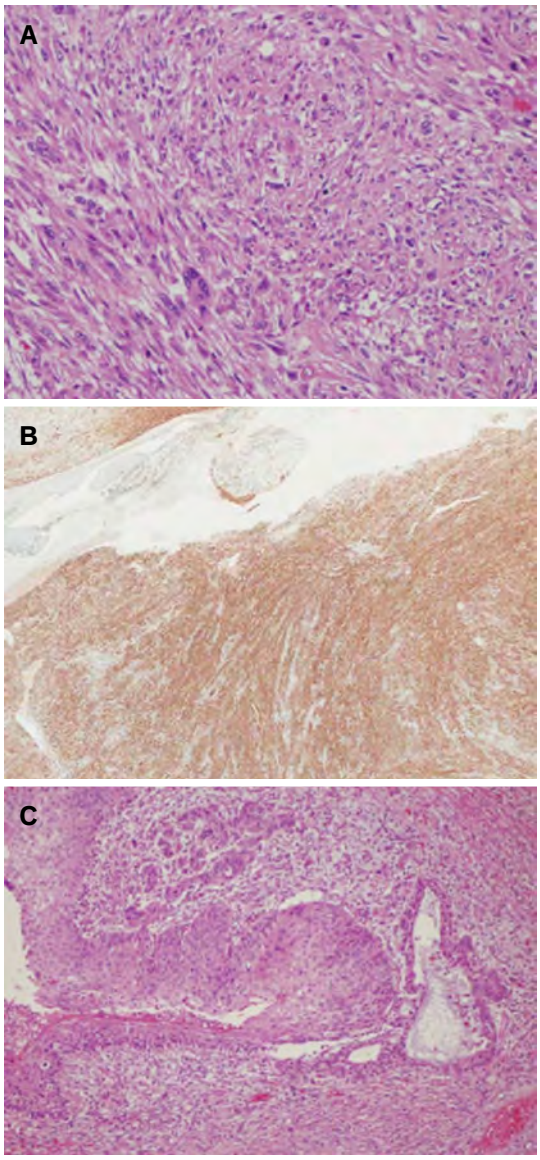

Figure 5 Pathologic images. A: Pleomorphic spindle cells showing mitosis and cell necrosis compatible with leiomyosarcoma [hematoxylin and eosin (HE) stain, × 200]; B: Immunohistochemical stain was positive for smooth muscle actin (× 12); C: Squamous severe dysplasia and focal stratified squamous epithelial invasion into lamina propria was noted in mucosa (HE stain, × 100).

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CASE REPORT

Intestinal Behçet's disease appearing during treatment with adalimumab in a patient with ankylosing spondylitis

Sook Hee Chung, Soo Jung Park, Sung Pil Hong, Jae Hee Cheon, Tae Il Kim, Won Ho Kim

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Author contributions: Chung SH wrote the manuscript; Park SJ designed the research and performed peer review; Cheon JH analyzed the clinical data and consulted about pathologic data and medical agents; Hong SP, Kim TI and Kim WH reviewed the manuscript.

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Core tip: Here, we report on a patient who was diagnosed with intestinal Behçet's disease despite treatment with the fully humanized tumor necrosis factor-α blocker (adalimumab) for underlying ankylosing spondylitis. This patient achieved clinical remission and complete mucosal healing through the addition of a steroid and azathioprine to the adalimumab regimen.

Chung SH, Park SJ, Hong SP, Cheon JH, Kim TI, Kim WH. Intestinal Behçet's disease appearing during treatment with adalimumab in a patient with ankylosing spondylitis. *World J Gastroenterol* 2013; 19(32): 5389-5392 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5389.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5389

Abstract

Behçet's disease (BD) is a chronic inflammatory disease affecting multiple organ systems, such as the skin, joints, blood vessels, central nervous system, and gastrointestinal tract. Intestinal BD is characterized by intestinal ulcerations and gastrointestinal symptoms. The medical treatment of intestinal BD includes corticosteroids and immunosupressants. There have been several reports of tumor necrosis factor-α (TNF-α) blockers being successful in treatment of refractory intestinal BD. Here, we report on a patient who was diagnosed with intestinal BD despite treatment with the fully humanized TNF-α blocker (adalimumab) for underlying ankylosing spondylitis. This patient achieved clinical remission and complete mucosal healing through the addition of a steroid and azathioprine to the adalimumab regimen.

Key words: Intestinal Behçet's disease; Tumor necrosis factor-α; Adalimumab

INTRODUCTION

Behçet's disease (BD) involves multiple organ systems, such as the skin, joints, blood vessels, central nervous system, and gastrointestinal (GI) tract[1]. Intestinal BD is characterized by intestinal ulcerations and gastrointestinal symptoms[2]. The incidence of BD involving the GI tract varies by country, ranging from 3% to 60% of cases of BD[3]. GI bleeding and perforation can be associated with intestinal BD, with resultant comorbidities[1]. The medical treatment for intestinal BD includes corticosteroids and immunosupressants. Unfortunately, surgical treatment, such as ileocecal resection, is sometimes necessary for intestinal BD with perforation, intractable pain, and hemorrhage which are refractory to conventional therapy[4]. There have been several reports of tumor necrosis factor-α (TNF-α) blockers being successful in refractory intestinal BD. Most of these reported on the efficacy of infliximab[4-9] and a few reported on the efficacy of adalimumab[10,11]. Here, we report on a patient who was diagnosed with intestinal BD despite being treated with the fully humanized TNF-α blocker (adalimumab) for

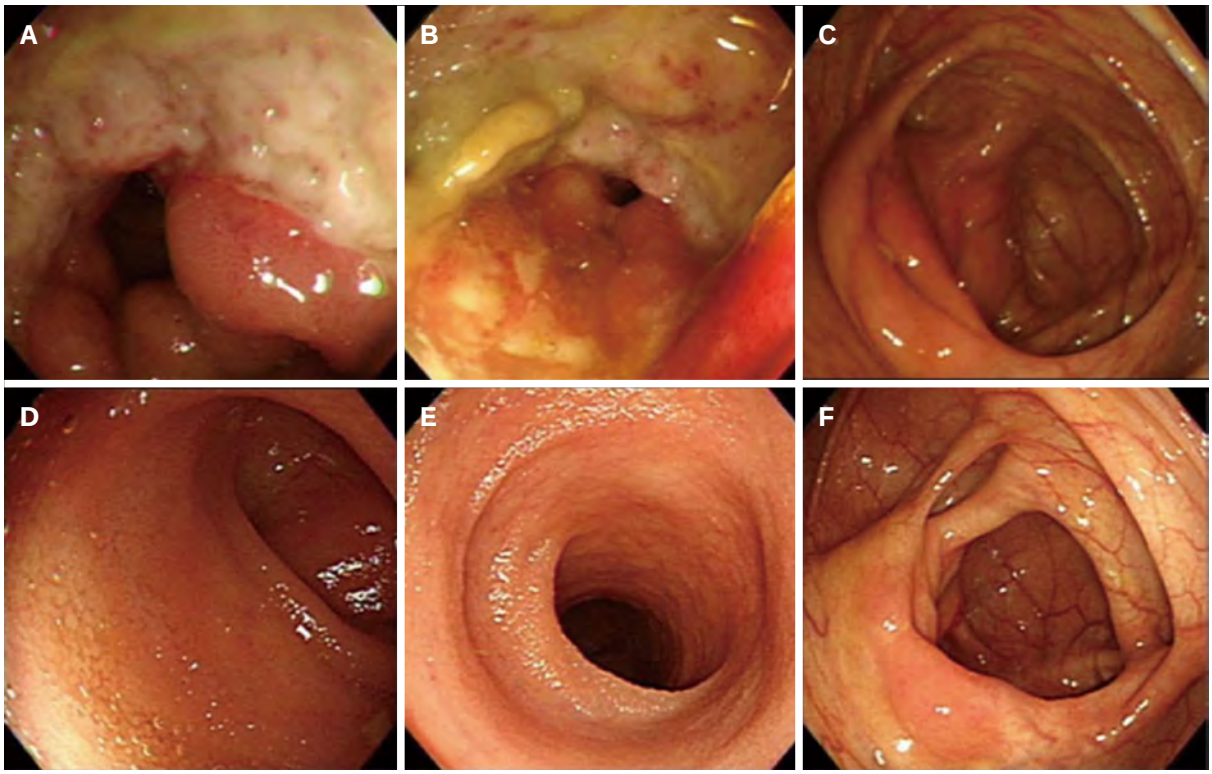

Figure 1 A colonoscopy on admission revealed a large, deep, well-demarcated ulcer with exudate, mucosal edema and erythema at the terminal ileum (A-C). On follow-up colonoscopy at 36 mo, the ulcer at the terminal ileum was replaced by normal mucosa (D-F) with complete mucosal healing.

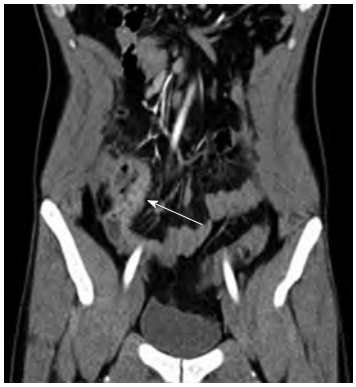

Figure 2 In computed tomography, arrow showed bowel wall thickening **and prominent enhancement with surrounding fat infiltration at the terminal ileum and cecum.** This was suggestive of active inflammation.

underlying ankylosing spondylitis. This patient achieved and maintained clinical remission and complete mucosal healing through the addition of a steroid and azathioprine to the adalimumab regimen for 43 mo.

CASE REPORT

A 29-year-old male patient was hospitalized due to severe right lower quadrant abdominal pain for the preceding 15 d. He had experienced recurrent oral ulcerations and arthralgia for 15 years and had had an erythematous papule on his back for the past 2 years. He had undergone appendectomy for appendicitis 17 years ago. He was diagnosed with ankylosing spondylitis 2 years ago because of lower back and shoulder pain. He had taken salazopyrine 1000 mg for 2 mo and had been injected with infliximab for his ankylosing spondylitis for 9 mo (5 mg/kg intravenously at 0, 2 and 6 wk; 5 mg/kg intravenously every 8 wk). The oral ulcerations, arthralgias, and erythematous papule on his back had improved, but his back pain had not been improved at that time. Therefore, the infliximab had been switched to adalimumab (40 mg subcutaneously every 2 wk) since 10 mo ago. On physical examination at admission, he appeared acutely ill, and had a blood pressure of 120/70 mmHg, a pulse of 84 beats/min, a respiratory rate of 24 breaths/min, and a temperature of 36.5 ℃. The abdomen was flat with direct tenderness in the right lower quadrant. Bowel sounds were normal. The results of laboratory tests showed a white blood cell count (WBC) of 20930/mm^3; hemoglobin, 13.9 g/dL; hematocrit, 41.7%; platelet count, 282000/mm^3; total protein, 7.2 g/dL; erythrocyte sedimentation rate increased to 33 mm/h; and C-reactive protein increased to 111 mg/L. A colonoscopy performed on admission showed a well-demarcated, large, deep ulcer with an exudate, mucosal edema, and erythema at the terminal ileum (Figure 1A-C). Colonic biopsies at the terminal ileum showed an ulcer with a necroinflammatory exudate. On computed tomography, bowel wall thickening and prominent enhancement with surrounding fat infiltration were noted at the terminal ileum and cecum, suggesting active inflammation (Figure 2). Finally he was diagnosed as intestinal BD according to the clinical symptoms and examination. The disease activity index for intestinal Behçet's disease (DAIBD) was 90, reflecting severe disease

Table 1 Clinical characteristics of patients with intestinal Behçet's disease receiving infliximab or adalimumab

Case	Age (yr)/ gender	Duration of disease (yr)	Anti TNF-α Ab for induction	Previous therapies	Maintenance therapy	Outcomes	Follow-up duration after achieving remission	Ref.
1	32/F	5	IFX	Steroids 6-MP	IFX 6-MP	Remission	9 mo	[14]
2	37/F	2	IFX	Mesalamine Steroids 6-MP	IFX 6-MP	Remission	16 mo	[14]
3	51/M	4	IFX	Steroids Methotrexate	IFX	Remission	3 yr	[14]
4	38/M	5	IFX	Steroids Colchicines Cyclosporine A	IFX	Remission	10 mo	[14]
5	43/F	6	IFX	Steroids Azathioprine	AZA	Surgery	6 mo	[14]
6	38/M	3	IFX	Steroids 6-MP	IFX 6-MP	Remission	2 yr	[14]
7	35/F	Over 20	IFX	Steroids Azathioprine	Methotrexate	Relapse	8 mo[1]	[7]
8	27 /F	2	IFX	Steroids Thalidomide	Steroids Thalidomide	Remission	17 mo	[5]
9	30 /F	3	IFX	Steroids Colchicines Cyclosporine	Thalidomide	Relapse	10 mo[1]	[5]
10	42/M	11	IFX	Steroids Colchicine	-	Remission	1 wk	[9]
11	47/M	20	IFX	Sulfasalazine Steroids Azathioprine	-	Remission	12 mo	[4]
12	30/F	-	IFX	Steroids Azathioprine	IFX Switch to adalimumab	Remission	22 mo	[10]
13	45/F	9	IFX	Mesalamine Steroids 6-MP	IFX	Remission	25 wk	[6]

[1]Duration between remission stage and the first relapse stage after infusion of anti tumor necrosis factor-α antibody (anti TNF-α Ab). F: Female; M: Male; IFX: Infliximab; 6-MP: 6-mercaptopurine.

activity[12]. Subsequently, the patient was treated with conventional medical therapy, including azathioprine 150 mg and 5-aminosalicylate (5-ASA, Pentasa) 3000 mg/d. His abdominal pain seemed to decrease after 10 d. However, the patient's severe right lower quadrant abdominal pain recurred after one month. The DAIBD score at the time of recurrent abdominal pain was 80, again reflecting severe disease activity[12]. In the early stages of treatment, clinical remission could not be obtained through combination therapy with azathioprine and 5-ASA. Thus, at the time of abdominal pain recurrence, intravenous hydrocortisone (300 mg/d) was administrated. Then the abdominal pain was improved 2 d after steroid injection. Intravenous hydrocortisone was slowly tapered to oral prednisolone for 2 mo. Finally, DAIBD score was 10. A follow-up colonoscopy after 36 mo demonstrated that the ulcer at the terminal ileum was replaced by normal mucosa (Figure 1D-F) with complete mucosal healing. Combination therapy with azathioprine, 5-ASA and adalimumab was continued for 43 mo with clinical and endoscopic remission.

DISCUSSION

We reported on a 29-year-old man with BD and ankylosing spondylitis who developed intestinal BD despite continuous use of adalimumab. We treated this patient with intravenous steroids to induce clinical remission and with azathioprine and 5-ASA for maintenance of remission. This case is unique in two ways. First, the terminal ileal ulcer characteristic of intestinal BD appeared while the patient was receiving adalimumab for ankylosing spondylitis. Second, mucosal healing was achieved and maintained through combination therapy with azathioprine and 5-ASA after induction of remission with intravenous steroids.

Conventional therapies such as mesalamine, corticosteroids, immunosuppressive agents, thalidomide, bowel rest, and total parenteral nutrition have been used in the treatment of intestinal BD[13]. However, in patients with intestinal BD unresponsive to conventional therapies, TNF-α blockers have been shown to improve symptoms[12]. Both infliximab and adalimumab can be used for treatment of intestinal BD because they are similar active biologics, monoclonal antibodies to TNF-α[10,11,14]. There has been no data available regarding the comparative efficacy of infliximab and adalimumab in intestinal BD. In Table 1 there have been many publications reporting on the effectiveness of infliximab[4-9]. However, there have been only a few reports of the efficacy of adalimumab

in treating intestinal BD[10,11]. Infliximab with combination therapies, such as 5-ASA and immunosuppressants, in patients with intestinal BD is effective for the induction and maintenance of remission[4-9,11,14]. In the case of adalimumab, adalimumab was reported to be effective in inducing complete remission as monotherapy[10]. We suggest three reasons why this patient may have developed an ulcer of the terminal ileum during the use of adalimumab, but not during the use of infliximab. First, the two medicines have different routes of injection, with adalimumab injected subcutaneously (SQ) and infliximab injected intravenously. When infliximab is injected intravenously, it enters the venous circulation directly with 100% bioavailability and no absorption phase, thereby reaching a more rapid therapeutic range than achieved with subcutaneous injection. Conversely, the bioavailability of an adalimumab 40 mg SQ dose has been estimated as 64%[15]. Second, there might be a difference in the effective dose between adalimumab and infliximab. Adalilmumab is used to be injected as fixed dose irrespective of body weight (40 mg subcutaneously every 2 wk) but infliximab is used to be injected according to the body weight (5 mg/kg at 0, 2 and 6 wk; 5 mg/kg every 8 wk). Compared to the infliximab, adalilmumab in fixed dose was supposed to be less effective in this patient with 22.8 kg/m^2 body mass index because of shortage of dose. Third, intravenous injection might be more potent than subcutaneous injection because BD is characterized by systemic vasculitis[16]. Infliximab and adalimumab are different medicines having unique pharmacokinetics. There was no head to head study for comparing effectiveness of infliximab and adalimumab. Further studies are warranted to compare the efficacy of adalimumab and infliximab in patients with intestinal BD.

In conclusion, this is the first case report of intestinal BD appearing despite the use of adalimumab. Furthermore, the subject in this case improved with the conventional combination of intravenous steroids for the induction of remission and azathioprine and 5-ASA for maintenance of remission. Despite the use of adalimumab, a conventional combination of therapies including intravenous steroids, azathioprine, and 5-ASA might be important in treating patients with intestinal BD.

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Favorable effect of modest alcohol consumption to fatty liver disease

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Abstract

We previously reported that modest alcohol consumption was significantly inversely associated with fatty liver disease. Feng *et al* pointed out a discrepancy of statistical significance between our current larger scale cohort and a previous cohort. However, the prevalence of non-alcoholic fatty liver disease was higher in non or minimal drinkers than those in light drinkers in both cohorts. They also argue that some potential co-factors such as soft drink consumption and genetic variations should be discussed.

Key words: Alcohol; Fatty liver disease; Obesity; Diabetes; Metabolic syndrome

Core tip: We reported the inversed association of modest alcohol consumption with fatty liver disease. However, other potential co-factors were argued to be important. Herein, we reply and discuss these important factors in this letter.

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TO THE EDITOR

Feng *et al* argued against the validity of the category of alcohol consumption we used, however this categorization was made in accordance with standardized methods[1,2]. They also pointed out the discrepancy of statistical significance between our current larger scale cohort and a previous cohort[3-5]. However, the prevalence of non-alcoholic fatty liver disease (NAFLD) was higher in non or minimal drinker than those in light drinker in both cohorts. In the current cohort, the prevalence of NAFLD was higher in non or minimal drinkers than those in light drinkers both in men (36.5%, 2248/6154 *vs* 26.4%, 457/1734) and in women (10.4%, 719/6893 *vs* 5.4%, 22/406)[4]. A similar trend was reported in the previous cohort as follows; the prevalence of NAFLD was higher in non or minimal drinkers than those in light drinkers both in men (28.6%, 170/595 *vs* 23.5%, 464/1977) and in women (10.7%, 105/981 *vs* 8.6%, 73/848)[5]. While statistical significance was not detected in the cohort used in our previous study, however we speculated that the smaller size of the cohort may have reduced the power of the statistical test and thus failed to detect the difference. Moreover, we previously reported that there is a favorable effect of modest alcohol consumption for the development of NAFLD in a prospective cohort study[6]. The adjusted odds ratio of light drinkers for the development of NAFLD was 0.82 (0.59-1.15, $P = 0.26$) in men, and 0.86 (0.51-1.45, $P = 0.56$) in women, respectively. The odds ratio was not statistically significant, but the

possibility of a favorable effect was speculated[6].

Feng *et al*[3] also argue that soft drink consumption or genetic variations may be potential co-factors for the favorable effect of modest alcohol consumption to fatty liver disease. Soft drink consumption is an important risk factor for NAFLD[7]. Unfortunately, in this case we had no data of soft drink consumption. However, a previous study has reported no association between alcohol consumption and soft drink consumption[8]. Thus, the association of NAFLD with soft drink consumption may be independent of those with alcohol consumption.

Genetic variations have been reported as important risk factors for NAFLD and non-alcoholic steatohepatitis[9-13]. The mechanisms of these genetic variations for development of NAFLD are still largely unknown. Thus, future investigations are needed; however, we appreciated Dr. Feng's extension of our discussion regarding the role of genetic variations in the pathophysiology of NAFLD.

In conclusion, we reported the inverted association of modest alcoholic consumption with fatty liver disease in a large scale cross-sectional study. Future large scale prospective studies and basic investigations are needed to clarify the effect of modest alcohol consumption in the development of fatty liver disease.

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