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EDITORIAL

Cell sheet technology for regeneration of esophageal mucosa

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Abstract

The progress of tissue-engineering technology has realized development of new therapies to treat various disorders by using cultured cells. Cell- and tissue-based therapies have been successfully applied to human patients, and several tissue-engineered products have been approved by the regulatory agencies and are commercially available. In the review article, we describe our experience of development and clinical application of cell sheet-based regenerative medicine. Endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD) have been shown to be useful for removal of gastrointestinal neoplasms with less invasiveness compared with open surgery, especially in esophageal surgery. However, postoperative inflammation and stenosis are major complications observed after intensive mucosal resection. Therefore, we have developed novel regenerative medicine to prevent such complications and promote wound healing of esophageal mucosa after EMR or ESD. Transplantable oral mucosal epithelial cell sheets were fabricated from patients' own oral mucosa. Immediately after EMR or ESD, fabricated autologous cell sheets were endoscopically transplanted to the ulcer sites. We performed a preclinical study with a canine model. In human clinical settings, cell culture and cell sheet fabrication were performed in clean rooms according to good manufacturing practice guidelines, and pharmaceutical drugs were used as supplements to culture medium in place of research regents used in animal study. We believe that cell-based regenerative medicine would be useful to improve quality of life of patients after EMR or ESD.

Key words: Cell sheet; Endoscopic resection; Esophageal stenosis; Oral mucosa; Good manufacturing practice

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INTRODUCTION

Endoscopic mucosal resection (EMR) is one of the standard techniques for removing superficial early-stage cancers in the gastrointestinal tract with low invasiveness compared with open surgery[1,2]. In the case of the esophagus, EMR is a useful operation for removing m1 (epithelial cancers restricted to the intraepithelial layer) and m2 (mucosal cancers with invasion of intermediate depth) carcinomas without esophageal reconstruction. To permit large *en bloc* resection of cancerous lesions without technical restriction of EMR, endoscopic submucosal dissection (ESD) was developed as a new mucosal resection technique with development of endoscopic devices[3-5]. Early-stage esophageal cancers are removed using a hook-knife, which is an endoscopic device used to perform ESD[6]. ESD is a more impressive operative procedure than EMR for reducing the recurrence of esophageal squamous cell carcinoma[7]. Although these developments of endoscopic surgery contribute low invasive cancer resection for patients suffering from esophageal cancer, there are some postoperative complications after ESD. Esophageal stenosis is a major complication caused by endoscopic resection, and the stenosis is significantly associated with the mucosal defect involving over three-fourths circumference of the esophagus lumen[8]. The esophageal stenosis caused by aggressive ESD considerably affects the patient's quality of life, since the patient has to receive treatment with balloon dilation or temporary stents to expand the esophageal stricture with further inflammation and postoperative pain. These physical dilations carry a risk of perforation[9]. Treatment with anti-inflammatory drugs after endoscopic resection may be an effective therapy for preventing stricture after ESD[10,11].

With recent progression of tissue engineering and regenerative medicine, there are some reports proposing new technologies using biological scaffolds[12] or cell suspensions[13,14] for preventing the esophageal stenosis caused by mucosal defects. We have developed a novel method of endoscopic transplantation of autologous epithelial cell sheets immediately after ESD to prevent the postoperative complications[15]. Transplantable tissue-like epithelial cell grafts are fabricated by cell sheet technology. On the basis of results obtained with canine and porcine models, we have used this technology with human patients since 2008.

CELL SHEET TECHNOLOGY FOR REGENERATION OF ESOPHAGEAL MUCOSA

Tissue engineering by using cell sheet technology

The concept of tissue engineering was originally proposed by Langer *et al*[16]. Conventionally, biodegradable polymer scaffolds have been used to reconstruct tissue architecture, and cells are seeded on them. The technique should be useful to reconstruct bone and cartilage, having a large amount of extracellular matrices (ECM) and few cells. However, scaffold-based tissue engineering would not be optimal for the regeneration of parenchymal tissues filled with a huge amount of cells and faint ECM. Therefore, we have proposed an alternative method of tissue reconstruction by using transplantable cell sheets to eliminate biodegradable scaffolds.

In order to fabricate transplantable cell sheets without any scaffolds, we employ temperature-responsive culture surfaces, onto which poly (N-isopropylacrylamide) is covalently immobilized to control cell adhesion/detachment with a simple temperature change[17]. Cells adhere, spread, and proliferate on temperature-responsive surfaces at 37 ℃, which is the normal temperature for mammalian cell culture. By reducing temperature below 32 ℃, cells spontaneously detach from the surfaces without proteolytic enzyme such as trypsin, since the grafted polymer becomes hydrophilic. When the temperature is reduced after cells reach confluence, all the cells are harvested as a single contiguous cell sheet. Because this technique eliminates trypsin for cell harvest, all the cell membrane proteins including growth factor receptors, ion channels and cell-to-cell junction proteins are intact even after cell and cell sheet harvest. Furthermore, ECM deposited during cell culture is retained under cell sheets[18]. Therefore, cell sheets easily integrate to transplanted sites. With this technique, many types of epithelial cell sheets are fabricated and subjected to regenerative medicine of skin[19], cornea[20], urinary bladder[21], and trachea[22].

Cultured epithelial cells for clinical application

Cultured autologous epidermal keratinocytes have been used as cell grafts to treat burns as the first therapy using cultured cells[23]. A transplantable epidermal cell graft is fabricated using murine 3T3 feeder layer cells[24]. Human keratinocytes co-cultured with 3T3 feeder cells proliferate and show stratification as *in vivo* with characteristics of native epithelial tissues including tonofilaments and desmosomes. Interestingly, human epidermal keratinocytes can be serially cultured to more than 150th passages *in vitro*[25]. The cultivation of human keratinocytes achieved clinical treatments for severe burns[26] and giant congenital nevi[27] by transplantation of cultured autologous keratinocyte grafts. Moreover, allogeneic keratinocyte grafts are also used for skin ulcers[28]. In these cases, keratinocyte sheets are harvested with bacteria-derived dispase treatment.

Oral mucosal epithelium is also stratified squamous epithelium, and on the basis of the feeder layer method, the epithelial cells have been used as a cell source to fabricate transplantable epithelial cell grafts for treating oral mucosa[29-31]. Moreover, since extraction of oral mucosal tissue is easy, cultured human oral mucosal epithelial cells are selected as useful cell grafts for ectopic transplantation, such as skin[30,32]. We have successfully applied cultured autologous oral mucosal epithelial cell sheets fabricated on temperature-responsive culture inserts to treat human patient cornea suffering from limbal epi-

thelial stem cell deficiency[33,34]. Furthermore, we utilized cultured autologous oral mucosal epithelial cell sheets for bladder reconstruction by the transplantation onto a demucosalized gastric flap in a canine model[35]. We believe that cultured oral mucosal epithelial cell sheets fabricated on temperature-responsive surfaces are useful for treatment of many types of epithelial diseases as well as reconstruction of defective tissues.

Fabrication of transplantable oral mucosal epithelial cell sheets in the clinical setting

To promote healing of esophageal ulcers after ESD for the prevention of postoperative complications, we performed endoscopic transplantation of cultured autologous oral mucosal epithelial cell sheets in the clinical setting. From the safety view point, animal-derived materials, such as bovine serum and 3T3 feeder cells should be eliminated as much as possible. For example, human embryonic stem cells cultured under a typical culture condition using animal-derived serum replacements and mouse feeder layer express an immunogenic nonhuman sialic acid[36]. Moreover, cells co-cultured with a mouse feeder layer are classified as xenogeneic products by United States Food and Drug Administration.

We reported that oral mucosal epithelial cells make transplantable stratified cell sheets on temperature-responsive cell culture inserts having micropores to supply culture medium from the basal cell surfaces even without a 3T3 feeder layer and allogeneic serum[37]. The method can, in principle, permit the cultured human oral mucosal epithelial cell sheets to be used as an autologous epithelial cell sheet graft for human patients[38]. Therefore, the autologous epithelial cell sheets are not, in principle, rejected after transplantation onto the esophageal ulcer and may be replaced by native epithelium in the esophagus. Moreover, the culture medium for the preparation of cultured oral mucosal epithelial cell sheets is modified to exclude laboratory reagents as much as possible for the clinical use[39].

Transplantable epithelial cell sheets are fabricated in a cell-processing center (CPC) with clean rooms according to the good manufacturing practice guideline in the clinical study. The cleanliness of the CPC is controlled by air conditioning systems using high efficiency particulate air filters, and standard operation procedures (SOP) are documented to keep a sterile environment of cell culture rooms in the CPC. The environment of the CPC is continuously monitored with various monitors including aerosol particles, temperature, and humidity, and various sterilization tests are performed to validate the cleanliness of the environment. In these tests culture rooms in the CPC were successfully kept sterile[40,41]. SOP of the culture method was also documented for the prevention of human error of operators to fabricate cell sheets highly reproducibly. Consequently, transplantable human epithelial cell sheets were successfully fabricated in a clinical study to treat ten human patients, and validation tests to demonstrate the quality assurance were performed one day before the ESD and transplantation.

From the average yield of the cells isolated from excised human oral mucosal tissues of healthy volunteer donors, we concluded that approximately 0.3 cm^2 of oral mucosal tissue is needed to fabricate one transplantable epithelial cell sheet having an area of 2.8 cm^2 (Figure 1A and B). The degree of cell stratification is an important point to enable reproducible cell sheet harvesting from temperature-responsive culture inserts. Figure 1C and D show the growth of seeded human oral mucosal epithelial cells. These cells became confluent on the surface of temperature-responsive culture inserts, and elevation of cell density accompanied with cell stratification was observed after a further 5 d.

Endoscopic transplantation of epithelial cell sheet onto esophageal ulcer immediately after ESD

Since 2008, we have performed a clinical study using cultured autologous human oral mucosal epithelial cell sheets to treat esophageal ulcer after endoscopic resection of a mucosal neoplasm. Endoscopic transplantation of the cultured epithelial cell sheets is performed using a support membrane to grasp by endoscopic forceps[42]. Epithelial cell sheets transplanted onto esophageal ulcer with a support membrane in a swine model are shown in Figure 2. One epithelial cell sheet fabricated on temperature-responsive culture insert (23 mm in diameter) covers the ulcer surface involving approximately one-third circumference of the esophagus lumen (Figure 2A and B), and three or four epithelial cell sheets were needed to cover the full circumferential ulcer (Figure 2C and D).

Although more research is needed for understanding the mechanism and correlation between ulcer size and number of transplanted epithelial cell sheets for preventing inflammation and postoperative stenosis, serial cultivation is a useful method of preparation of epithelial cells from small tissue for fabricating cell grafts to transplant onto a large ulcer after aggressive ESD. Serial culture using low calcium concentration medium (LCM) is a useful method to amplify a cell number of stratified squamous epithelial cells without using 3T3 feeder cells[43,44]. Interestingly, although normal human epidermal keratinocytes cultured in LCM show some mesenchymal-like phenotype, the keratinocytes can express the differentiation marker and stratify in differentiation-inducing culture conditions[45]. Since the conventional culture medium for serial cultivation of epithelial cells typically includes animal-derived material, the medium is not useful for clinical use. The modification of the LCM would be important for fabricating epithelial cell sheets to transplant onto large esophageal ulcers after aggressive ESD without any complications.

CONCLUSION

This article introduces cell sheet technology to fabricate stratified epithelial cell sheet grafts for preventing postoperative inflammation and stenosis after endoscopic re-

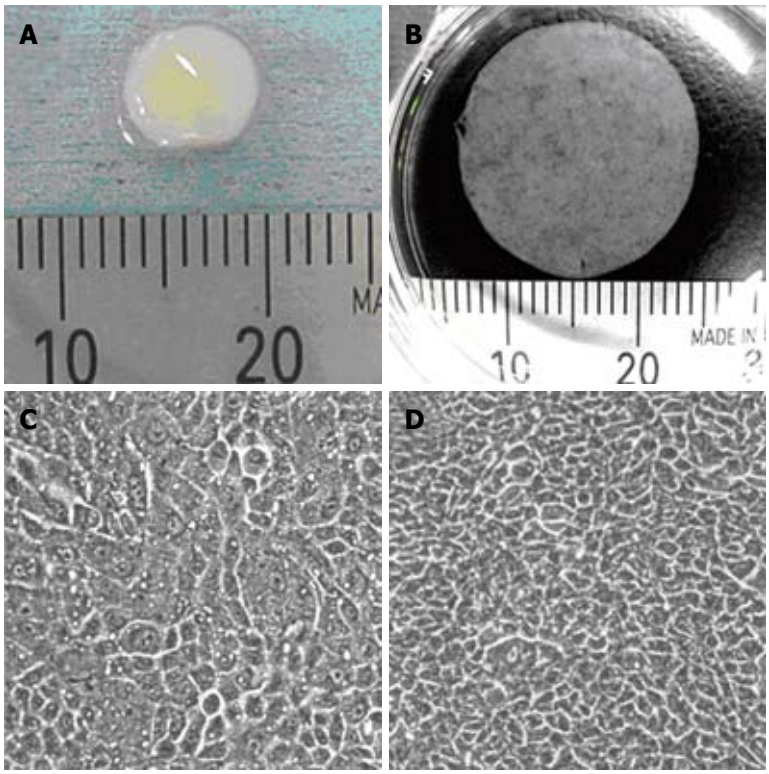

Figure 1 Transplantable oral mucosal epithelial cell sheet. A: Swine buccal oral mucosal tissue taken by punch biopsy; B: Human oral mucosal epithelial cell sheet harvested from temperature-responsive cell culture insert; C: Cultured human oral mucosal epithelial cells just become confluent on the surface of temperature responsive culture insert; D: The oral mucosal epithelial cells cultured for more than 5 d.

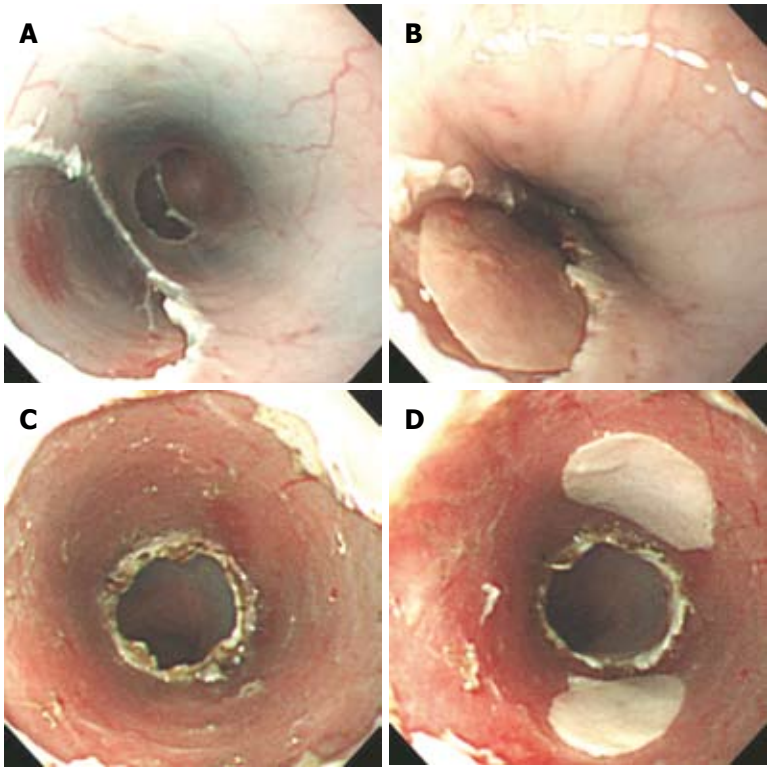

Figure 2 Transplantation of oral mucosal epithelial cell sheets on esophageal ulcer surface in animal model. A: Swine esophageal ulcer after endoscopic mucosal resection (EMR); B: Swine oral mucosal epithelial cell sheet transplanted on esophageal ulcer after EMR; C: Swine esophageal ulcer after endoscopic submucosal dissection (ESD); D: Two swine oral mucosal epithelial cell sheets transplanted on esophageal ulcer after ESD.

section for removing early-stage cancers from the esophagus. The efficacy of the oral mucosal epithelial cell sheet has been demonstrated, and the method for fabricating the cell sheet has also developed to use the cell graft as a clinical application for treating esophageal ulcers. This new therapy has been developed by integration of endoscopic technology and cell sheet technology. We believe that cell sheet technology will meet new technology and then the integration will create new therapies for treating patients without any specific therapies.

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GUIDELINES FOR CLINICAL PRACTICE

Irritable bowel syndrome: Diagnosis and pathogenesis

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Abstract

Irritable bowel syndrome (IBS) is a common gastrointestinal (GI) disorder that considerably reduces the quality of life. It further represents an economic burden on society due to the high consumption of healthcare resources and the non-productivity of IBS patients. The diagnosis of IBS is based on symptom assessment and the Rome Ⅲ criteria. A combination of the Rome Ⅲ criteria, a physical examination, blood tests, gastroscopy and colonoscopy with biopsies is believed to be necessary for diagnosis. Duodenal chromogranin A cell density is a promising biomarker for the diagnosis of IBS. The pathogenesis of IBS seems to be multifactorial, with the following factors playing a central role in the pathogenesis of IBS: heritability and genetics, dietary/intestinal microbiota, low-grade inflammation, and disturbances in the neuroendocrine system (NES) of the gut. One hypothesis proposes that the cause of IBS is an altered NES, which would cause abnormal GI motility, secretions and sensation. All of these abnormalities are characteristic of IBS. Alterations in the NES could be the result of one or more of the following: genetic factors, dietary intake, intestinal flora, or low-grade inflammation. Post-infectious IBS (PI-IBS) and inflammatory bowel disease-associated IBS (IBD-IBS) represent a considerable subset of IBS cases. Patients with PI- and IBD-IBS exhibit low-grade mucosal inflammation, as well as abnormalities in the NES of the gut.

Key words: Cholecystokinin; Chromogranin A; Diagnosis; Diet; Endocrine cells; Intestinal flora; Hereditary; Low-grade inflammation; Peptide YY; Serotonin

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INTRODUCTION

Irritable bowel syndrome (IBS) affects as many as 5%-20% of individuals worldwide (Figure 1)[1-31]. The annual incidence of IBS is between 196 and 260 per 100 000[32,33], with IBS occurring more often in women than in men, and being more commonly diagnosed in patients younger than 50 years of age[14,34-44]. IBS symptoms range from diarrhoea to constipation, or a combination of the two, with abdominal pain or discomfort existing alongside abdominal distension[45]. The degree of symptoms varies in different patients from tolerable to severe, and the time pattern and discomfort varies immensely from patient to patient[14,34-44]. Some patients complain of daily symptoms, while others report intermittent symptoms at intervals of weeks or months. IBS is not known to be associated with the development of serious disease or with excess mortality[46,47]. However, IBS causes a reduced quality of life with the same degree of impairment as major chronic diseases, such as diabetes, congestive heart failure, renal insufficiency and hepatic cirrhosis[48-50]. Although a minority (10%-50%) of IBS patients seek healthcare, they generate a substantial workload in both primary and secondary care[51-53]. The annual costs in the United States, both direct and indirect,

for the management of patients with IBS are estimated at 15-30 billion USD[37,54,55].

The treatment options for IBS have included pharmacological symptomatic relief of symptoms such as pain, diarrhoea or constipation. Evidence of the long-term benefit of pharmacological agents has been sparse, and new agents that have proven to be effective have raised issues concerning safety[56,57]. Alternative therapies, such as cognitive behavioural therapy and gut-directed hypnotherapy, have been used with good results[58]. Other non-pharmacological approaches have been also tried with proven effects on symptoms and the quality of life in patients with IBS[58].

The present review is an attempt to give an update on the diagnosis and pathogenesis of IBS, and to discuss some controversial issues in both the diagnosis and pathogenesis of IBS.

DIAGNOSIS

There is currently no biochemical, histopathological or radiological diagnostic test for IBS, with the diagnosis of IBS being based mainly on symptom assessment. Over the last few years, Rome working parties have generated detailed, accurate, and clinically useful definitions of the syndrome. As a result, the Rome criteria (Ⅰ, Ⅱ and Ⅲ) have been established (Table 1)[59,60]. In addition to these criteria, warning symptoms or red flags, such as age over 50 years, a short history of symptoms, nocturnal symptoms, weight loss, rectal bleeding, anaemia, and the presence of markers for inflammation or infections, should be excluded. IBS patients are sub-grouped on the basis of differences in the predominant bowel pattern as diarrhoea-predominant (IBS-D), constipation-predominant (IBS-C), or a mixture of both diarrhoea and constipation (IBS-M), and un-subtyped IBS in patients with an insufficient abnormality of stool consistency to meet the criteria for IBS C, D or M (Table 2). It has been reported that around one third of patients have IBS-D, one third have IBS-C, and the remainder have IBS-M[61-63]. The division of IBS patients into subtypes is useful for clinical practice and symptomatic treatment, but it is common for IBS patients to switch from one subtype to another over time. These patients are known as "alternators". More than 75% of IBS patients change to either of the other 2 subtypes at least once over a 1-year period[63].

The majority of gastroenterologists believe that a symptom-based diagnosis, such as that based on the Rome Ⅲ criteria, without red flags is enough for the diagnosis of IBS and that no further investigations are needed. The use of red flags in combination with Rome criteria has been found to be highly specific, but not particularly sensitive[64]. The American College of Gastroenterology Task Force does not recommend routine colonoscopy in patients younger than 50 years of age without any associated alarming symptoms[65]. The guidelines of the of the British Society of Gastroenterology go further, however, by recommending an examination of the colon earlier if there is a first degree relative affected by colorectal cancer who is younger than 45 years, or two first degree relatives of any age[66]. The British Society Of Gastroenterology also recommended further investigations in IBS-D due to the overlap with other diarrhoea diseases, such as coeliac and inflammatory bowel disease (IBDs)[66]. These recommendations seem to be suitable for detecting and diagnosing colorectal cancer in this group of patients, but not in other organic gastrointestinal (GI) diseases. It is rather difficult to clinically distinguish IBS from adult-onset coeliac disease (CD)[67-73], as the breadth of the spectrum of symptoms associated with IBS results in a potential for overlap of IBS and CD symptomatologies. The situation is further complicated by the fact that the abdominal symptoms of both IBS and CD patients are triggered by the ingestion of wheat products. In CD patients, this is due to a gluten allergy, while in IBS the effect is attributed to the long sugar polymer fructan in the wheat[74]. The prevalence of CD in IBS varies in different studies and varies from 0.04% to 4.7%[72,73,75-84]. Regardless of the number of CD patients among patients diagnosed with IBS, I believe that IBS patients from all subtypes should be routinely screened for CD, which is in line with current opinions in the field[84-86]. Distinguishing IBD from IBS, especially with mild disease activity, can be difficult[87]. Furthermore,

Table 1 Rome Ⅲ criteria for the diagnosis of irritable bowel syndrome[1]

Recurrent abdominal pain or discomfort with onset at least 6 mo prior to diagnosis, associated with 2 or more of the following, at least 3 d/mo in the last 3 mo
Improvement with defecation
Onset associated with change in frequency of stool
Onset associated with change in form (appearance) of stool
Symptoms that cumulatively support the diagnosis are:
Abnormal stool frequency (greater than 3 bowel movements per day or less than 3 bowels movements per week)
Abnormal stool form (lump/hard or loose/watery stool)
Abnormal stool passage (straining, urgency or feeling of incomplete evacuation)
Passage of mucous
Bloating or feeling of abdominal distension

[1]Adapted from reference [1] with the permission from Nova Science Publisher, Inc.

Table 2 Subtyping of irritable bowel syndrome[1]

IBS with constipation-hard or lumpy stools > 25% and loos or watery stools < 25% of bowel movements
IBS with diarrhea-loos or watery stools > 25% and hard or lumpy stools stools < 25% of bowel movements
Mixed IBS-loos or watery stools > 25% and hard or lumpy stools stools > 25% of bowel movements
Unsubtyped IBS-insufficient abnormality of stool consistency to meet criteria for IBS-C,D or M

[1]Adapted from reference [1] with the permission from Nova Science Publisher, Inc. IBS: Irritable bowel syndrome; IBS-C: IBS with constipation; IBS-D: IBS with diarrhea-loos; IBS-M: IBS with a mixture of both diarrhoea and constipation.

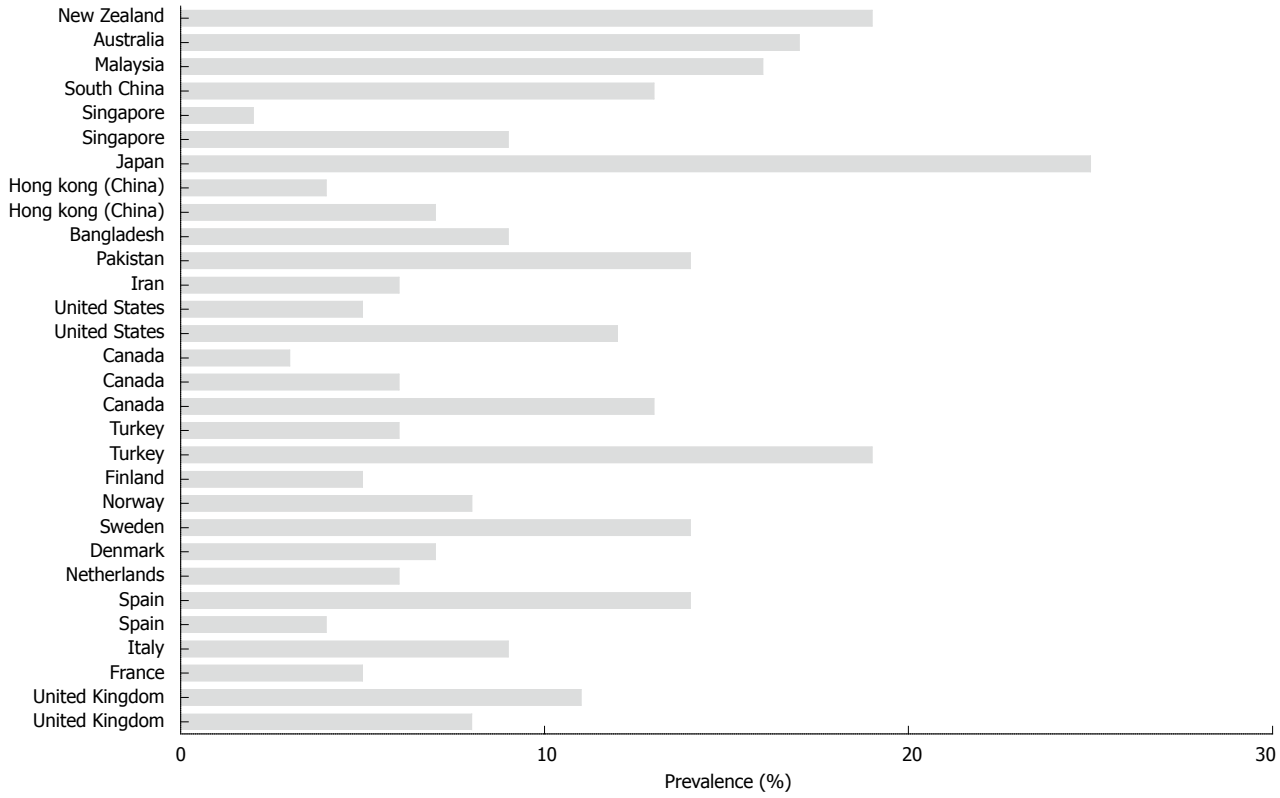

Figure 1 The prevalence of irritable bowel syndrome according to Rome criteria in different countries. Reproduced from reference [1] with permission from Nova Science Publisher, Inc.

IBS-like symptoms are frequently reported before the diagnosis of IBD[87-90]. Microscopic colitis (MC) and IBS have similar symptoms and a normal endoscopic appearance[91-101], and the diagnostic overlap between IBS, IBD and MC is important because of a potentially different treatment for each disorder. The prevalence of IBD in patients that fulfilled the Rome criteria without alarming symptoms varies between 0.4% and 1.9%[96-100], and MC from 0.7% to 1.5%[90-97]. It is conceivable, therefore, to conclude that symptom-based diagnosis of IBS may lead to a number of other GI disorders that require quite different management than IBS being missed. Sigmoidoscopy in IBS patients might be insufficient, however, as a considerable number of MC patients may not be identified without mucosal biopsies from the right colon[101]. Moreover, performing a sigmoidoscopy would not exclude Crohn's disease lesions in the terminal ileum, making ileocolonoscopy prefered, especially in IBS-D patients. This seems, at first sight, to add more economic burden to healthcare, which is already suffering from a lack of resources. IBS patients are already consuming a large amount of healthcare resources. However, performing an ileocolonoscopy would reassure IBS patients and prevent them from seeking a new examination, which would not increase the economic burden of this patient group on society, but instead use the existing resources effectively.

Several biomarkers for the diagnosis of IBS have been considered, but only gut transit measured by radio-isotope markers meets the criteria for reproducibility and availability[102]. However, radio-isotope tests themselves are expensive and of limited availability[102]. It has been reported that the chromogranin A-containing cell density is low in the duodenum of IBS patients (Figure 2)[103,104]. As chromogranin A is a general marker for endocrine cells[105,106], this finding indicates a general reduction in small intestinal endocrine cells in these patients. It has been proposed that the quantification of duodenal chromogranin A cell density could be used as a histopathological marker for the diagnosis of IBS[103,104]. Receiver-operator characteristic curves for chromogranin A cell density in the duodenum is given in Figure 3. The sensitivity and specificity at the cut-off < 31 cells/mm^2 in the duodenum are 91% and 89%, respectively. Screening of IBS patients for CD is now widely accepted. Thus, gastroscopy with duodenal biopsies can be used for excluding or confirming CD instead of blood tests, and the same biopsies can be used for the diagnosis of IBS. The duodenal endocrine cell types affected and their role in the pathogenesis of IBS is discussed in the next section.

PATHOGENESIS

Patients with IBS typically present with GI complaints for which physicians can find no organic cause. It is natural and understandable to make comparisons with hysteria, which is also predominant in women. Hysteria has been replaced in modern psychiatry by somatisation disorders and conversion disorders. The notion that IBS is a psychiatric disorder is deeply rooted in clinical practice.

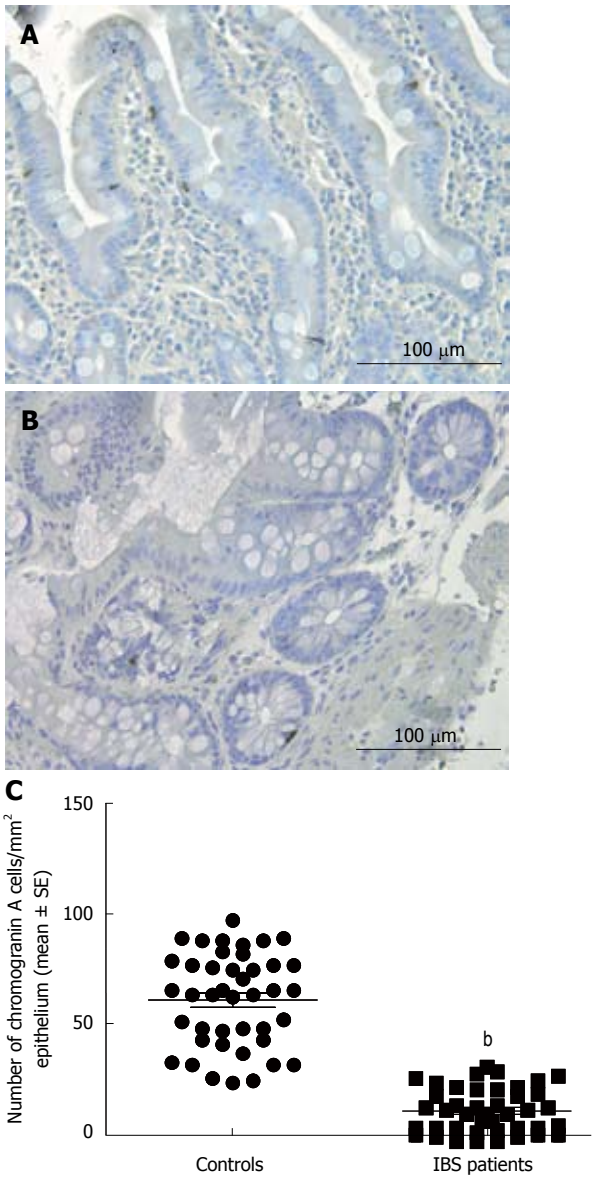

Figure 2 Chromogranin A cells in the duodenum. A: A healthy subject; B: A patient with irritable bowel syndrome (IBS); C: Controls and IBS patients. Reproduced from reference [1] with permission from Nova Science Publisher, Inc. $^{b}P < 0.01$ *vs* control group.

This situation was not improved by the huge number of publications on a selected group of IBS patients, which show that IBS patients are more likely to be psychiatrically ill and sexually or physically abused than the general population[107-121]. Many patients with IBS ignore their symptoms and regard them as a normal part of everyday life. IBS patients with anxiety, depression, somatisation or hypochondria are more liable to seek healthcare than other IBS patients. Unless this is borne in mind, incorrect conclusions can be drawn. A hospital-based case-control study showed that patients with IBS have a comparable health-related quality of life, level of psychological distress and occurrence of recent stressful life events to age-matched IBD patients[122]. These findings are interesting as IBD patients receive effective treatment and are treated with sympathy, understanding and support by their doctors as well as society. In contrast, IBS patients are offered non-effective treatments, are treated with mistrust and neglect by their doctors, feel that they are labelled as hypochondriacs and believe that they receive no support from society. It could be expected that IBS patients would be more anxious and depressed than IBD patients, but this is not the case. Two percent of patients diagnosed with IBS among the adult residents of Olmsted County, Minnesota, United States, were found to suffer from depression compared to the 16.2% incidence of depression in the entire population of the United States[122,123]. In conclusion, there is no convincing evidence to show that psychological factors play a role in the onset and/or progression of IBS[66].

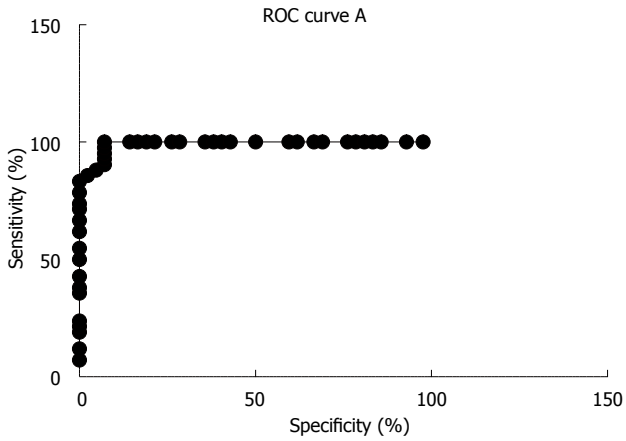

Figure 3 Receiver-operator characteristic for chromogranin A cell density in the duodenum. Reproduced from reference [1] with permission from Nova Science Publisher, Inc. ROC: Receiver-operator characteristic.

The pathogenesis of IBS appears to be multifactorial. There is evidence to show that the following factors play a central role in the pathogenesis of IBS: heritability and genetics, environment and social learning, dietary or intestinal microbiota, low-grade inflammation and disturbances in the neuroendocrine system (NES) of the gut.

Heritability and genetics

Up to 33% of patients with IBS had a family history of IBS compared to 2% of the controls[124]. In a study of a family cluster from Olmsted County, United States, a significant association was reported between having a first degree family member with bowel symptoms and presenting with IBS. In contrast, those who reported having a spouse with bowel symptoms were no more likely to present with IBS than the general population[125]. It was further shown that the prevalence of IBS was 17% in the relatives of patients compared to 7% in the relatives of spouses[126]. Another study showed that patients with IBS were more likely to present a family history of IBS than controls (33.9% and 12.6%, respectively). Moreover, 21.1% of IBS non-consulter patients reported a family history of IBS, in comparison with 12.6% of the control subjects[127].

In twin studies, a higher rate of IBS was reported in monozygotic twins than in dizygotic twins (33.3% *vs*

13.3%). Moreover, 56.9% of the variance was attributed to additive genetic factors, indicating a substantial genetic component in IBS[128-132]. In contrast, a study performed on British twin pairs did not show any significance in the rates of IBS between monozygotic and dizygotic twins[133].

The serotonin transporter (*SERT*) gene encoding the SERT protein is located on chromosome 17q11.1-q12. A functional polymorphism is the insertion or a deletion of 44 base pairs in the *SERT*-gene-linked polymorphic region[134]. An association was reported between a functional polymorphism in the *SERT* gene and diarrhoea-predominant IBS[135,136]. Individuals with a long allele genotype of the *SERT* gene have been shown to be vulnerable to developing IBS with constipation[137]. Other studies, however, did not show such association between *SERT*-gene polymorphism and IBS[136]. A polymorphism in the CCK1 receptor *CCKAR* gene (779T>C) has also been found to be associated with IBS[138,139].

Environment and social learning

Parental modelling and the reinforcement of illness behaviour can contribute to the causes of IBS[140-144]. Having a mother with IBS has been shown to account for as much variance as having an identical set of genes as a co-twin who has IBS. This suggests that the contribution of social learning to IBS is at least as great as the contribution of heredity[144].

Dietary and intestinal flora

Patients with IBS believe that their diet has a significant influence on their symptoms and they are interested in finding out which foods they should avoid[145-148]. About 60% of IBS patients report a worsening of symptoms following food ingestion: 28% within 15 min after eating and 93% within 3 h[148]. Many IBS patients report specific foods as triggers, most commonly implicating milk and dairy products, wheat products, onion, peas and beans, hot spices, cabbage, certain meats, smoked products, fried food and caffeine as the offending foods[149]. However, dietary composition among IBS patients in the community does not differ from community controls[150-153]. In a recent study, IBS patients were reported to have made a conscious choice to avoid certain food items, some of which belong to fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs). However, they reported a higher consumption of other food items that are rich in FODMAPs. Patients also reportedly avoided other food sources that are important for health, which result in a low intake of calcium, phosphorus and vitamin B2[153].

There is no documented evidence showing that a food allergy or intolerance plays a role in IBS symptoms[1]. The reaction of IBS patients to certain food items has been attributed to a number of short-chain carbohydrates that are poorly absorbed so that a significant portion of the ingested carbohydrates enters the distal small bowel and colon. Once there they increase the osmotic pressure and provide a substrate for bacterial fermentation with the production of gas, distension of the large intestine and abdominal discomfort or pain. These carbohydrates are FODMAPs and include fructose, lactose, fructans, galactans and sugar alcohols, such as sorbitol, maltitol, mannitol, xylitol and ismalt. Fructose and lactose are present in apples, pears, watermelon, honey, fruit juices, dried fruits, milk and dairy products. Polyols are used in low calorie food products. Galactans and fructans are present in common dietary constituents, such as wheat, rye, garlic, onions, legumes, cabbage, artichokes, leeks, asparagus, lentils, inulin, soy, Brussels sprouts and broccoli[78,147].

A deficiency in dietary fibre was widely believed to be the primary cause of IBS[154]. Although increasing the amount of dietary fibre continues to be a standard recommendation for patients with IBS, clinical practice has shown that increased fibre intake in these patients increases abdominal pain, bloating and distension. IBS patients assigned to the fibre treatment showed persistent symptoms or no improvement in symptoms after treatment compared to patients taking the placebo or a low-fibre diet. Other studies have shown that whilst a water-insoluble fibre intake did not improve IBS symptoms, soluble-fibre intake was effective in improving overall IBS symptoms[155,156]. It is noteworthy that the role of FODMAPs and fibre on IBS symptoms is associated with intestinal flora. The presence of bacteria that break down FODMAPs and fibre and produce gas, such as *Clostridia spp.*, can cause distension of the large intestine with abdominal discomfort or pain.

Most bacteria in the GI tract exist in the colon. The colon of each individual contains between 300 and 500 different species of bacteria[1], and each person has his own unique intestinal flora. The intestinal flora is affected by several factors, such as diet, climate changes, stress, illness, aging and antibiotic treatment[1]. The intestinal flora in IBS patients has been found to differ considerably from that of healthy controls, as IBS patients have fewer *Lactobacillus* and *Bifdobacterium spp.* than healthy subjects[157]. These bacteria bind to epithelial cells and inhibit pathogen binding as well as enhancing barrier functioning[158]. Furthermore, these bacterial species do not produce gas upon fermenting carbohydrates, which is an effect that would be amplified as they also inhibit the *Clostridia spp.*[158]. Probiotics alter colonic fermentation and stabilise the colonic microbiota, and several studies on probiotics have shown improvements in flatulence and abdominal distension, with a reduction in the composite IBS symptom score[158-160].

Low-grade inflammation

In a subset of IBS patients GI symptoms appear following gastroenteritis, with about 25% of patients showing IBS-D symptoms 6 mo post-infection and approximately 10% developing persistent symptoms[161-164]. Post-infectious (PI)-IBS has been reported after viral, bacterial, protozoa and nematode infections[1], with the incidence of PI-IBS varying between 7% and 31%, although the largest studies suggest this number is about 10%[161-164]. One study showed that 6% to 17% of sporadic (un-

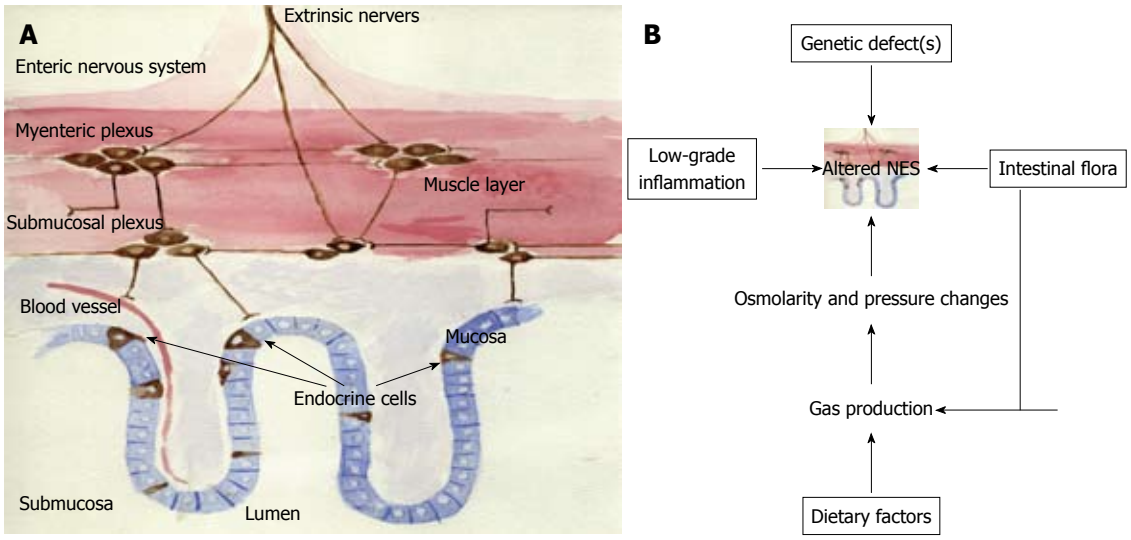

Figure 4 Schematic drawing to illustrate the neuroendocrine system of the gut and the possible pathogenesis of irritable bowel syndrome. A: Schematic drawing of neuroendocrine system; B: Possible pathogenesis of irritable bowel syndrome. Reproduced from reference [1] with permission from Nova Science Publisher, Inc. NES: Neuroendocrine system.

selected) IBS patients believed that their symptoms began with an infection[1]. Following infection, the initial inflammatory response shows an increase in CD3 lymphocytes, CD8 intraepithelial lymphocytes and calprotectin-positive macrophages[161]. These changes rapidly decrease in most subjects but a small number with persistent symptoms fail to show this decline[165]. Furthermore, the number of serotonin cells was shown to increase in subjects with persistent symptoms[165]. There are several pieces of evidence showing that inflammation and immune cells affect the NES of the gut, which controls and regulates GI motility and sensitivity[166]. Thus, serotonin secretion by enterochromaffin (EC) cells can be enhanced or attenuated by the secretory products of immune cells such as CD4+T[167]. Furthermore, serotonin modulates the immune response[167]. The EC cells are in contact with or very close to CD3+ and CD20+ lymphocytes, and several serotonergic receptors have been characterised in lymphocytes, monocytes, macrophages and dendritic cells[168]. Moreover, immune cells in the small and large intestine show receptors for substance P and vasoactive intestinal polypeptide[169].

IBS occurs in 32%-46% of patients with ulcerative colitis (UC) and in 42%-60% of Crohn's disease patients who are in remission[170-174]. Faecal calprotectin has been found to be significantly elevated in UC and Crohn's disease patients with criteria for IBS, compared to those without IBS-type symptoms, indicating the presence of occult inflammation[174].

Abnormalities in the NEC of the gut in IBS

The NES of the gut consists of two parts: endocrine cells scattered among the epithelial cells of the mucosa facing the gut lumen, and peptidergic, serotonergic and nitric oxide-containing nerves of the enteric nervous system (ENS) in the gut wall (Figure 4A)[1]. This system regulates several functions of the GI tract, such as motility, secretion, absorption, microcirculation in the gut, local immune defence and cell proliferation[1]. This regulatory system includes a large number of neuroendocrine peptides/amines, which exert their effects *via* a number of actions: an endocrine mode of action, by circulating in the blood to reach distant targets, an autocrine/paracrine mode, which is a local action, and *via* synaptic signalling or *via* neuroendocrine means, which involve the release from synapses into the circulating blood. The different parts of this system interact and integrate with each other and with afferent and efferent nerve fibres of the central nervous system, in particular the autonomic nervous system. There are at least 14 different populations of endocrine or paracrine cells in the GI tract[1]. The ENS comprises a large variety of neurotransmitters and associated receptors. Almost every known neurotransmitter can be found in the ENS, and most of the receptors associated with these neurotransmitters are also expressed there[1].

In the stomach of patients with IBS, the density of ghrelin-immunoreactive cells in the oxyntic mucosa was found to be significantly lower in IBS-constipation patients and significantly higher in IBS-diarrhoea patients compared to healthy controls[175]. However, the levels of total or active ghrelin in plasma and stomach tissue extracts from IBS patients did not differ from those of healthy subjects[175,176]. Ghrelin is a 28-amino acid peptide hormone that was originally isolated from the stomach[177]. Ghrelin mostly originates from endocrine cells in the oxyntic mucosa of the stomach, but small amounts are expressed in the small intestine, large intestine and in the arcuated nucleus of the hypothalamus[177]. Ghrelin has several functions, including a role in regulating growth hormone (GH) release from the pituitary, where it acts synergistically with the GH-releasing hormone[178,179]. Ghrelin also increases appetite and feeding and plays a major role in energy metabolism[178-181]. Furthermore, this hormone has been found to accelerate gastric and

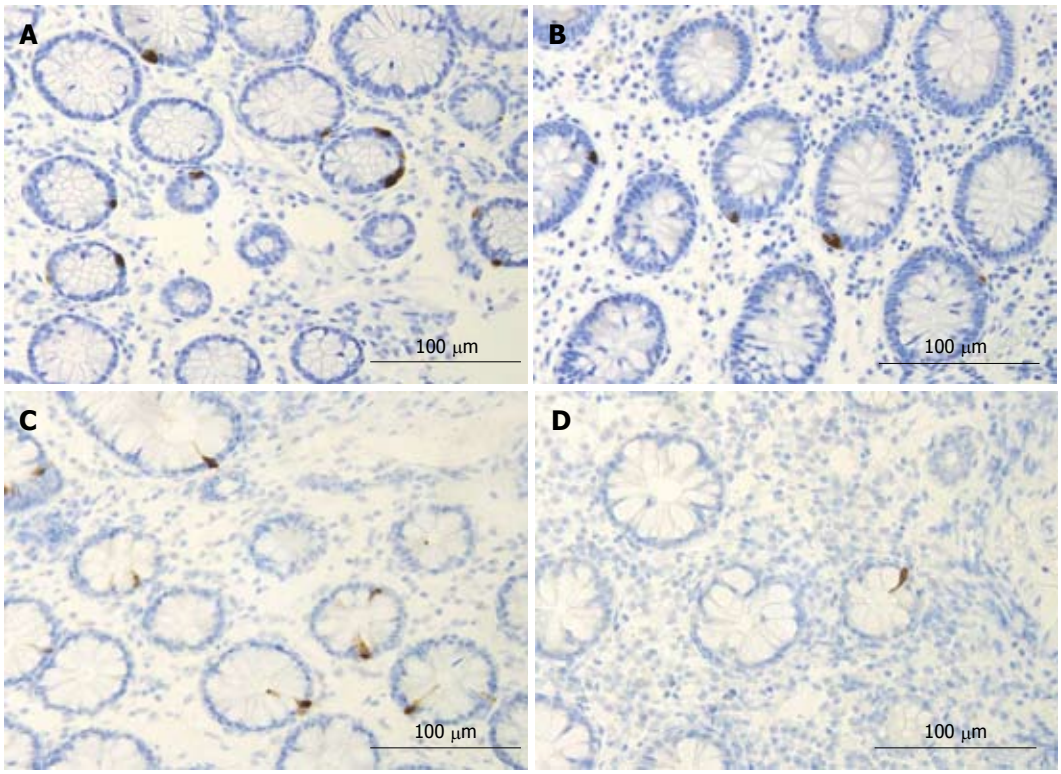

Figure 5 Serotonin cells and polypeptide YY immunoreactive cells in the colon. A: A healthy control in serotonin cells; B: A patient with irritable bowel syndrome in serotonin cells; C: A healthy subject in polypeptide YY (PYY) immunoreactive cells; D: An irritable bowel syndrome patient in PYY immunoreactive cells. Reproduced from reference 1 with permission from Nova Science Publisher, Inc.

small and large intestinal motility[181-192], as well as having anti-inflammatory actions and protecting the gut against a wide range of insults. The density of neuropeptide-expressing cells is altered in the small intestine of IBS patients. Thus, the density of cells expressing gastric inhibitory polypeptide and somatostatin is decreased in patients with both diarrhoea- and constipation-predominant IBS subtypes[193]. The densities of secretin and cholecystokinin (CCK)-expressing cells are decreased in the diarrhoea-predominant subtype, but not in the constipation-predominant subtype. Serotonin cell density has also been found to be unchanged in the duodenum of IBS patients, regardless of the subtype[193], which is interesting as serotonin cells were previously reported to be affected in the small intestine of IBS patients[194-196]. These peptides all play important roles in secretion and gastric motility. In the large intestine, serotonin and polypeptide YY (PYY) cell densities have been found to be low in both IBS-constipation and IBS-diarrhoea patients (Figure 5)[197]. Furthermore, the mucosal 5-HT concentration has also been reported to be low in IBS patients[197], which is in line with current observations. In PI-IBS, the number of CCK and serotonin cells has been reported to be increased in the small intestine[198], and serotonin and PYY cell numbers were found to be increased in the large intestine[199-202].

HYPOTHESIS

As described above, abnormalities in the neuroendocrine peptides/amines of the gut have been reported. These abnormalities could cause disturbances in digestion, GI motility and visceral hypersensitivity. These abnormalities appear to contribute to symptom development and could play a central role in the pathogenesis of IBS. Genetic differences have been found between IBS patients and healthy subjects in genes controlling the serotonin signalling system and CCK. Moreover, differences in the diet, intestinal flora and inflammation affect the NES of the gut. The release of different gut hormones depends on the composition and quantity of ingested food, as the food content of FODMAPs and fibre, intestinal flora and the subsequent fermentation can increase intestinal osmotic pressure. This change in intestinal pressure can stimulate hormonal release, such as the release of serotonin. Likewise, inflammation and the release of secretory products from immune cells effects hormonal release and the proliferation of gut endocrine cells.

Therefore, it is feasible to hypothesise that the cause of IBS is an altered NES (Figure 4B). An altered NES would cause abnormal GI motility, secretion and sensation, all of which are characteristic of IBS[203-216]. The alteration in NES could be a result of one or more of the following: genetic factors, dietary intake, intestinal flora or low-grade inflammation.

CONCLUSION

The diagnosis of IBS is based on symptom assessment and the Rome Ⅲ criteria. Whereas the latter has been

widely used in scientific studies and in GI congresses in the past 10 years, it is not, however, used by most clinicians consulted by IBS patients[217-220]. This is not because these clinicians are unaware of the Rome Ⅲ criteria, but because of the reality in the clinic. IBS patients that seek advice from a doctor are worried and want to be investigated, and are rarely satisfied until this is done, so they will repeatedly seek healthcare until they are investigated. I believe, therefore, that the Rome Ⅲ criteria should be combined with a physical examination, blood tests, gastroscopy, duodenal biopsies and colonoscopy with segmental biopsies. These examinations and tests, in addition to the Rome Ⅲ criteria, would reassure the patient and exclude CD, IBD, MC and cancer. Furthermore, performing these examinations and tests would remove the pressure applied by some patients to perform these examinations repeatedly, as the need for further investigations can always be argued against if there are no new symptoms. Duodenal chromogranin A cell density also appears to be a promising biomarker for the diagnosis of IBS.

The pathogenesis of IBS appears to be multifactorial. There is evidence to suggest that the following factors play a central role in the pathogenesis of IBS: heritability and genetics, dietary and intestinal microbiota, low-grade inflammation and disturbances in the NEC of the gut. Several authors have tried to connect these factors in a logical cause-effect pattern, but it is my belief that the proposed hypothesis presented in this review is the most logical.

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GUIDELINES FOR CLINICAL PRACTICE

Gastric carcinogenesis

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Abstract

Gastric cancer is the second most common cancer worldwide and the second most common cause of cancer-related deaths. Despite complete resection of gastric cancer and lymph node dissection, as well as improvements in chemotherapy and radiotherapy, there are still 700 000 gastric cancer-related deaths per year worldwide and more than 80% of patients with advanced gastric cancer die of the disease or recurrent disease within 1 year after diagnosis. None of the treatment modalities we have been applying today can influence the overall survival rates: at present, the overall 5-year relative survival rate for gastric cancer is about 28%. Cellular metaplasia due to chronic inflammation, injury and repair are the most documented processes for neoplasia. It appears that chronic inflammation stimulates tumor development and plays a critical role in initiating, sustaining and advancing tumor growth. It is also evident that not all inflammation is tumorigenic. Additional mutations can be acquired, and this leads to the cancer cell gaining a further growth advantage and acquiring a more malignant phenotype. Intestinalization of gastric units, which is called "intestinal metaplasia"; phenotypic antralization of fundic units, which is called "spasmolytic polypeptide-expressing metaplasia"; and the development directly from the stem/progenitor cell zone are three pathways that have been described for gastric carcinogenesis. Also, an important factor for the development of gastrointestinal cancers is peritumoral stroma. However, the initiating cellular event in gastric metaplasia is still controversial. Understanding gastric carcinogenesis and its precursor lesions has been under intense investigation, and our paper attempts to highlight recent progress in this field of cancer research.

Key words: Gastric Cancer; Cancer Stem Cell; Carcinogenesis; Oncogenesis; Tumorigenesis

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GASTRIC CARCINOMA

Cancer is a major public health problem and at the beginning of the 19th century, gastric cancer was the second most common cancer worldwide[1]. Every year there are 900 000 new cases and 700 000 gastric cancer-related deaths in the world[2]. Although chemotherapy improves life expectancy, and despite seemingly complete resection of gastric cancer (R0) *via* gastrectomy, more than 80% of patients with advanced gastric cancer die of the disease or recurrent disease within 1 year after diagnosis. This situation suggests that standard treatment protocols are ineffective in a considerable number of cases[3]. Thus, the understanding of the mechanism underlying the progression of gastric carcinoma is essential for the management of this disease.

RISK FACTORS

A number of risk factors are known for gastric cancer (Table 1), but study results regarding some factors, especially salt intake, vitamin C, alcohol, occupational exposure to nitrosamines and inorganic dusts, have been inconsistent[4-9].

HISTOLOGY AND PATHOLOGY

The majority of gastric cancer patients have adenocarcinoma (90%); the remaining 10% have lymphoma or gastrointestinal stromal tumor. There are two general types of gastric adenocarcinoma: the intestinal type (50%) and the diffuse type (33%) according to the Lauren classification system[9]. The remaining 17% are mixed or unclassified type[10]. The intestinal type is more common and is more often located in the distal part of the stomach. In contrast, the diffuse type has a poorer prognosis; generally occurs in younger patients; and can occur anywhere in the stomach, but especially in the cardia. The intestinal type is frequently accompanied by liver metastasis, whereas because the diffuse type has an increased propensity for intra- and trans-mural spread, it has been associated with peritoneal dissemination and poorer prognosis[1]. The diffuse type of gastric cancer shows more poorly differentiated cells than the intestinal type[11]. Intestinal-type adenocarcinoma is preceded by metaplastic changes, whereas diffuse-type adenocarcinoma is thought to arise in normal gastric mucosa.

Gastric adenocarcinoma can also be divided into two groups, known as "differentiated" and "undifferentiated", using the Nakamura classification system[12]. Intestinal-type adenocarcinoma is considered to be essentially equivalent to differentiated adenocarcinoma, as is diffuse-type equivalent to the undifferentiated adenocarcinoma. However, some cases of intestinal-type adenocarcinoma also arise from the gastric mucosa without intestinal metaplasia (IM). So based on the type of IM, some authors suggest that gastric cancer phenotypes can be classified into four groups depending on the marker combinations as: complete intestinal type, incomplete intestinal type, gastric type and unclassified type. Gastric-type differentiated adenocarcinomas can be distinguished from other types of differentiated adenocarcinomas on the basis of their increased malignant potential in the incipient phase of invasion and metastasis[13].

The mucous epithelium of the stomach represents a major barrier to the various noxious agents by means of intercellular tight junctions. This epithelium and its components are also vital for complex communications and physiological functions[14]. Histologically, the human gastric mucosa is divided into three regions: cardia, fundus-corpus and antrum-pylorus. Also, a transitional zone separates the stereotypic corpus and antral/pyloric epithelia and has features of each. The epithelium of these regions is composed of millions of glands that are surrounded by supporting stromal cells which are derived from mesenchyme. In the corpus, glands are long and composed of several epithelial cell types, including surface mucous foveolar cells (pit cells), acid-making oxyntic (parietal) cells, mucous neck cells (intermediate progenitor for chief cells), zymogenic (chief) cells, and hormone-secreting endocrine cells. In the antrum, the shorter glands are composed mainly of mucus-secreting cells and endocrine cells that secrete hormones such as gastrin and somatostatin. The stomach mesenchymal compartment surrounding the glands is less studied and little understood[15-18].

The human stomach mucosal tubular glands are further subdivided into foveolus, isthmus, neck and base regions. The gastric glands open into the bottom of the pits, on an average with 4 to 5 glands per pit. Fundic glands are quite straight, whereas antral glands are branched and coiled in their basal ends. Fundic and antral units (combination of a pit and a gland) differ very much in their cell characteristics and turnover rates (the human antral mucosa is known to have a much higher turnover rate). The gastric glands which contain 'surface mucous' cells and "mucous neck" cells (in the foveola), pepsinogen-secreting zymogenic (chief) cells (at the base of the glands), acid-secreting oxyntic (parietal) cells (at the base of the glands), and endocrine cells including the histamine-producing enterochromaffin-like (ECL) cells are located in the fundus; the zymogenic (chief) cells, oxyntic (parietal) cells and ECL cells are also found in the corpus of the stomach. The antral unit contains surface mucous foveolar cells, antral gland cells, endocrine cells (mainly gastrin-producing G-cells, but also EC and somatostatin-producing D cells), and occasional oxyntic cells. In the pylorus, the gastric glands contain many more mucinous cells, no zymogenic cells and few oxyntic cells (Figure 1)[19-23].

In addition, it should be noted that the subepithelial mesenchymal cells and their secreted basement membrane factors compose the lamina propria. This constitutes a structural support while regulating epithelial cell function and epithelial cell networks[24,25].

MOLECULAR TARGETS AND SIGNALING PATHWAYS

Some of the earliest observations in cancer biology as well as recent advances in molecular analyses contribute to our knowledge about the multistep process of gastric carcinogenesis[26-28].

The gastrointestinal tract has rapid epithelial turnover and exposure to injury by infections and dietary toxins. These conditions create very high cancer prevalence. Intestinalization of gastric units, which is called "IM"; phenotypic antralization of fundic units, which is called "spasmolytic polypeptide-expressing metaplasia (SPEM)"; and the development directly from the stem/progenitor cell zone, are three pathways that have been described for gastric carcinogenesis[29-31].

Neoplasia can follow cellular metaplasia due to chronic inflammation, injury and repair[32]. This is the most documented process for gastric cancer[33-35]. An accept-

Table 1 Risk factors for gastric cancer

Genetic factors	Environmental factors	Other factors
Sex	*Helicobacter pylori*	Gastric adenomas
Familial adenomatous polyposis	Epstein-Barr virus	Barrett's esophagus
Hereditary nonpolyposis colorectal cancer (Lynch Ⅱ)	Nitrites	Hamartomas
Genetic diffuse gastric cancer (E-cadherin - CDH1 mutation)	Excess alcohol ingestion	Ménétrier's disease
Genetic polymorphisms for pro- and anti-inflammatory cytokines	High intake of salted, pickled, or smoked foods	Chronic atrophic gastritis
Polymorphisms for cell receptors of innate immune response	Low intake of fiber, fruits and vegetables	Gastric metaplasia
Peutz-Jeghers syndrome	Antioxidant consumption (especially ascorbic acid, carotenoids, folates and tocopherols)	Pernicious anemia
	Tobacco smoking (adenocarcinoma of cardia)	Benign gastric ulcers
		Fundic gland polyps
		Hyperplastic polyps
		Gastric biopsy revealing high-grade dysplasia
		History of subtotal gastrectomy (> 20 yr)

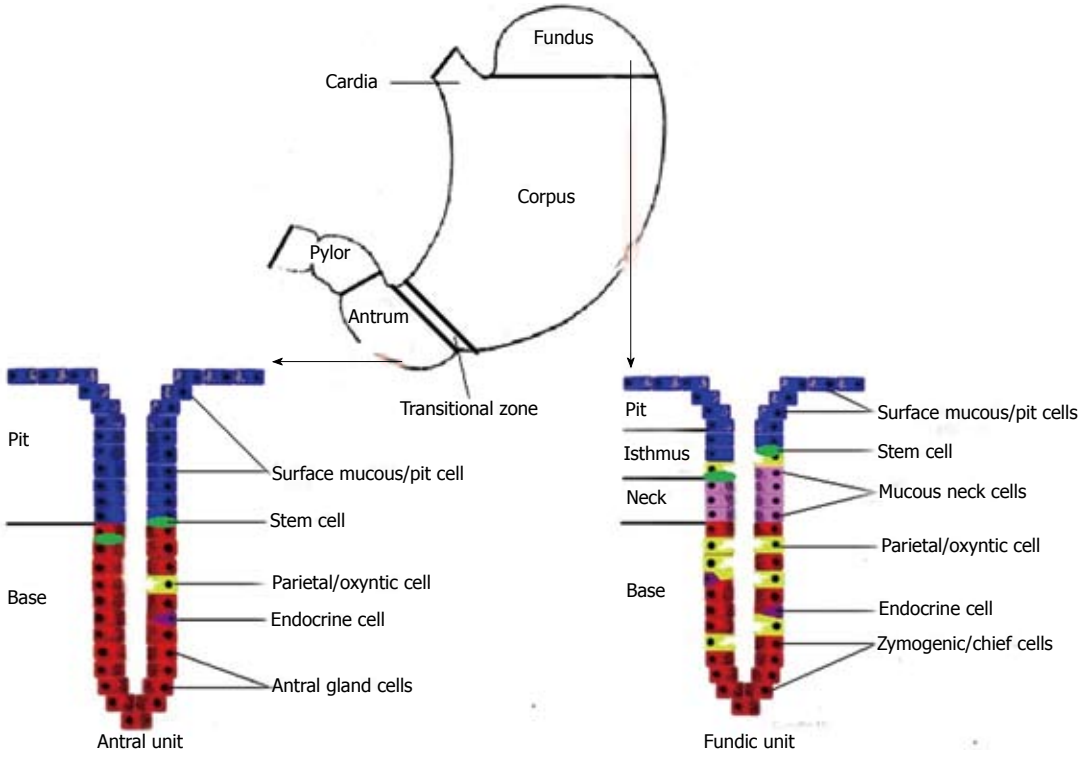

Figure 1 Schematic explanation of fundic and antral units.

able concept is that there are two corner-stones with regard to this process. Firstly, the initial observation of Rudolf Virchow in 1863 about leucocytes in neoplastic tissues and the connection between inflammation and cancer[36]; secondly, about 15 years ago, researchers' evidence about the relationship between stomach cancer and infection by *Helicobacter pylori* (*H. pylori*) (isolated by Drs. Marshall and Warren in 1984[37]. Also, we must note that Epstein-Barr virus has been detected in stomach tissues in approximately 10% of gastric carcinoma cases[38].

Beginning with some of the earliest observations in cancer biology, it appears that chronic inflammation stimulates tumor development and plays a critical role in initiating, sustaining and advancing tumor growth[39,40]. Direct effect of the viral pathogens on neoplastic transformation of epithelial cells has been shown; however, it is also evident that not all inflammation is tumorigenic[41]. It can be suggested that either the tumor alters the immune response by reactive oxygen species and cytokines or chronic inflammation plays a primary role in transforming tissue cells (especially mentioned in "stem cell theory") into tumor cells. In the acute phase of inflammation, the release of endogenous reactive oxygen and nitrogen species (O_2^-, H_2O_2, NO, OH, $ONOO^-$, HOCl) from such innate immune cells as macrophages and leukocytes plays an important role in the elimination of

pathogens[42]. However, when present chronically, this can induce DNA damage in proliferating cells. In addition, it is also possible for other bacteria to colonize the stomach and additionally trigger carcinogenesis by gastric atrophy (result of chronic inflammation) which represents a loss of gastric glands and associated lower acidity of gastric juice[43,44]. Hypoacidity associated with *H. pylori* infection induces gastric mucosal atrophy to advance multistage carcinogenesis in the stomach. Interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) are elevated in gastric mucosa with *H. pylori* infection. Gastrin is upregulated and acid secretion from parietal cells is inhibited mainly due to pro-inflammatory cytokines IL-1β and TNF-α[45,46]. TNF-α and IL-1β are essential in the initiation of chronic inflammation. Recent works have shown that IL-1β overexpression, in the absence of *Helicobacter* infection, is sufficient to cause gastric cancer and it is one of the essential proinflammatory cytokines modulated during *H. pylori* infection that directs the mucosa toward atrophy, metaplasia, and neoplastic transformation[47-49]. Another important point that should be added is that *H. pylori* has been consistently associated with higher risk of gastric noncardia cancer. The inverse association of *H. pylori* with gastric cardia cancer or esophageal adenocarcinoma has been shown in several studies, especially in Western populations[50]. Furthermore, mast cells in particular play an important role in attracting inflammatory cells by releasing inflammatory mediators. Monocytes differentiate into macrophages, and become activated in response to local chemokine and cytokine interactions[51]. Also, the correlation between tumor-associated macrophage abundance and poor prognosis has been shown[52]. Furthermore, macrophage-deficient mice display reduced progression of tumors to a more malignant phenotype[53]. Recently, direct evidence has also linked IL-6 to inflammation-mediated tumor initiation and proliferation in colon cancer[54]. IL-6 can inhibit dendritic cell maturation and, together with the NF-κB-activating cytokines IL-1 and TNF, can promote tumor progression. Cytokines also affect cell death and cell cycle pathways[55,56]. TNF-α is produced mainly by macrophages. It is also produced by tumor cells. TNF-α is associated with tissue destruction and plays a role in destroying tumor blood supply. However, if it is produced chronically, it can act as a tumor promoter by contributing to tissue remodeling and stromal development[57,58]. Nuclear factor (NF)-κB and STAT3 pathways have emerged as key regulators of the release of these pro-inflammatory cytokines, and important mediators of both tumor proliferation and persistence of chronic inflammation. The activation of these pathways results in further cytokine release[57,59,60].

Activation of the innate immune system is followed by the adaptive immune response. Th1 response and its accompanying mediators (IFN-γ) are not only necessary for *Helicobacter*-induced inflammation but also for the development of atrophy or metaplasia and SPEM; however a Th2 response and its mediators (i.e., IL-4) appear to be protective. The presence of a Th1, rather than a Th2, immune response is also associated with better survival in gastric cancer patients[36].

Although the subsequent pathways are different, chronic inflammation is the first step in both the intestinal and the diffuse type of gastric cancer. While the intestinal type has a sequence of multifocal atrophic gastritis, IM and dysplasia, which advances to carcinoma, the diffuse type tends to be primarily genetic in origin[61,62]. The progress from IM to gastric cancer has a wide range of molecular alterations affecting transcription factors, such as CDX1 and CDX2, telomerases, microsatellite instability, mutations of p53 protein, overexpression of COX-2, cyclin D2, and decreased expression of p27[63]. The next step is gastric dysplasia. During the progression of normal tissue through the metaplasia-dysplasia sequence, there are mutations in genes including *p53*, also loss of heterozygosity of the adenomatous polyposis coli gene, overexpression of the antiapoptotic gene *bcl-2* and a mixture of polyploidy and aneuploidy[63].

Inflammation also plays an important role in the ability of tumor cells to invade and metastasize. The ability of epithelial tumor cells which metastasize to express specific chemokine receptors has been shown[64]. Paracrine secretion of pro-inflammatory cytokines (i.e., IL-1β, IL-6, TNF-α) and certain autocrine cytokine production support this process[65]. During the later stages, additional mutations can be acquired, and this leads to the cancer cell gaining a further growth advantage and acquiring a more malignant phenotype[66,67].

THERAPEUTICS AND OUTCOME

In recent studies investigators have found out that *K-ras* activation resulted in an inflammatory response and enhanced the expression of COX-2 in the glandular stomach. COX2 is upregulated in the gastric epithelium and in the infiltrating inflammatory cells in the stomach during gastritis[68-70]. Furthermore, it has been shown that sulindac, a nonsteroidal anti-inflammatory drug, suppresses the progression of gastric cancer in mice[71]. Hence, a *K-ras* activation-induced inflammatory response may facilitate the formation of IM and promote the progression of gastric cancer.

SPEM is associated more commonly with gastric cancer than IM[72,73]. It can be defined as a corpus lesion. Nevertheless, IM and SPEM often occur together[74,75]. Increase in mucus and loss of mature parietal and chief cells in humans correlates with SPEM (Figure 2)[73]. SPEM is characterized by expression of TFF2 (spasmolytic polypeptide) which is normally a product of mucous neck cells and antral gland cells[72]. SPEM also arises from a second proliferative zone at the bases of metaplastic fundic units, either by transdifferentiation of chief cells or activation of an unknown basal crypt progenitor[76,77]. However, it is not clear whether these cells are related to the gastric progenitor cells[78].

It must be noted that an important factor for the development of gastrointestinal cancers is peritumoral stro-

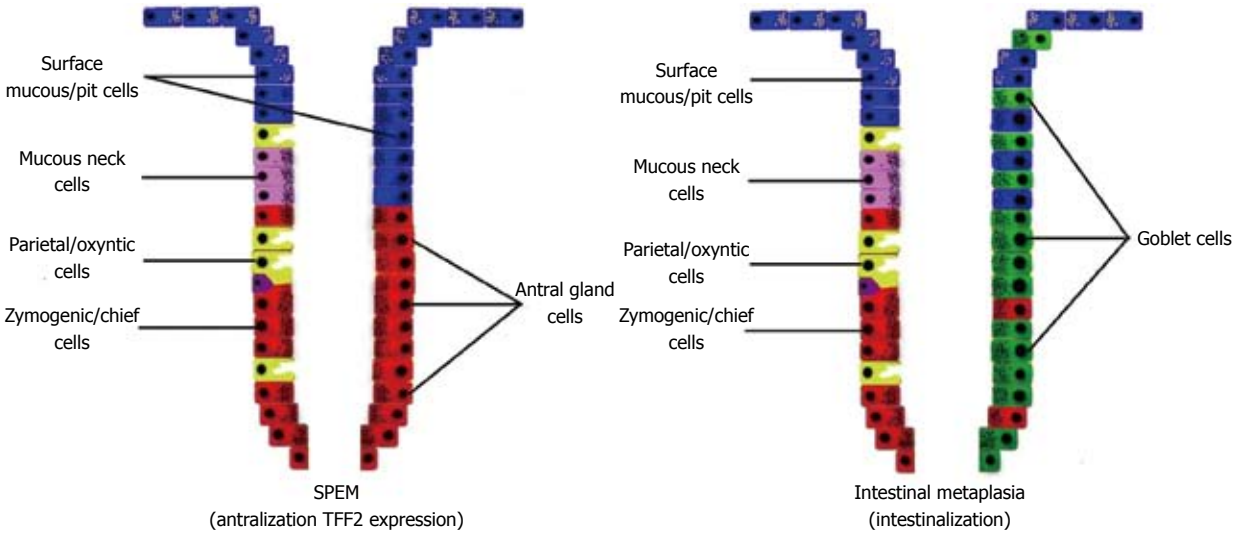

Figure 2 Schematic explanation of spasmolytic polypeptide-expressing metaplasia and intestinal metaplasia. SPEM: Spasmolytic polypeptide-expressing metaplasia; TFF-2: Trefoil family factor 2.

ma. Activated fibroblasts within the stroma can help to create an environment containing vessels and infiltrating inflammatory cells and it is the interaction between these different cell types which is permissive of tumor growth, angiogenesis, and invasion[79-81].

The question that must be answered is: what is the initiating cellular event in gastric metaplasia? The interpretation that the metaplasia is an intermediate step in the development of gastric cancer may be facile, because different types of IM have different degrees of association with malignancy, and early stage gastric cancers can arise in nonintestinalized epithelium[82-84]. Investigators have reported that solid cancers might originate from differentiated cells and they have reported the possible existence of cancer stem cells (CSCs) or tumor initiating cells in solid malignant tumors[85,86]. However, based on the assessment of the differentiation status of tumor cells, they appear to deviate little from their normal progenitors and to show similar differentiation programs. Studies on tissues undergoing continuous cell renewal suggest that cancer cells may originate from a stem cell compartment[87]. The origin of human gastric CSCs has yet to be elucidated, but data obtained from a mouse model of *Helicobacter*-induced gastric cancer have implicated bone marrow-derived cells as a potential candidate. Further studies focusing on the identification and characterization of CSCs in gastric cancer may lead to novel diagnostic and therapeutic tools, dramatically improving the prognosis of gastric cancer patients.

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REVIEW

KRAS mutation testing in metastatic colorectal cancer

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Abstract

The *KRAS* oncogene is mutated in approximately 35%-45% of colorectal cancers, and *KRAS* mutational status testing has been highlighted in recent years. The most frequent mutations in this gene, point substitutions in codons 12 and 13, were validated as negative predictors of response to anti-epidermal growth factor receptor antibodies. Therefore, determining the *KRAS* mutational status of tumor samples has become an essential tool for managing patients with colorectal cancers. Currently, a variety of detection methods have been established to analyze the mutation status in the key regions of the *KRAS* gene; however, several challenges remain related to standardized and uniform testing, including the selection of tumor samples, tumor sample processing and optimal testing methods. Moreover, new testing strategies, in combination with the mutation analysis of *BRAF*, *PIK3CA* and loss of *PTEN* proposed by many researchers and pathologists, should be promoted. In addition, we recommend that microsatellite instability, a prognostic factor, be added to the abovementioned concomitant analysis. This review provides an overview of KRAS biology and the recent advances in *KRAS* mutation testing. This review also addresses other aspects of status testing for determining the appropriate treatment and offers insight into the potential drawbacks of mutational testing.

Key words: *KRAS*; Epidermal growth factor receptor; Metastatic colorectal cancer; Testing status; Biomarker

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers worldwide. In the United States, approximately 102 900 cases of colon cancer and 39 670 cases of rectal cancer were diagnosed in 2010, and approximately 51 370 patients died of CRC in the same year, accounting for about 9% of all cancer deaths[1]. With the emergence of two anti-epidermal growth factor receptor (EGFR)-targeted antibodies, cetuximab (Erbitux) and panitumumab (Vectibix), the treatment of metastatic CRC has entered into the era of personalized treatment. Of the two antibodies, one is a human-mouse chimeric IgG1 monoclonal that was approved by the United States Food and Drug Administration (FDA) in 2004 as a second-line treatment of CRC; the other is a human IgG2 k monoclonal antibody that was approved by the FDA as a third-line drug in 2007. However, EGFR, the target of these drugs, which is overexpressed in approximately 80% of colorectal carcinomas, failed to predict a therapeutic response when used clinically[2,3]. Therefore, downstream signaling effectors were sought to help predict the efficacy of anti-EGFR treatment. The *KRAS* gene, which has

been extensively studied for more than three decades, has been demonstrated to be a strong negative predictive biomarker to indicate whether a CRC patient will respond to anti-EGFR treatment. As the target treatment may also be toxic and expensive, *KRAS* mutation status detection has become a crucial diagnostic factor for treating metastatic CRC patients.

KRAS GENE AND ITS ROLE IN EGFR SIGNALING

The *RAS* gene was initially identified as a viral gene homologous to the transforming gene from the Kirsten rat sarcoma virus[4,5]. Mutations in *RAS* are found in approximately 30% of all human cancers, making it one of the most commonly mutated genes in cancer[6]. The KRAS protein, also called p21, is a member of the *Ras* superfamily of proteins, is located on human chromosome 12 and encoded by 189 amino acids, and contains four coding exons and a 5' non-coding exon[7]. KRAS is a membrane-anchored guanosine triphosphate/guanosine diphosphate (GTP/GDP)-binding protein and is widely expressed in most human cells. As a small GTPase (GTP cleaving enzyme), KRAS is involved in intracellular signal transduction and mainly responsible for EGFR-signaling activation. The exchange of the active GTP-bound state and the inactive GDP-bound state is tightly controlled by GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors[8]. Under normal physiological conditions, upstream signals activate wild-type *KRAS* by promoting the exchange of bound GDP for GTP. This process is transient because of GAP-mediated GTP hydrolysis. However, this process becomes altered when the *KRAS* gene is mutated.

Mutant *KRAS* is found in about 35%-45% of CRCs[9-15], and codon 12 and 13 are two hotspots, which account for about 95% of all mutation types, with approximately 80% occurring in codon 12 and 15% in codon 13. Other mutations in codons 61, 146 and 154 occur less frequently in CRC, accounting for 5% of all mutation type[16]. Referring to the Catalogue of Somatic Mutations in Cancer Database, more than 5000 mutations have been found in the *KRAS* gene in CRC samples.

KRAS mutations are almost single nucleotide point mutations as reported, and the most common patterns are G12D, G12A, G12R, G12C, G12S, G12V and G13D. In the codon 12 mutation, p.G12D, pG12V is the most frequent, and in codon 13, the substitution of glycine for aspartate (p.G13D) is the most frequent[17].

These mutations impair the intrinsic GTPase activity of KRAS and prevent GAPs from promoting GTP hydrolysis by KRAS, therefore causing KRAS proteins to accumulate in the GTP-bound, active form. In this manner, mutant *KRAS* results in a constitutively active GTP-bound state and the activation of downstream proproliferative signaling pathways[18,19]. Therefore, *KRAS* mutations play a critical role in human tumorigenesis and are the most prevalent in pancreatic, thyroid, colorectal and lung cancers.

SIGNIFICANCE OF *KRAS* MUTATION TESTING

KRAS as a prognostic factor

It has been suggested that prognostic and predictive factors should be clarified; the former (including traditional clinical markers like lymph node involvement, the histological grade of the tumor, and molecular biomarkers, *etc*.) often refers to the outcome of the natural history of the tumor, while the latter predicts the response to the therapies. Until recently, the prognostic value of *KRAS* mutation was in dispute. Two canonical trials have demonstrated that the *KRAS* mutation may be prognostic of treatment outcomes for patients with CRC. The Kisten Ras in Colorectal Cancer Collaborative Group Study (RASCAL study)[20], with 2721 patient samples collected from 13 different nations, indicated that the presence of a *KRAS* mutation increased the risk of recurrence and death, especially in a guanine (G) to thymine (T) mutation. Moreover, the expanded RASCAL Ⅱ study suggested that the prognostic role of the *KRAS* mutation, limited only to a glycine to valine mutation, was found in 8.6% of all patients and had a statistically significant effect on failure-free survival [P = 0.004, hazard ratio (HR) 1.3] and overall survival (OS) (P = 0.008, HR 1.29)[21]. However, in a translational study of PETACC3[22], a randomized phase Ⅲ trial showed that the *KRAS* mutation status does not have major prognostic value in stages Ⅱ and Ⅲ colon cancer. The difference in results may be largely due to the difference in sample size. The results from other trials are also not consistant[23].

KRAS as a predictive factor

Because *KRAS* is the most frequently mutated factor downstream of the EGFR signaling pathway, it was considered a candidate molecular biomarker for anti-EGFR therapy. In 2006, for the first time, the predictive value of *KRAS* was validated in a study by Lièvre *et al*[24] in which the *KRAS*-mutated patients showed no response to cetuximab and had a poorer OS compared with the wild-type *KRAS* patients. Later, a series of single-arm studies confirmed this result[25-29]. Then, not only cetuximab but also panitumumab were demonstrated to only be effective for wild-type *KRAS* patients[30,31]. These trials demonstrated that the outcomes of patients with wild-type *KRAS* were clearly better than those of the *KRAS*-mutant patients, although these were all retrospective analyses. The publication of two large, multicenter, randomized phase Ⅲ clinical trials unequivocally demonstrated the predictive value of *KRAS* for anti-EGFR therapy (Table 1). In these two trials, panitumumab or cetuximab *vs* best supportive care (BSC) was given to patients with chemorefractory CRC compared with BSC alone. Amado *et al*[10] demonstrated that the response rate of panitumumab was 17% and 0% for the wild-type *KRAS* group and the mutant group, respectively (P < 0.0001). In addition, when combined with chemotherapy [5-fluorouracil, leucovorin and irinotecan (FOLFIRI) or 5-fluorouracil, leucovorin and oxaliplatin], anti-EGFR

Table 1 Predictive value of *KRAS* for anti-epidermal growth factor receptor therapy in metastatic colorectal cancer

Reference	Regimen	Treatment line	Phase	*n*	Mutation status (%)	Method	Remarkable results
Monotherapy							
Karapetis *et al*[9], 2008	Cetuximab *vs* BSC	Chemotherapy refractory	III	394	42.3	Sequencing	Cetuximab alone works on patient with WT KRAS tumors
Amado *et al*[10], 2008	Panitumumab *vs* BSC	Chemotherapy refractory	III	427	43	Allele-specific PCR (DxS, United Kingdom)	Panitumumab alone works on patient with WT KRAS tumors
Combined with chemotherapy							
Van Cutsem *et al*[11], 2009	Cetuximab + FOLFIRI, FOLFIRI	First-line CRYSTAL trial	III	540	35.6	PCR clamping and HRM (TIB MolBioL, Germany)	Cetuximab plus FOLFIRI, reduced the risk of progression of metastatic colorectal cancer
Bokemeyer *et al*[12], 2009	Cetuximab + FOLFOX, FOLFOX	First-line, OPUS trial	II	233	42	PCR clamping and HRM (TIB MolBioL, Germany)	Significantly increased ORR in patients with WT KRAS tumors
Peeters *et al*[13], 2010	Panitumumab + FOLFIRI FOLFIRI	Second-line	III	1083	45	Allele-specific PCR (DxS, United Kingdom)	Significantly improved PFS in patients with WT KRAS tumors
Douillard *et al*[14], 2010	Panitumumab + FOLFOX FOLFOX	First-line	III	1096	40	Allele-specific PCR (DxS, United Kingdom)	Significantly improved PFS in patients with WT KRAS tumors
Van Cutsem *et al*[15], 2011	Cetuximab + FOLFIRI, FOLFIRI	First-line	III	1063	37	PCR clamping and HRM (TIB MolBioL, Germany)	Significantly improved OS in patients with WT KRAS tumors

BSC: Best supportive care; WT: Wild type; ORR: Overall response rate; FOLFIRI: 5-fluorouracil, leucovorin and irinotecan; FOLFOX: 5-fluorouracil, leucovorin and oxaliplatin; PFS: Progression-free survival; OS: Overall survival; PCR: Polymerase chain reaction; HRM: High-resolution melting.

antibodies (cetuximab or panitumumab)-treated patients had a better response rate and progression-free survival (PFS) or OS alone in the wild-type *KRAS* group, regardless of the treatment line[11-15]. Recently, better OS (median, 23.5 mo *vs* 20.0 mo; HR 0.796, $P = 0.0093$) was found in the cetuximab plus FOLFIRI-treated wild-type *KRAS* patients compared with the FOLFIRI-treated KRAS-mutated patients[15]. According to a recent meta-analysis of 11 studies conducted between 1966 and 2010[32], the *KRAS* status and the adding of anti-EGFR antibodies to standard chemotherapy were closely related to PFS [95% confidence interval (CI): 57%-90%, $P = 0.005$] and response rate (95% CI: 8.22%, $P < 0.001$).

On the basis of these results, National Comprehensive Cancer Network (NCCN), American Society of Clinical Oncology (ASCO) and European Medicines Evaluation Agency recommended testing for *KRAS* gene mutations in advanced CRC patients. The NCCN added *KRAS* testing to their 2009 clinical practice guidelines for colon and rectal cancers[33,34] and stipulated that only patients with wild-type (normal) *KRAS* genes should receive treatment with cetuximab (Erbitux) or panitumumab (Vectibix). The ASCO, in the same year, proposed a provisional clinical opinion (PCO)[35] demonstrating that testing for *KRAS* mutations should be performed prior to anti-EGFR monoclonal antibody therapy and that patients with KRAS mutations in either codon 12 or 13 should not receive this therapy as part of their treatment. This recommendation is slightly different from the NCCN guideline because the use of anti-EGFR therapy in the *KRAS*-mutated patients may be toxic.

KRAS TESTING STATUS

Frequency of testing

In a recent three cross-sectional survey performed in Europe, Latin America and Asia[36], physicians completed questionnaires on four patients per year. An analysis of 3800 samples per year showed that the *KRAS* testing frequency in metastatic CRC patients increased from 3% in 2008 to 47% in 2009 and 69% in 2010. It appears that the importance of *KRAS* mutation testing has become progressively understood by physicians and oncologists. Because implementation of the testing in the clinical practice has begun, it is essential to identify testing performance, as there are no set criteria for the process of *KRAS* detection, i.e., the selection of tissue specimens, specimen preparation, the timing of testing and the best method.

External quality assessment

A *KRAS* external quality assessment protocol was established in 59 laboratories throughout eight different European countries[37]. In the first assessment round, the results were unsatisfactory. The samples, including unstained sections of 10 invasive CRC with a known *KRAS* mutation status, were tested by each laboratory using their own preferred method for histological evaluation, DNA isolation, and mutation analysis. The test results were centrally validated by one of two reference laboratories. Only 70% of the laboratories correctly identified the *KRAS* mutational status in all samples, and the reports often lacked essential information. In another quality assessment for *KRAS* testing in Italy, five CRC specimens with known *KRAS* mutations were sent to be tested in 59 centers[38]. The limit to pass the assessment was set at 100% true responses. Only two centers failed in both the first round and the second round of testing. In Canada, until recently, there has been no such quality assessment. However, a guideline was developed according to a Canadian consensus conference held in Montreal in April 2010, in which the expert group provided recommenda-

tions on *KRAS* testing in the treatment of CRC[39]. In the United States, there is currently no FDA-approved standardized test. However, the PCO provided recommendations to the *KRAS* testing clinics. In Asia, there has been no external quality assessment system as yet, and it is critical to fulfill this objective.

Mutation status

As reported in 2011, the *KRAS* mutation frequencies in Asia, Europe, Latin American were 24%, 36% and 40%, respectively ($P < 0.0001$)[36]. It is unclear why a lower incidence is observed in Asian patients. In China, *KRAS* mutations were detected in 33.3% (30/90) of the CRC tumor samples using the nucleotide sequence analysis method[40]. These results significantly correlated with the response rate and survival time of cetuximab-treated patients. The difference of mutation status may result from many aspects, such as the tissue, the percent of tumor cells, the extracted DNA quality, the testing methods and the testing target.

Testing target

Currently, in most of the *KRAS* detection methods, only mutations of codon 12 or 13 are certified as informative for selecting non-responders to the anti-EGFR treatment in large clinical trials[15]. Therefore, mutation analysis of these sites is recommended. However, recent research has revealed new findings. Mutations in exons 3 and 4 are also effective in predicting the efficacy of EGFR-antibodies[41,42]. Codon 61 was found to account for 2% of all *KRAS* mutations and, similar to some of the codon 12 mutations, had predictive value[43]. Therefore, codon 61 may be useful in *KRAS* mutation testing. In contrast, not all mutations in codon 13 appear to be informative. In a recent analysis, cetuximab surprisingly worked on patients with chemotherapy-refractory CRC with p.G13D-mutated tumors, and these patients have a longer overall and PFS compared with those with the *KRAS*-mutated tumors[44]. Therefore, efforts are still required to confirm the importance of various mutations of the *KRAS* gene.

Sample selection

The most widely used tissue for *KRAS* testing is formalin-fixed paraffin-embedded (FFPE) tissue blocks[45], which are easy to obtain and convenient to preserve. However, DNA extracted from FFPE is time consuming and may be of poor quality, which can also result in false-positive or false-negative results due to an incomplete tissue fixation or tissue overfixation. Another specimen type is frozen tissue. Studies that compared the mutation detection rates in frozen and FFPE samples from the same tissue have found that the mutation rate in frozen samples is higher than that detected in FFPE samples[46]. The use of frozen tissue is suggested to be the gold standard for analysis, but the associated expense and technical difficulty of using frozen tissue make this method unsuitable for routine testing. In contrast, a high concordance was observed between primary tumors and metastatic locations (91.7%-96.4%)[47-49]. Therefore, the *KRAS* status in a primary site can be used for selecting patients who would benefit from anti-EGFR therapy. However, *KRAS* status can be heterogeneous within a primary tumor, and thus, different parts of such tumors should be examined to accurately predict the *KRAS* status in metastatic lesions.

Beyond the selection of tissue, other choices, such as peripheral blood, have been studied. Yen *et al*[50] detected circulating tumor cells with *KRAS* oncogenes using membrane arrays; *KRAS* mutations were identified in 39.5% (30/76) of peripheral blood samples, which is similar to that in tumors (43.4%). According to a review concerning the validation of *KRAS* mutation testing in CRC blood samples which summarizes the studies that detect *KRAS* status using tissue or plasma/serum[51], a positive *KRAS* mutation in plasma or serum suggests a *KRAS* mutation in the tumor whereas the absence of a *KRAS* mutation in the plasma or serum does not necessarily prove a lack of a similar mutation in the CRC tumor tissue. Further studies are needed in this field.

Methods

A number of methods can be used in *KRAS* mutation testing, with different sensitivity, turnaround time, and cost. In the NCCN guideline or ASCO PCO, no explicit method was assigned. Therefore, the use of assays worldwide is somewhat chaotic. In the Italian quality assessment for *KRAS* testing[38], five CRC specimens were sent to 59 centers, which were asked to use their own preferred method for DNA extraction and mutational analysis. Of these 59 centers, polymerase chain reaction (PCR) sequencing was the predominant method for mutational analysis, as 48 (81.3%) centers used this methodology. Among the remaining centers, 5 centers (8.5%) used pyrosequencing, 3 centers (5.1%) used Real-Time PCR (Therascreen kit), 2 centers (3.4%) used restriction fragment length polymorphism (RFLP) analysis and 1 center (1.7%) used the KRAS strip assay. In the United States, the amplification refractory mutation system was used by most laboratories[52].

The traditional methods used for mutation testing are hybridization and DNA sequencing. These methods are complex and time consuming. The emergence of polymerase chain reaction (PCR) sheds new light on this field. Currently, mutation testing methods are almost exclusively based on this technology, including PCR-based sequencing, high resolution melting analysis (HRMA), amplification refractory mutation system (ARMS), and cleaved amplification polymorphism sequence-tagged sites (PCR-RFLP).

Among these methods, DNA sequencing, also called Sanger sequencing or dideoxy sequencing, is considered the gold standard because this methodology analyzes the DNA sequence nucleotide by nucleotide and can identify all possible mutations in the analyzed *KRAS* gene segment, including base substitutions, insertions and deletions. However, this approach has a low sensitivity of about 20%, and is laborious and time consuming. An alternative approach to this methodology, i.e.,

Table 2 Methods used for *KRAS* mutation testing[45,55,60-62]

Method	Sensitivity (mutant/wild-type) (%)	Turnaround time	Main advantages	Main disadvantages
Sanger sequencing	20–30	Slow (4 d to 2 wk)	Detects all possible mutations, cost-effective	Insensitive, time consuming, open PCR system is easily contaminated
Pyrosequencing	5	Rapid	Detects all possible mutations, sensitive	Open PCR system is easily contaminated
Real-time PCR with HRMA	5	Rapid	Rapid, closed PCR system, detects all possible mutations (heterozygous and homozygous)	Occasionally difficult to distinguish between mutation types
Allele-specific real-time PCR	10	Rapid	Rapid, closed PCR system	Detects only the 7 most common mutations, requires more tissue for analysis compared with other methods
RFLP with sequencing	0.1	Slow (4 d to 2 wk)	Sensitive	Requires confirmation by sequencing, complicated
DxS (ARMS/S)	1	Rapid	Sensitive, time-saving	Expensive, detects specific mutations targeted by the designed primers
COLD-PCR with sequencing	1-2.5	Rapid	Sensitive, cost-effective, detects all possible mutations	-

ARMS: Amplification refractory mutation system; RFLP: Restriction fragment length polymorphism; PCR: Polymerase chain reaction; COLD-PCR: Coamplification at lower denaturation temperature PCR.

pyrosequencing, has a sensitivity proven to be approximately 5%-10% and has commercialized the detection of *KRAS* mutations; corresponding commercial kits, the PyroMark® (Qiagen, Valencia, CA, United States), have been developed[53,54].

Of the non-sequencing methods, ARMS[55], real-time PCR analysis with HRMA[56], RFLP[57] and allele-specific real-time PCR[58], most of which are based on real-time PCR technology, have been well studied in the past three years with Sanger sequencing as the reference, demonstrating the effectiveness and availability of these methods for *KRAS* status testing. A multicenter study[59], which evaluated six different *KRAS* mutation detection methods, including pyrosequencing, HRMA, dideoxy sequencing, and two commercial kits, showed a concordant *KRAS* status in 66/80 (83%) of frozen tissue samples and 71/74 (96%) of paraffin tissues using the five best performing assays. Each of the assays has its advantage and limitations, and as details have been described in previous publications, we have summarized some notable features in Table 2[45,55,60-62]. The HRMA assay, often based on real-time PCR, detects the mutant sequence through measuring changes in the melting of a DNA duplex with the aid of intercalating dyes. This method is fast and sensitive but has been reported to have a false-positive rate of 20%[63]. Therefore, this method requires sequencing confirmation and cannot show the concrete mutation pattern. The allele-specific Real-Time PCR and ARMS can only detect the limited mutation sites of the *KRAS* gene, which makes these methods less feasible in clinical practice. The ARMS-based commercial kit, TheraScreen® (DxS Ltd, Manchester, United Kingdom), however, has been widely used in laboratories[64]. This kit has a real-time PCR-based assay that combines the ARMS with Scorpion probes (seven probes for seven different mutations in *KRAS*), eliminating the need for post-PCR confirmation by direct sequencing, and is thought to be the most sensitive method until recently with a sensitivity of 1%[45].

Recently, more sensitive methods have been utilized in *KRAS* detection. One method is the PCR-clamp assay, and the other is coamplification at lower denaturation temperature PCR (COLD-PCR). The PCR-clamp assay utilizes mutation-specific hybridization probes and another wild-type-complementary peptide nucleic acid probe to suppress the amplification of the normal sequence and can detect less than 1% of the allele[65,66]. A commercial kit (KRAS LightMix) by TIB MolBiol (Berlin) uses this technology and a melting curve analysis and has been used in multicenter, phase Ⅲ clinical trials in which patients were treated with the anti-EGFR antibody, cetuximab[11,12,15]. COLD-PCR is another selective amplifying system that enriches the "minority alleles" from the mixed DNA sequences based on the lower melting temperature of mutant homoduplexes as compared with wild-type ones. Therefore, in COLD-PCR, the denaturation temperature is set at 80 ℃ whereas the denaturation temperature in conventional PCR is approximately 94 ℃. Using this principle, this technology does not require special equipment or reagents or time-consuming procedures. As a sensitive DNA enrichment method, COLD-PCR is often followed with HRMA or pyrosequencing. Mancini *et al*[67] demonstrated that COLD-PCR combined with HRM can improve the limit of detection of *KRAS* and *BRAF* mutations in CRC, increasing the percentage of mutated CRCs from 40% (47/117) to 48.7% (57/117) compared with traditional PCR and direct sequencing. In another study by Zuo *et al*[68], COLD-PCR combined with pyrosequencing detected all the mutations in 50 samples, including DNA extracted from either fresh or FFPE tissue specimens that were confirmed positive by conventional PCR, and the mutation detection sensitivity was certified as 1.5%.

In addition, COLD-PCR combined with HRMA

assay does not require expensive and time-consuming procedures; thus, in clinical settings, this procedure has the potential to be used to select those patients who are eligible for EGFR-targeted therapies.

Our recommendation

Currently, it is accepted that the DNA fragmentation caused by improper fixation, heterogeneous somatic *KRAS* gene mutations, and the influence of stromal cells can cause false-positive *KRAS* mutation testing results. Fortunately, the technique refinements and sufficient tissue selected can reduce this limitation. It is suggested that at least 300 tumor cells or 30 ng of template DNA are required for *KRAS* status analysis. However, the appropriate method to extend to the clinic is still unclear. Molinari *et al*[69] found that highly sensitive methods could improve the accuracy of predictions of anti-EGFR monoclonal antibody efficacy. Therefore, assay sensitivity when detecting *KRAS* mutations is a key issue for correctly analyzing tumor specimens. However, Carotenuto *et al*[70] demonstrated that in samples with more than 30% tumor cells, the DxS assay and PCR-sequencing, which are the most sensitive and non-sensitive methods, respectively, showed no difference in identifying *KRAS* mutations. Therefore, more effective and sensitive methods are required for inconclusive samples and those with a low number of tumor cells. Upon considering the sensitive detection methods, as previously described, pyrosequencing is a new, robust but expensive technology. The DxS assay (ARMS/S) is now widely used in clinical labs but can only detect the seven common mutations, and it is costly. COLD-PCR, which can enrich the mutant alleles, is considered a simple method that increases *KRAS* testing sensitivity. Therefore, we recommend the use of this assay combined with HRM or sequencing for determining *KRAS* status; although, this approach should be validated by further large sample studies.

CONCOMITANT ANALYSIS WITH OTHER FACTORS

Unfortunately, *KRAS* mutations account for approximately 35% of the nonresponsive patients that receive anti-EGFR treatment[35]. Therefore, using *KRAS* as a predictor of clinical outcomes is not always useful. These results have led researchers back to the molecular mechanisms of cetuximab and panitumumab resistance to find other powerful prognostic markers. *BRAF*, which is another member of EGFR signaling cascade, is located downstream of *KRAS* and is considered the most promising marker for predicting anti-EGFR treatment resistance apart from *KRAS* gene. *BRAF* mutations mainly occur at exon 15 with a frequency of approximately 5% to 10% and the common V600E pattern. It is notable that *BRAF* and *KRAS* mutations are mutually exclusive ($P < 10^{-6}$)[71]. Therefore, *BRAF* mutation analysis is recommended when the *KRAS* gene is the wild type. Di Nicolantonio *et al*[72] found in a retrospective study that none of the *BRAF*-mutated patients responded to cetuximab or panitumumab and that none of the responders carried *BRAF* mutations ($P = 0.029$). In addition, *BRAF*-mutated patients had a significantly shorter PFS ($P = 0.011$) and OS ($P < 0.0001$) compared with wild-type patients. On the basis of these results, the NCCN clinical guidelines in 2010 currently recommend *BRAF* mutational status assessment of metastatic CRC patients with a wild-type *KRAS* to guide the therapeutic use of cetuximab and panitumumab.

Apart from the *KRAS* and *BRAF* gene mutations, other genetic aberrations, such as *PIK3CA* and *PTEN*, were demonstrated to be helpful in predicting the resistance to anti-EGFR treatment[40,73]. In addition, many oncologists and pathologists have proposed that combining the analysis of these factors simultaneously will provide a clearer overall prognostic indication for EGFR inhibitor status. The recent data from a retrospective analysis demonstrated that when the loss of *PTEN* expression and mutations of *KRAS*, *BRAF* and *PIK3CA* are concomitantly ascertained, as many as 70% of the metastatic CRC patients can be identified as unlikely to respond to anti-EGFR therapies[74]. Therefore, CRCs lacking alterations in *KRAS*, *BRAF*, *PTEN* and *PIK3CA*, which may have the highest probability of response to anti-EGFR therapies, are defined as "quadruple negative"[74,75].

In addition, in a retrospective consortium analysis[43], the largest series to date according to our knowledge, the effects of *KRAS*, *BRAF*, *NRAS* and *PIK3CA* mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal was studied. In total, 1022 tumor DNA samples were tested, of which 40.0% (299/747) harbored a *KRAS* mutation, 14.5% had a *PIK3CA* mutation, 4.7% had a *BRAF* mutation, and 2.64% *NRAS* mutation, and carriers of the four mutations had a lower response rate to the cetuximab plus chemotherapy treatment compared with those lacking any of the four mutations. A multivariate analysis also confirmed that if *KRAS* is unmutated, assessing the *BRAF*, *NRAS*, and *PIK3CA* exon 20 mutations provides additional information about patient outcomes. It is notable that while *NRAS* accounts for only 2.64% of these molecular alterations, this mutation is associated with unresponsiveness to panitumumab treatment.

It is obvious that *KRAS* mutational status analysis is insufficient for predicting the efficacy of anti-EGFR therapy, and adding the concomitant analysis of downstream factors can be helpful in selecting the correct patient for this personal treatment. In addition, we suggest that microsatellite inestability (MSI) be added to this concomitant analysis.

Microsatellite instability, defined as small deletions or expansions within short tandem repeats in tumor DNA resulted from the inactivation of the DNA mismatch repair system, has been found in up to 90% of the tumors of the hereditary nonpolyposis CRC and in approximately 20% of sporadic colorectal tumors[76,77]. Using a panel of 5 microsatellites recommended by the National

Cancer Institute, i.e., BAT 25 and BAT 26 (mononucleotide repeats), D2S123, D5S346 and D17S250 (dinucleotide repeats), CRC tumors are classified as MSI-high (MSI-H), MSI-low (MSI-L) and microsatellite stability (MSS), and the MSI-H was thought to indicate a more favorable prognosis[78]. However, with regard to predicting therapy response, the role of MSI is conflicting. Recently, some researchers have combined *KRAS* and MSI in their study[79] and found that both genes are prognostic of CRC. In another study[80], the combined analysis of specific *KRAS* and *BRAF* mutations, and microsatellite instability were used to identify prognostic subgroups of sporadic and hereditary CRC. As the result, 3 distinct prognostic subgroups were observed in univariate (P = 0.006) and multivariable (P = 0.051) analysis: group 1 consisted of patients with *KRAS G12D* or *G12V* or *BRAFV600E* mutations independent of MSI status; they had a poor survival time and suffered more patient deaths. Group 2 included patients with either wild-type *KRAS*/*BRAFV600E* or *KRAS* G13D mutations in the MSS/MSI-L tumors and had a more favorable outcome. Finally, the patients with MSI-H cancers and simultaneous *G13D* mutations were observed to have the worst outcomes. The survival times for groups 1-3 varied significantly (P = 0.006). Therefore, we recommend the concomitant analysis of *KRAS, BRAF, PIK3CA,* and *PTEN* combined with MSI, which can facilitate selecting the appropriate patients for anti-EGFR treatment while also indicating the outcome of CRC patients.

CONCLUSION

KRAS, an important member of the EGFR signaling cascade, can acquire activating mutations in codons 12 and 13 of exon 2 in approximately 35%-45% of the CRC cases, rendering EGFR inhibitors ineffective. Though the prognostic value of *KRAS* is conflicting, it is a promising predictive biomarker of personalized treatment. Numerous clinical trials have clarified the significant benefit of outcomes in patients with wild-type *KRAS* for anti-EGFR therapy, despite the treatment line. Therefore, *KRAS* status testing has been recommended by national organizations, including NCCN, American Society for Clinical Oncology and European Medicines Agency. In recent years, *KRAS* testing is administered with a high frequency; however, standards are desired worldwide, including the selection and processing of the tumor sample and the choice of the appropriate detection method, which may affect the accuracy of the testing results. COLD-PCR is a simple assay that can increase *KRAS* testing sensitivity by enriching the mutant alleles. This technology combined with HRM or sequencing is potentially useful in *KRAS* detection in a clinic practice. In addition, concomitant analysis with other factors, such as BRAF, PIK3CA, PTEN and MSI, is helpful in supporting *KRAS* as predictive and prognostic factors, but further efforts are needed prior to implementation.

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ORIGINAL ARTICLE

Effect of double-balloon enteroscopy on pancreas: An experimental porcine model

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Abstract

AIM: To evaluate the effect of double-balloon enteroscopy (DBE) on pancreas histology and levels of pancreatic enzymes.

METHODS: Conventional upper gastrointestinal endoscopy was performed on five control pigs. Oral DBE was performed with an EN-450T5 enteroscope on 20 pigs. Two experimental groups (10 pigs each) were defined according to DBE duration: 90 min for Group 1 and 140 min for Group 2. During oral insertion, the balloons were not inflated in the descending part of the duodenum to avoid the minor duodenal papilla. Serum amylase, lipase and C-reactive protein (CRP) levels were monitored before the procedure and repeated every 30 min until the exploration was finished, as well as 24 h and 7 d after. After the procedure and for a total of 7 d, the pigs were observed twice a day for signs of decreased activity, irritability, vomiting or anorexia. Gross and microscopic examination of the pancreas was performed on day 7.

RESULTS: All animals tolerated DBE without clinical manifestations of acute pancreatitis. Experimental groups had higher levels of enzymes than the control group at 24 h. Throughout the exploration, the amylase levels increased significantly above the baseline 24 h after DBE, although the increase was not statistically significant and did not reach 20% of the baseline. An increase in lipase and CRP was observed at 24 h after the procedure, although by day 7, all enzymatic levels had returned to baseline. No differences between Groups 1 and 2 were found for any enzyme and sampling site during and after the procedure. Similarly, no correlation between insertion depth and enzyme levels was observed. Direct *in situ* and post-removal inspection of the pancreas did not show any evidence of fluid collection, abscesses or hemorrhage. Histological examination of the pancreas from Groups 1 and 2 revealed the existence of focal areas (0.14-0.26 mm^2) of ischemic necrosis in 47.4% of the animals. In the pigs with damaged pancreas, the left lobe (tail) was always affected. However, this only happened in 83.3% of the samples from the right lobe (head) and in 33.3% of the samples from the body of the pancreas. Significant differences were found between the left lobe (tail) and the body for the percentage of affected pancreas. Both the size of the lesions and the percentage of affected pancreas were higher in the left pancreatic lobe (tail). The presence of the lesions was not related to the exploration length.

CONCLUSION: The increase in pancreatic enzymes

after DBE could be related to focal points of pancreatic ischemic necrosis due to mechanical stress.

Key words: Endoscopy; Pancreatitis; Double-balloon enteroscopy; Experimental study; Pig model

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INTRODUCTION

Double-balloon enteroscopy (DBE) has enabled endoscopic diagnosis and treatment in the small intestine, which had been very difficult for many years[1]. Yamamoto *et al*[2] introduced DBE in 2001 and its usefulness is already recognized in many countries. DBE is considered a well-tolerated and safe endoscopic technique[3-8], but an increase in pancreatic enzymes and potential pancreatitis are recognized as complications directly attributed to the procedure[1,7,9-12].

The mechanism for post-DBE pancreatitis remains unclear. Potential explanations might include: (1) pancreatic duct obstruction by direct oppression of the papilla with the inflated balloon[7,9]; (2) reflux of intestinal fluid into the pancreatic duct owing to an increase in intraduodenal pressure because of mechanical strain[12,13]; or (3) prolonged mechanical injury or ischemia on the pancreas as a result of repeated stretching and shortening of the endoscope and overtube[7,14-16].

Unfortunately, levels of pancreatic enzymes several days after the procedure and evaluation of potential lesions in the pancreas under normal clinical conditions are unknown in humans. Also, to the best of our knowledge, there have been no studies in animal models to clarify the etiology of pancreatic hyperamylasemia and pancreatitis post-DBE. This study was aimed at determining the effects of the DBE technique on the pancreatic enzymes and histology under nonpathological conditions. To assess if the timing of DBE influences the pancreatic enzyme markers, two experimental groups with different DBE duration (90 or 140 min) were established.

MATERIALS AND METHODS

Animals and protocols

Twenty-five Large White pigs (35-40 kg) were used. The day before DBE, animals were fasted, with no liquid restrictions, and given a laxative preparation. Animals were prepared and anesthetized for the endoscopic procedures. Intravenous saline solution was administrated to secure basic hydration during the DBE procedure. After 24 h fasting, each pig was intramuscularly premedicated with diazepam 0.1 mg/kg, ketamine 10 mg/kg and atropine 0.01 mg/kg. General anesthesia was induced with propofol 2 mg/kg intravenously and maintained with sevofluorane 1.8%-2% delivered *via* an endotracheal tube. Animals from the control group (n = 5) underwent conventional upper gastrointestinal (GI) endoscopy. In the remaining 20 pigs DBE was performed with an EN-450T5 enteroscope (Fujinon, Japan) by experienced endoscopists. The exploration depth was estimated according to the methodology established by May *et al*[17].

Two experimental groups (10 pigs each) were defined according to DBE duration: 90 min for Group 1 and 140 min for Group 2. During the oral insertion of the scope and overtube, the balloons were not inflated in the descending part of the duodenum to avoid the major and minor duodenal papilla. Blood samples were taken before the procedure and during the exploration at 20 (control group only) 30, 60, 90 and 140 (Group 2 only) min, and also at 24 h and 7 d after DBE (Groups 1 and 2) and 24 h and 7 d after GI endoscopy (control group) to evaluate the serum concentrations of amylase, lipase and C-reactive protein (CRP). Animals were allowed to feed 24 h after DBE. After the procedure and for a total of 7 d, the pigs were observed twice a day for signs of decreased activity, irritability, vomiting or anorexia. On day 7, all the animals were euthanized with a pentobarbital overdose and the pancreases were removed. Each pancreas was examined *in situ*, palpated, removed from the cadaver and then sectioned to identify gross alterations. The right lobe (head) and left lobe (tail), as well as the body of the pancreas were preserved in 10% buffered formalin, trimmed into 1 cm × 1 cm × 1 cm tissue blocks (18-22 blocks per pancreas) and processed for histopathology after hematoxylin and eosin staining. Histology sections were studied under light microscopy (three fields per block of tissue) and when lesions were observed, the cross-sectional areas were measured with the SigmaScan Pro 5.0 program (Systat Software Inc., San Jose, CA, United States).

Ethical approval

All animals received humane care in compliance with the European Communities Council Directive (86/609/EEC). Protocols were approved by the local government Ethics Committee for Animal Research. The endoscopic equipment used was for research with animals only.

Statistical analysis

Data of enzyme levels were included in a spreadsheet and analyzed with SPSS 17.0 (SPSS, Chicago, IL, United States). Descriptive statistics were obtained and all the

Table 1 Comparison of serum enzyme levels between double-balloon enteroscopy (Group 1) and control group at different sampling intervals

	Amylase (U/L)	Lipase (U/L)	CRP (U/L)
t_0			
Control	1295.74 ± 120.55	14.66 ± 2.16	11.02 ± 3.92
DBE	2074.42 ± 296.82	17.74 ± 2.44	39.14 ± 13.02
t_{end}			
Control	1290.62 ± 158.58	9.38 ± 1.02	11.86 ± 3.8
DBE	2070.99 ± 281.31	8.84 ± 1.15	37.16 ± 12.1
$t_{24\ h}$			
Control	1311.02 ± 88.56	19.48 ± 3.6	51.46 ± 26.02
DBE	2487.18 ± 364.46[b]	26.73 ± 6.63	114.81 ± 31.84
$t_{168\ h}$			
Control	1474.42 ± 143.16	8.86 ± 2.21	20.36 ± 10.52
DBE	2337.9 ± 300.72	5.71 ± 0.51	78.07 ± 31.6

t_0: Samples before the procedure; t_{end}: Samples at the end of the procedure [gastrointestinal endocopy (GI) in control group and double-balloon enteroscopy (DBE) in experimental group]; $t_{24\ h}$: Samples 24 h after the procedure (GI in control group and DBE in experimental group); t_{168h}: Samples 7 d after the procedure (GI in control group and DBE in experimental group). [b]$P < 0.01$ *vs* control group.

variables tested for normality (Kolmogorov-Smirnov test) before being subjected to analysis of variance (ANOVA) (linear model with repeated measures). Within-subject factors were the different timing of blood sampling and the inter-subject factor was the duration of the exploration (90 or 140 min). Tukey and Bonferroni tests were used to ascertain *post hoc* differences. The possible association between the experimental groups and the presence of lesions in the pancreas was checked with the χ^2 test. In addition, the nonparametric Mann-Whitney test was used to ascertain any dependence between the size of the lesions and the portion of the pancreas.

RESULTS

Procedure evaluation

All the animals tolerated the procedure without any clinical manifestations of pancreatitis or distress. During the endoscopic exploration, passing the endoscope and the overtube into the duodenum was not difficult (< 3 min). Remnants of food in stomach did not make DBE more difficult. During the 7 d observation period after the procedure, the activity and dietary intake were normal in all the animals.

Estimations were calculated using the depth of insertion technique described by May *et al*[5]. The average insertion depth in Group 1 was lower than in Group 2: 268 cm (range: 209-336 cm) and 333 cm (range: 230-488 cm), respectively. Nevertheless, due to high data variability, the ANOVA for the insertion depth between the two groups was not significant ($P = 0.181$).

Biochemical evaluation

No statistical differences between sampling states in the control group were found. Experimental Group 1 had higher levels of enzymes than the control group at 24 h (Table 1). These differences were also present between the control group and Group 2.

Enzyme serum levels at the different sampling stages are displayed in Figure 1. To simplify notation, values at 30 and 60 min of the procedure are omitted because they were always similar to time 0.

All the animals had similar basal amylase levels before the procedure (approximately 2000 IU). Throughout the exploration, no significant changes in the amylase levels were noted (Figure 1A). However, the amylase levels increased significantly above the baseline 24 h after DBE, although the increase did not reach 20% of the baseline level. On day 7 after the procedure, the amylase level decreased progressively to the baseline, but it was still significantly higher in Group 1.

Lipase levels showed a variable trend during and after the exploration (Figure 1B). This is well illustrated in Group 1, where there was a significant decrease during the exploration, peak levels at 24 h after the procedure, and the lowest levels 7 d later.

CRP levels were significantly higher 24 h after DBE (more than twice the initial levels) (Figure 1C). However, CRP concentrations then decreased progressively towards the baseline, so no significant differences were found between the initial levels and 7 d after DBE.

No differences between Groups 1 and 2 were found for any enzyme and sampling site during and after the procedure. Similarly, no correlation between insertion depth and enzyme levels was observed (P for Pearson coefficient was always > 0.3).

Histology evaluation

Direct *in situ* and post-removal inspection of the pancreas did not show any evidence of fluid collection, abscesses or hemorrhage (Figure 2).

Light microscopy examination of tissue samples from the control group showed occasional small areas with infiltration and edema. However, the tissue samples from Groups 1 and 2 revealed the existence of limited areas of ischemic necrosis scattered throughout the parenchyma (Figure 3). This was observed in nine of the 19 pigs (47.4%). It should be noted that the histology samples from one pig were not included in the analysis due to bad processing. Some of the necrotic areas showed slight inflammatory cell infiltration around the sites of necrosis, but alterations to the pancreatic duct system were rare. In the nine pigs with damaged pancreas, the left lobe (tail) was always affected. However, this only happened in 83.3% of the samples from the right lobe (head) and in 33.3% of the samples from the body of the pancreas. The average area of lesions (μm^2) related to each portion was: right lobe (head): 198274 ± 23952, body: 136782 ± 24163 and left lobe (tail): 260516 ± 32819. The percentage (%) of affected pancreas in each portion was: right lobe (head): 1.83, body: 0.60 and left lobe (tail): 3.11. Although the overall differences were not statistically significant, the average values for both parameters were higher in the left

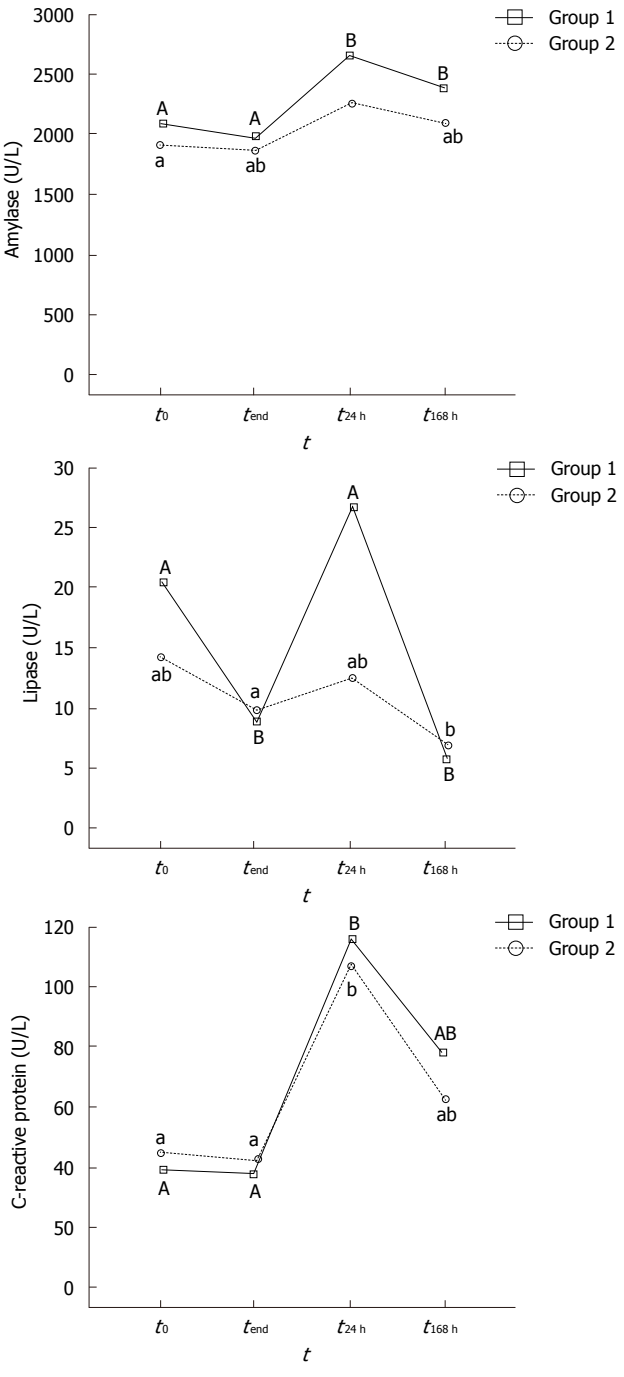

Figure 1 Plots representing the serum levels of amylase (A), lipase (B) and C-reactive protein (C) for Groups 1 and 2 at different sampling intervals. t_0: Samples before the procedure; t_{end}: Samples at the end of the procedure [gastrointestinal endocopy (GI) in control group and double-balloon enteroscopy (DBE) in experimental group]; $t_{24\ h}$: Samples 24 h after the procedure (GI in control group and DBE in experimental group); $t_{168\ h}$: Samples 7 d after the procedure (GI in control group and DBE in experimental group). Within-group differences: sampling stages with no coincident capital (Group 1) or normal case (Group 2) letters were significantly different ($P < 0.05$). No significantly different results between Groups 1 and 2 were found at any sampling stage.

pancreatic lobe (tail). Significant differences were found between the left lobe and the body for the percentage of affected pancreas. Interestingly, the presence of the lesions was not related to the exploration length (90 or 140 min; Pearson χ^2 and Fisher's exact tests > 0.3).

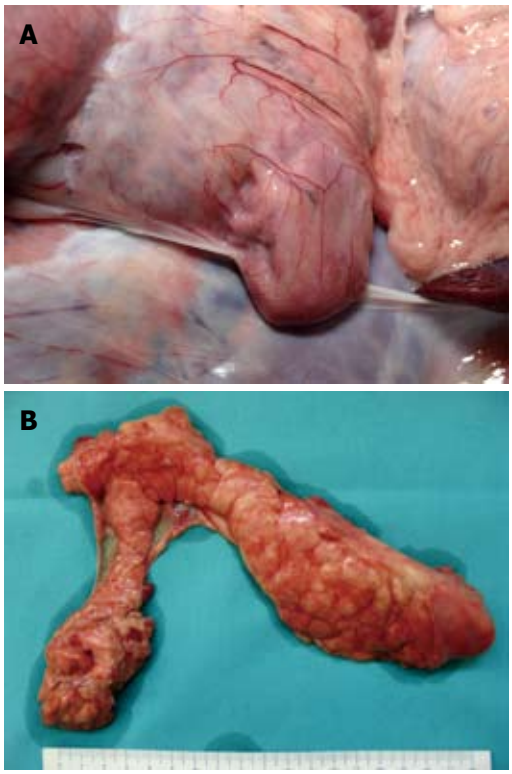

Figure 2 Gross anatomy of the porcine pancreas after double-balloon enteroscopy. A: *In situ* image of the left lobe (tail); B: Aspect of the whole pancreas immediately after removal from cadaver.

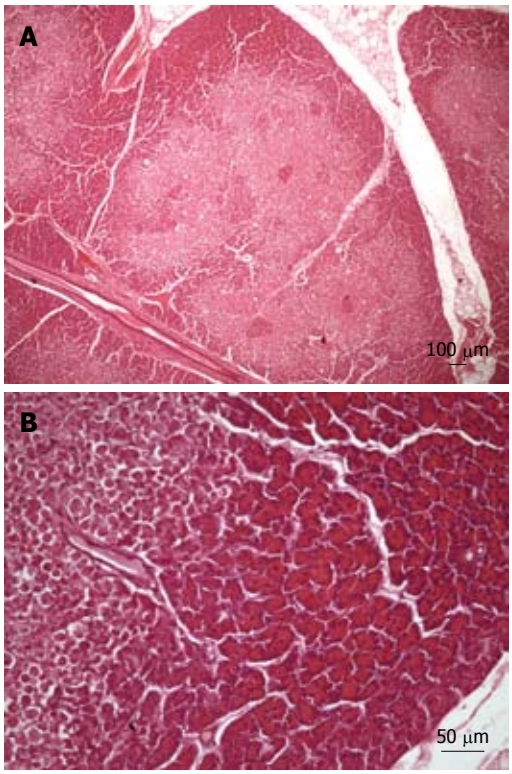

Figure 3 Light microscopy pictures of the porcine pancreas after double-balloon enteroscopy. A: Light microscopy of porcine pancreas after double-balloon enteroscopy showing located ischemic necrosis in pancreatic interlobular tissue; B: Magnification of previous image, view of margin between necrosis and viable tissue.

DISCUSSION

A low incidence (0.3%) of acute pancreatitis after diagnostic DBE has been reported in retrospective studies in Europe and Asia[18], and also in the United States[19,20]. However, latent hyperamylasemia without the development of pancreatitis occurs after peroral DBE more frequently than was previously thought[9,10,21].

The physiology and anatomy of the porcine pancreas is similar to that in humans, that is, it is a partially retroperitoneal organ and the pancreatic body wraps the portal vein. The firmness of the pancreatic parenchyma in swine is also similar to the gland in humans[22]. Thus, the porcine model has been used in many types of studies related to the pancreas such as endoscopic approaches to the pancreas[23-25], or experimental obstructive pancreatitis[26]. An *ex vivo* model for training has also been developed on porcine intestine[5,27]. Recently, the swine model has been validated for both DBE training and research, altogether improving the safety conditions of DBE in humans[28].

The characteristics, in terms of duration and insertion depth, of this study have been designed for comparison with DBE in humans. The DBE time length in Group 1 (90 min) and Group 2 (140 min) was selected in accordance with published average values from prospective studies in humans. Thus, Mehdizadeh *et al*[29] referred to a duration of 109.1 ± 44.6 min for the first 10 cases and 92.4 ± 37.6 min for subsequent cases. Similar times were referred to by other authors, namely, 75 min[14], 95 ± 42 min,[7] 115 ± 9 min[21], and 148 min[10,16]. Our results for the average insertion depth in both groups of animals were within the same range as those reported in previous works: 240 ± 100 cm[5], 250 ± 170 cm[30], 220 ± 90 cm[6], 270 ± 100 cm[7], and 351 ± 108 cm[21].

According to the literature, three authors have specifically measured amylase levels in patients before and after oral DBE[9,10,21]. Honda *et al*[9] found that 46% of patients undergoing DBE developed hyperamylasemia. Kopácová *et al*[10] investigated the levels of serum amylase, lipase and CRP both before and after DBE (4 h and 24 h). They found increased levels of amylase and lipase in 51.4% of the patients 24 h after the procedure. However, only 2.8% of them suffered acute pancreatitis. Pata *et al*[21] also checked levels of serum amylase and lipase both before and 4 h and 12 h after DBE. Just 4 h after the procedure, they found 25% of the patients had hyperamylasemia and hyperlipasemia, and 12.5% of the patients had pancreatitis. It is important to pay attention to the fact that the increases in the serum pancreatic amylase described in those three previous studies were twice or even three times higher than normal levels. In contrast, serum levels of amylase and lipase in the present work never reached twice the baseline level, and this could be related to the fact that the balloons were always inflated after the site of the pancreatic duct opening in the duodenum. In support of this, Pata *et al*[21] have described that amylase levels after DBE are negatively correlated to the depth at which the balloons are first inflated. Interestingly, no changes in the levels of serum amylase and lipase have been reported in patients when the first inflation of the balloons was done after reaching the ligament of Treitz[14,31,32]. On the other hand, some authors have described that substantial hyperamylasemia tended to be associated with longer duration of DBE[10,15,21,32]. However, we did not find such a difference, and the enzyme levels of Groups 1 and 2 did not vary significantly at any stage. Similar results have been reported by others authors[9,16].

The mechanical stress to the small intestine, mesentery and pancreas has also been suspected[7,9] to cause increased levels of amylase, lipase and CRP. Thus, a plausible explanation for the increased levels of enzymes[9,12,13] is an effect of the increased intraluminal pressure on the pancreatic ducts allowing intestinal fluid to progress towards the pancreas, and as such, should be kept in mind. However, the histological injuries found in this study are more likely related to an ischemic process in the vascular supply to the pancreas. The continuous pressure of the small intestine and the mesentery during the push and pull maneuvers could compromise the vascular supply to the pancreas, resulting in an increase in the pancreatic enzymes and unspecific inflammatory factors such as CRP. Along these lines, several works have reported pancreatic vascular restriction as a potential mechanism for hyperamylasemia after oral DBE[11,16,33]. On the other hand, the larger and more frequent areas of ischemic necrosis in the left lobe (tail) of the pig pancreas seem to be related to the particular vasculature of this pancreatic portion. In pigs, the left lobe (tail) is supplied by a single artery, which is a branch of the splenic artery. A similar situation is found in humans where the main artery of the tail of the pancreas is the major pancreatic artery. Such anatomical particularity predisposes the left pancreatic lobe to suffer from hypoxia or even ischemia if there is any mechanical restriction to the blood supply through this artery. Although this explanation requires further specific research, it is interesting to highlight that computed tomography has revealed that human pancreatitis is predominantly located in the tail of pancreas[15,34]. Considering the variance of the enzyme levels and that this was a non-survival animal model study, the number of animals could be a limitation of this work.

In conclusion, the inflation of the balloons after the duodenal papilla diminished the iatrogenic effects on the pancreas. However, minor enzymatic alterations and focal lesions in the pancreas remained, which on the other hand, failed to cause any clinical signs of pancreatitis. A vascular component is probably involved in the etiology of DBE-related pancreatic alterations, but this topic needs further research aimed at evaluating the effects of DBE exploration maneuvers on the vascular supply to the pancreas.

COMMENTS

Background

Double-balloon enteroscopy (DBE) has enabled endoscopic diagnosis and treatment in the small intestine, but an increase in pancreatic enzymes and

potential pancreatitis are recognized as complications directly attributed to the procedure.

Research frontiers

Unfortunately, levels of pancreatic enzymes several days after the DBE procedure and evaluation of potential lesions in the pancreas under normal clinical conditions are unknown in humans. In this study, the authors demonstrated that focal ischemic lesions in the pancreatic parenchyma, and minor enzymatic alterations were related with DBE procedure in a porcine model.

Innovations and breakthroughs

Previous reports have highlighted the importance of the amylase levels in patients before and after oral DBE. Increased levels of amylase and lipase in 51.4% of the patients 24 h after the procedure have been reported. However, only 2.8% of them suffered acute pancreatitis. This is believed to be the first study in an animal model aimed at clarifying the etiology of pancreatic hyperamylasemia and pancreatitis after DBE. Furthermore, the study suggested that a vascular component was probably involved in the etiology of pancreatic alterations after DBE.

Applications

By understanding the etiology of post-DBE pancreatic hyperamylasemia, this study demonstrates the need for further research aimed at evaluating the effects of DBE on the vascular supply to the pancreas.

Terminology

DBE is recognized as the gold standard method for total exploration of the small intestine. Based on the already existent push endoscopy, DBE is a form of deep endoscopy that not only allows the exploration but also treatment of the most common digestive disorders of the small intestine, such as obscure gastrointestinal bleeding, tumors, Crohn's disease and polyps. The equipment consists of an endoscope and an overtube; both of them with a latex balloon attached to the tip. The two balloons are inflated and deflated in an alternating sequence so as to allow the endoscope to progress (pushing phase) or fold the explored intestine behind the balloons (pulling phase).

Peer review

This is an important issue because DBE might be associated with an increase of serum pancreatic enzymes or even complicated by acute pancreatitis. The mechanism of post-DBE pancreatitis has not been fully explained yet. That is why such an experimental study is important to understand possible pathogenetic mechanisms.

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ORIGINAL ARTICLE

Serum levels of microRNAs can specifically predict liver injury of chronic hepatitis B

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Abstract

AIM: To investigate whether circulating microRNAs (miRNAs) can serve as molecular markers to predict liver injury resulted from chronic hepatitis B (CHB).

METHODS: The profiles of serum miRNA expression were first generated with serum samples collected from 10 patients with CHB and 10 healthy donors (Ctrls) by microarray analysis. The levels of several miRNAs were further quantitated by real-time reverse transcription polymerase chain reaction with serum samples from another 24 CHB patients and 24 Ctrls. Serum samples of 20 patients with nonalcohlic steatohepatitis (NASH) were also included for comparison. The comparison in the levels of miRNAs between groups (CHB, NASH and Ctrl) was analyzed with Mann-Whitney *U*-test. The correlation between miRNAs and clinical pathoparameters was analyzed using Spearman correlation analysis or canonical correlation analysis. The receiver-operator characteristic (ROC) curves were also generated to determine the specificity and sensitivity of each individual miRNA in distinguishing patients with CHB from Ctrls.

RESULTS: miRNA profile analysis showed that 34 miRNAs were differentially expressed between CHB and Ctrl subjects, in which 12 were up-regulated and 22 down-regulated in CHB subject (fold change > 2.0 and $P < 0.01$). The median levels of miR-122, -572, -575 and -638 were significantly higher ($P < 1.00 \times 10^{-5}$) while miR-744 significantly lower ($P < 1.00 \times 10^{-6}$) in CHB compared with the Ctrl. The levels of miR-122, -572 and -638 were also higher ($P < 1.00 \times 10^{-3}$) while the level of miR-744 lower in CHB ($P < 0.05$) than in NASH, although the difference between them was not as significant as that between CHB and Ctrl. ROC curve analysis revealed that the levels of miR-122, -572, -575, -638 and -744 in serum were sensitive and specific enough to distinguish CHB, NASH and Ctrl. Multivariate analysis further showed that the levels of these miRNAs were correlated with the liver function parameters. Most significantly, it was the scatter plot of principal component with the levels of these miRNAs, but not the parameters of liver function, which clearly distinguished CHB, NASH and Ctrl subjects.

CONCLUSION: Serum levels of miR-122, -572, -575, -638 and -744 are deregulated in patients with CHB or NASH. The levels of these miRNAs may serve as potential biomarkers for liver injury caused by CHB and NASH.

Key words: Chronic hepatitis B; Nonalcohlic steatohepatitis; Serum microRNAs; Liver injury

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Zhang H, Li QY, Guo ZZ, Guan Y, Du J, Lu YY, Hu YY, Liu P, Huang S, Su SB. Serum levels of microRNAs can specifically predict liver injury of chronic hepatitis B. *World J Gastroenterol* 2012; 18(37): 5188-5196 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i37/5188.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i37.5188

INTRODUCTION

Hepatitis B virus (HBV) infection is one of the major health problems in China[1]. Of the 350 million individuals worldwide infected with the HBV, one-third are from China[2]. HBV infection results in chronic hepatitis B (CHB) and patients with CHB exhibit a high risk of developing liver cirrhosis and hepatocellular carcinoma[3]. Although HBV itself is noncytopathic, host immune response often causes liver damage in patients with HBV infection. Currently, the most commonly used markers of liver injury are the enzymatic activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in blood; however, these markers are devoid of sufficient sensitivity and specificity to diagnose virus-induced liver damages[4,5]. Therefore, assessing the severity of HBV-induced damages and monitoring the progression of CHB are major clinical challenges.

microRNAs (miRNAs) are evolutionarily conserved, and are small (typically ~22 nt in size) regulatory RNA molecules that modulate the levels of specific targets, and are thus actively involved in a wide range of physiologic and pathologic processes[6,7]. Interestingly, miRNAs are very stable in circulation systems, and tissue or organ-specific intracellular miRNAs can often be detected in blood under pathological conditions[8-12]. The elevated levels of these miRNAs in blood are most likely caused by their release into the circulation system in the processes accompanied with cell death, such as cell turnover, cell destruction and pathological injury[13-16]. For example, the levels of miR-1, a muscle and heart-specific miRNA, is elevated in blood during acute myocardial infarction[13]. miR-141, a miRNA highly expressed in prostate cancer cells, is present at a significantly higher level in prostate cancer patients than healthy donors[14]. In addition, the levels of serum miRNAs can also be associated with different physiological conditions. For instance, miRNAs of presumed placental origin were detected at high levels in the plasma of pregnant women[17]. CHB is an infectious illness, and the host immune response to HBV infection is thus expected to cause both hepatocellular damage and viral clearance[18]. In fact, CHB progresses with significant apoptosis and necrosis of hepatocytes[19]. It is of interest to determine whether particular miRNAs are released to blood of CHB patients and can serve as predictor for CHB liver injury.

The goal of this study is to investigate whether the circulating miRNAs can be used as molecular biomarkers to monitor the pathological development of CHB. As liver cells are also damaged along the progression of nonalcoholic steatohepatitis (NASH) and caused by the build-up of fat cell in the liver[20], we included samples of NASH patients in this study. Through the comparison of serum miRNA expression profiles among CHB and NASH patients and healthy donors, we found that the expression of miR-122, -572, -575, -638 and -744 was deregulated in both CHB and NASH patients. The expression of these five miRNAs was significantly correlated with pathological parameters of liver. We, therefore, conclude that these five miRNAs may serve as potential biomarkers for CHB and NASH-induced liver injury.

MATERIALS AND METHODS

Study subjects and clinical parameters

Sera collected from 34 CHB, 20 NASH patients and 34 healthy donors (Ctrls) were included in this study. Samples from 10 CHB patients and 10 Ctrls were subjected to miRNA microarray analysis to obtain serum miRNA profiles. Those miRNAs with altered levels were further measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) with the samples from the remaining 24 CHB patients and 24 Ctrls. To determine the specificity of miRNA level change, serum samples from 20 NASH patients were also included for qRT-PCR analysis. Serum samples of Ctrls were randomly selected from a collection of 120 individuals who had annual physical examination at Shanghai Shuguang Hospital, Shanghai, China. Samples of CHB and NASH were from patients seeking treatment in Shanghai Shuguang Hospital. The diagnostic criteria for CHB followed the guidelines that defined by the Chinese Society of Hepatology and Chinese Society of Infectious Diseases in 2005[21]. The diagnosis of NASH was based on the guidelines for diagnosis and treatment of nonalcoholic fatty liver diseases that issued by Fatty Liver and Alcoholic Liver Disease Study Group of the Chinese Liver Disease Association in 2006[22]. The clinical parameters of these patients are listed in Table 1. This study was approved by the Institutional Review Board of Shanghai Shuguang Hospital.

Serum sample collection and RNA isolation

All serum samples were derived from freshly-drawn blood and stored at -80 ℃. RNA in serum was isolated using a miRVana PARIS kit (Ambion, Austin, TX, United States) according to the manufacturer's protocol followed by the treatment of RNase-free DNase I (Promega, Madison, WI, United States) to eliminate DNA contamination. The concentration of RNAs extracted from serum ranged from 1.5 to 12 ng/μL.

Table 1 Clinical characteristics of participants in validation

Group	CHB	NASH	Ctrl
Individuals (*n*)	24	20	24
Gender (*n*)			
Male	21	17	19
Female	3	3	5
Age (yr)	37.6 ± 9.0	39.4 ± 9.6	35.6 ± 10.2
ALT (IU/L)	82.6 (14-412)	51.7 (18-203)	21.5 (14-43)
AST (IU/L)	62.6 (9-206)	31.55 (17-62)	21.4 (16-49)
GGT (IU/L)	69.4 (10-580)	45.8 (13-124)	20.8 (12-33)
ALP (IU/L)	95.8 (51-211)	66.0 (41-90)	62.3 (42-96)
TBIL (μmol/L)	19.2 (10.2-50.7)	19.5 (13.1-31.7)	15.9 (6.9-27.1)
HBV DNA	7 377 395 (0-94 800 000)	0	0
Bile acid (μmol/L)	6.9 (1.4-13)	17.9 (3-118.7)	8.4 (4.1-12)
HBV status (*n*)			
$HBsAg^+$	24	0	0
$HBsAg^-$	0	20	24

Bile acid were given as medians (range). ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-glutamyltransferase; ALP: Alkaline phosphatase; TBIL: Total bilirubinand; CHB: Chronic hepatitis B; NASH: Nonalcohlic steatohepatitis; Ctrl: Healthy donor; HBsAg: Hepatitis B surface antigen.

Serum miRNA profiling and data analysis

The profiles of serum miRNAs of 10 CHB patients and 10 Ctrls were generated using Agilent Human miRNA microarray V3 (Agilent Technologies Inc, Santa Clara, CA, United States). The microarray chip is comprised of 2371 different probes for a total of 851 human miRNAs. One hundred nanograms of serum RNA was used for each array. The arrays were read using the Agilent microarray scanner and the data were extracted using Feature Extraction V10.7 (Agilent Technologies, CA, United States). All data were transformed to log base 2. The differences between samples were calculated using unsupervised analysis (SAS system, Shanghai Biochip, Shanghai, China). Only the miRNAs with the fold difference > 2.0 and $P < 0.01$ were considered significant.

Validation of internal reference for serum miRNA quantification

There has been no consensus on the reference genes for qRT-PCR analysis of serum miRNAs. However, 6 miRNAs, RNU6B[23], miR-24[14,24], miR-16[15], miR-181a[25], miR-454[26] and miR-638[27], have been reported to be consistently present in human serum. Therefore, these miRNAs were empirically analyzed by qRT-PCR in samples from all patients and Ctrls. The cycle threshold (Ct) values were converted into relative quantities for analysis with geNorm software[28], which selects the optimal number of the most stable genes for normalization. To calculate the expression stability of a given gene (gene stability measure *M*), the program uses an algorithm based on the mean of the pairwise variation of a given reference gene compared to all other control genes. The higher the value of *M* is, the more the expression variability of the corresponding reference gene is. The least stable gene, i.e., the gene with the highest *M* value, was excluded from the subsequent analysis. The remaining genes was recalculated for *M* values and the gene with highest *M* was again excluded until the two most stable genes were left.

Quantification of serum miRNAs

qRT-PCR-based quantification of miRNAs (200 μL of serum from each participant) was performed with Bulge-Loop™ miRNA qPCR Primer Set (Guangzhou Ribobio, Guangzhou, China) and SYBR Green PCR Master Mixture (TOYOBO, LTD, Japan) according to the manufacturer's instructions using a Rotor-Gene 6000 Real-time PCR machine (Corbett Life Science, Sydney, Australia). The specificity of each PCR products was validated by melting curve analysis at the end of PCR cycles. All samples were analyzed in triplicate and the Ct was defined as the number of cycles required for the fluorescent signal to reach the threshold. The levels of miRNAs in serum were calculated using the formula $2^{\Delta Ct}$ where ΔCt = Ct of internal reference -Ct of target miRNA.

Establishment of receiver-operator characteristic curves

Receiver-operator characteristic (ROC) curves were established to evaluate the difference in the levels of serum miRNAs among CHB, NASH and Ctrls. Statistical significance for correlations was calculated using Spearman's non-parametric rank test and the correlation coefficient R generated by Spearman correlation formula.

Statistical analysis

Comparisons between groups were analyzed using Mann-Whitney *U*-test, Pearson χ^2 test, canonical correlation analysis or Spearman correlation analysis wherever appropriate. All tests were two-tailed and $P < 0.05$ was considered statistically significant.

RESULTS

Serum miRNA profiles of CHB patients are distinct from those of Ctrls or NASH patients

To determine whether there was difference in serum miRNA profiles between people with or without CHB, we performed miRNA microarray with RNAs isolated from the sera of 10 CHB patients and 10 Ctrls. Among a total of 851 miRNAs analyzed, 34 of them were differentially expressed between CHB patients and Ctrls (fold change > 2.0 and $P < 0.01$) (Table 2).

In order to validate the serum miRNA profiles generated from microarray, we initially turned our attention to identifying a particular serum miRNA that can be used as an internal control. As the levels of RNU6B, miR-24, -16, -181a, -454 and -638 were previously reported be relatively consistent[14,15,23-26], we measured their levels in 16 serum samples (4 CHB, 4 NASH and 8 Ctrls). We employed GeNorm to calculate the stability values (*M*-values) for these candidate miRNAs and excluded the candidates with the lowest stability (the highest *M* value). The stability value was recalculated until the two most stable miRNAs were predicted. Defining *M*-values below 1.5 as the critical limit, GeNorm data analysis showed that miR-24

Table 2 Differentially expressed miRNAs in chronic hepatitis B patients and healthy donors

miRNA	Fold change	*P* value	CHB (log_2) mean	Ctrls (log_2) mean
hsa-miR-122	8.29	2.99E-03	8.14	5.09
hsa-miR-138	4.23	5.69E-03	2.68	0.60
hsa-miR-638	4.18	2.43E-03	12.62	10.55
hsv1-miR-H1	3.93	7.92E-03	7.67	5.70
hsa-miR-575	3.67	5.69E-03	9.59	7.71
hsa-miR-572	3.36	3.98E-03	7.58	5.83
kshv-miR-K12-3	3.34	2.60E-03	10.19	8.45
hsa-miR-1915	3.12	5.22E-03	11.30	9.66
hsa-miR-623	3.07	4.69E-03	6.85	5.23
hsa-miR-1268	2.81	6.43E-03	9.94	8.45
hsa-miR-939	2.63	3.94E-03	8.81	7.42
hsa-miR-498	2.29	4.30E-03	6.07	4.87
hsa-miR-421	0.37	4.05E-03	0.93	2.38
hsa-miR-598	0.35	5.24E-04	0.67	2.20
hsa-miR-155	0.34	6.40E-03	2.40	3.94
hsa-miR-424	0.33	9.76E-03	3.67	5.26
hsa-miR-23b	0.28	8.42E-03	5.44	7.29
hsa-miR-195	0.27	1.17E-03	1.20	3.10
hsa-miR-487b	0.26	5.46E-03	1.49	3.44
hsa-miR-224	0.25	3.71E-03	1.45	3.45
hsa-miR-495	0.24	2.50E-03	1.16	3.21
hsa-miR-181c	0.22	6.78E-03	1.88	4.03
hsa-miR-654-3p	0.21	8.27E-03	1.99	4.22
hsa-let-7e	0.21	2.52E-03	0.75	2.99
hsa-miR-382	0.21	9.11E-03	1.78	4.02
hsa-miR-17[1]	0.19	9.66E-03	2.47	4.89
hsa-miR-128	0.18	6.17E-03	2.38	4.82
hsa-miR-625	0.18	2.70E-04	2.14	4.61
hsa-miR-30e[1]	0.16	2.94E-03	1.89	4.51
hsa-miR-139-5p	0.16	3.10E-03	2.39	5.03
hsa-miR-30c	0.16	8.92E-03	3.66	6.32
hsa-miR-744	0.15	9.63E-03	2.40	5.10
hsa-miR-374b	0.12	3.05E-03	2.35	5.44
hsa-miR-376c	0.11	4.32E-03	2.91	6.04

[1]MicroRNA (miRNA) cloning studies sometimes identify two about 22 nt sequences miRNAs which originate from the same predicted precursor. When the relative abundancies clearly indicate which is the predominantly expressed miRNA, the mature sequences are assigned names of the form miRNA (the predominant product) and miRNA* (from the opposite arm of the precursor). For example, miR-123 and miR-123* would share a pre-miRNA hairpin, but more miR-123 would be found in the cell. In the past, this distinction was also made with "s" (sense) and "as" (antisense). CHB: Chronic hepatitis B; Ctrl: Healthy donor.

and -181a had the least *M* value (0.656) among these candidate miRNAs, implicating that they were the most stable ones. We thus selected miR-24 as the internal control to standardize differentially presented serum miRNAs in qRT-PCR quantification.

Microarray analysis with 10 CHB and 10 Ctrl samples showed that the median levels of serum miR-122, -572, -575 and -638 were higher while median levels of miR-30c and -744 were lower in CHB patients than those in Ctrls (Table 2). In order to validate these microarray-generated results, RNA was prepared from serum samples of another 24 CHB patients and 24 Ctrls and was subsequently subjected to qRT-PCR to measure the levels of miR-122, -572, -575, -638 and -744. Identical to what we observed with microarray analysis, the levels of miR-122, -572, -575 and -638 were 83.40-, 43.17-, 15.24- and 12.95-fold higher in the sera of CHB patients than those of Ctrls ($P = 1.61 \times 10^{-8}$, $P = 1.20 \times 10^{-6}$, $P = 8.27 \times 10^{-8}$, $P = 2.88 \times 10^{-9}$, respectively) (Figure 1A-D) while the level of miR-744 was 5.11-fold lower in CHB patients than that in Ctrls ($P = 1.04 \times 10^{-7}$) (Figure 1E). The level of miR-30c was a little higher in CHB patients than that in Ctrls although it was not statistically significant (Figure 1F).

To determine how specific these altered serum miRNAs were to CHB, we next examined the levels of these miRNA in serum samples of NASH patients. qRT-PCR showed that the levels of serum miR-122, -638, -575 and -572 were 3.04-, 16.32-, 4.27- and 5.62-fold higher ($P = 6.89 \times 10^{-4}$, $P = 7.50 \times 10^{-5}$, $P = 6.72 \times 10^{-6}$ and $P = 1.14 \times 10^{-7}$, respectively) while the level of miR-744 was 3.75-fold lower in NASH patients than in Ctrls ($P = 2.15 \times 10^{-7}$) (Figure 1A-E). When comparing the levels of these serum miRNAs between CHB and NASH samples, we found that the levels of miR-122, -572 and miR-638 were 27.42-, 2.65- and 3.57-fold higher ($P = 5.83 \times 10^{-7}$, $P = 4.18 \times 10^{-3}$, $P = 8.89 \times 10^{-4}$, respectively) while the level of miR-744 was 1.36-fold lower in CHB patients than in NASH patients ($P = 4.8 \times 10^{-2}$) (Figure 1A-C, E). In contrast, no significant difference was found in the levels of miR-575 and -30c between the serum samples of CHB and NASH patients (Figure 1D and F). These results demonstrated that a subset of miRNAs was differentially present in the sera of CHB patients.

Levels of a subset of serum miRNAs can be used to distinguish CHB patients from NASH patients or Ctrls

To determine whether the levels of serum miRNAs can be used to distinguish patients with CHB from those with NASH or Ctrls, we established ROC curves to analyze the difference in the levels of serum miR-122, -638, -575, -572 and -744 between groups. Comparing CHB subjects with Ctrls, ROC curve areas of miR-122, -638, -575, -572 and -744 were found to be 0.98 (95% CI: 0.88-1.00), 1.00 (95% CI: 0.93-1.00), 0.91 (95% CI: 0.79-0.97), 0.95 (95% CI: 0.85-0.99) and 0.95 (95% CI: 0.84-0.99), respectively. The sensitivity and the specificity of each of these miRNAs were 87.5% and 100%, 100% and 100%, 83.3% and 83.3%, 79.2% and 100%, 91.7% and 95.8%, respectively in the CHB subjects and Ctrls (Figure 2A). These results clearly showed that the levels of serum miR-122, -638, -575, -572 and -744 can distinguish patients with CHB from Ctrls.

We next compared the levels of these serum miRNAs between NASH subjects and Ctrls. ROC curve areas of miR-122, -638, -575, -572 and -744 were 0.80 (95% CI: 0.65-0.91), 0.97 (95% CI: 0.87-0.99), 0.90 (95% CI: 0.77-0.97), 0.85 (95% CI: 0.71-0.94), and 0.96 (95% CI: 0.85-0.99). The sensitivity and the specificity were 95.0% and 62.5%, 95.0% and 95.8%, 90.0% and 79.2%, 100.0% and 66.7%, 100.0% and 95.8%, respectively in NASH subjects and Ctrls (Figure 2B). These results also demonstrated that the levels of these five miRNAs can distinguish patients with NASH from Ctrls. Interestingly, comparison of CHB subjects with NASH subjects implicated that the levels of miR-122, -638, -572 and -744 were use-

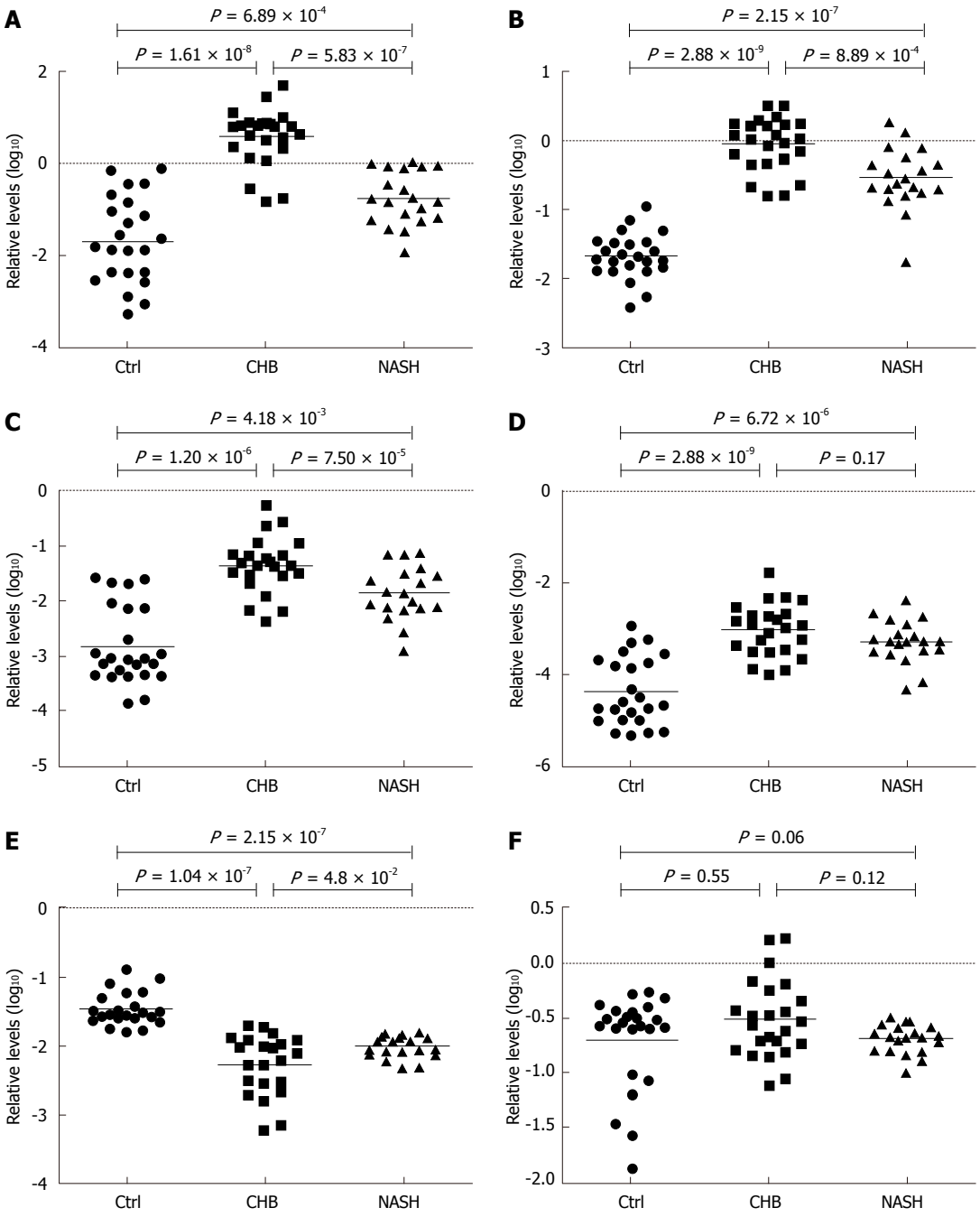

Figure 1 Serum levels of microRNAs in chronic hepatitis B, nonalcohlic steatohepatitis and healthy donors. The levels of serum miR-122 (A), miR-638 (B), miR-572 (C), miR-575 (D), miR-744 (E) and miR-30c (F) in patients with chronic hepatitis B (CHB) (n = 24), with nonalcohlic steatohepatitis (NASH) (n = 20) and healthy donors (Ctrl) (n = 24) were measured by quantitative reverse transcription polymerase chain reaction. The line at each group represents the median value of indicated miRNA. The values are normalized to miR-24 and shown in $\log_{10}$ scale at y-axis. P values on the top are NASH *vs* Ctrl, on the left are CHB *vs* Ctrl and on the right are NASH *vs* Ctrl.

ful markers for discriminating patients with CHB from those with NASH because ROC curve area of miR-122, -638, -572 and -744 were 0.94 (95% CI: 0.83-0.99), 0.79 (95% CI: 0.65-0.90), 0.75 (95% CI: 0.60-0.87) and 0.68 (95% CI: 0.52-0.81) and the sensitivity and the specificity were 87.5% and 100.0%, 83.3% and 70.0%, 75.0% and 75.0%, 50.0% and 85%, respectively, in the two groups (Figure 2C). Together, these results demonstrated that the levels of miR-122, -638, -572 and -744 in serum can be used to distinguish CHB, NASH and Ctrls.

Aberrant levels of serum miR-122, -572, -575, -638 and -744 correlate with the liver pathological parameters

To investigate whether the levels of serum miR-122, -572, -575, -638 and -744 can be used as independent molecular indicators of CHB- or NASH-induced liver injury, we first determined the potential correlation of these five miRNAs in themselves among serum samples from patients with CHB, NASH and Ctrls. Spearman correlation analysis showed that the levels of these five miRNAs in sera were highly correlated among them-

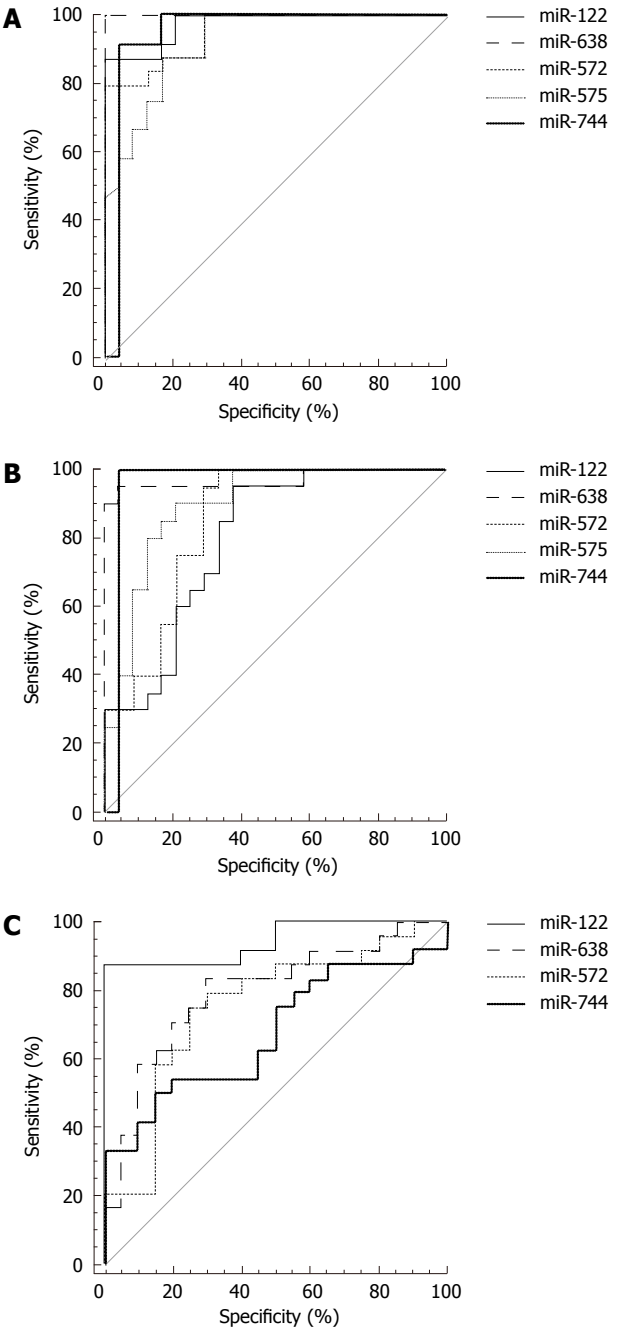

Figure 2 Receiver-operator characteristic curve analyses. Receiver-operator characteristic curves of the miR-122, -638, -572, -575 and -744 were established to discriminate chronic hepatitis B (CHB) from healthy donor (Ctrl) (A), nonalcohlic steatohepatitis (NASH) from Ctrl (B) and CHB from NASH (C).

selves ($r \geqslant 0.57$, $P = 1.00 \times 10^{-4}$; Table 3). We next analyzed the potential correlations between each of these five miRNAs and each of the clinical liver pathological parameters. Spearman correlation analysis showed that correlation only existed between selected miRNAs and selected liver function parameters (Table 3). For example, miR-122 was significantly correlated with both ALT ($r = 0.559$, $P = 1.00 \times 10^{-6}$) and AST ($r = 0.692$, $P = 1.00 \times 10^{-6}$; Table 3). However, none of these miRNAs was correlated with markers of hepatitis viruses including HBsAg, HBeAg and HBV DNA (data not shown).

Table 3 Coefficient of Spearman correlation between microRNA variables and liver function parameter variables (all 68 samples)

Variables	miR-122	miR-638	miR-572	miR-575	miR-744
miR-122	1.000	0.757[b]	0.780[b]	0.614[b]	-0.669[b]
miR-638	0.757[b]	1.000	0.876[b]	0.822[b]	-0.733[b]
miR-572	0.780[b]	0.876[b]	1.000	0.794[b]	-0.639[b]
miR-575	0.614[b]	0.822[b]	0.794[b]	1.000	-0.570[b]
miR-744	-0.669[b]	-0.733[b]	-0.639[b]	-0.570[b]	1.000
ALT	0.559[d]	0.431[d]	0.375[d]	0.299[c]	-0.413[d]
AST	0.692[d]	0.474[d]	0.465[d]	0.324[d]	-0.434[d]
GGT	0.421[d]	0.371[d]	0.280[c]	0.214	-0.355[d]
ALP	0.358[d]	0.312[d]	0.320[d]	0.306[c]	-0.180
TBIL	0.034	0.041	-0.114	-0.074	-0.068
Bile acid	0.068	-0.020	0.008	-0.023	0.090

[b]$P < 0.01$ microRNA (miRNA) *vs* miRNA without superscript b in the same column (2-tailed test); [c]$P < 0.05$ level miRNA *vs* liver function parameter (2-tailed test); [d]$P < 0.01$ level miRNA *vs* liver function parameter (2-tailed test). ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-glutamyltransferase; ALP: Alkaline phosphatase; TBIL: Total bilirubin and bile.

In the subsequent studies, we considered both levels of miRNAs and liver pathological parameters as multiple factors and analyzed their correlation using multivariate analysis (canonical correlation analysis). As shown in Figure 3A, the levels of miR-122, -638, -572, -575 and -744 were apparently correlated with liver functional parameters (ALT, AST, γ-glutamyltransferase, ALP, TBIL and bile acid) ($r = 0.74$, $P < 1.00 \times 10^{-4}$). Moreover, the changes in the levels of these miRNAs were greater than those in the values of ALT or AST in the CHB and NASH subjects (Figure 3B). Most significantly, the scatter plot of principal component with the levels of these miRNAs clearly distinguished CHB, NASH and Ctrl subjects (Figure 3C). In contrast, identical analysis with the values of liver functional parameters was unable to distinguish the three groups of subjects (Figure 3D). These data indicated that the profile of miR-122, -572, -575, -638 and -744 in the serum was a better indicator than those well-established liver functional markers for liver injury caused by CHB or NASH.

DISCUSSION

miRNAs can be released into circulating system through damaged cells and tissues. Circulating miRNAs are very stable in plasma and can be found in lipid or lipoprotein complexes[29], apoptotic bodies[30], microvesicles[31] or exosomes[32]. Recent studies have shown that the levels of circulating miRNAs can alter significantly at different physiological stages and pathological conditions. For example, the level of miR-122 (liver specific), miR-133a (muscle specific), and miR-124 (brain specific) are respectively elevated in blood of patients with liver, muscle, and brain injury[9]. Moreover, the level of miR-141 is significantly higher in patients with prostate cancer than in healthy controls[14]. These observations suggest that circulating miRNAs may represent a new class of

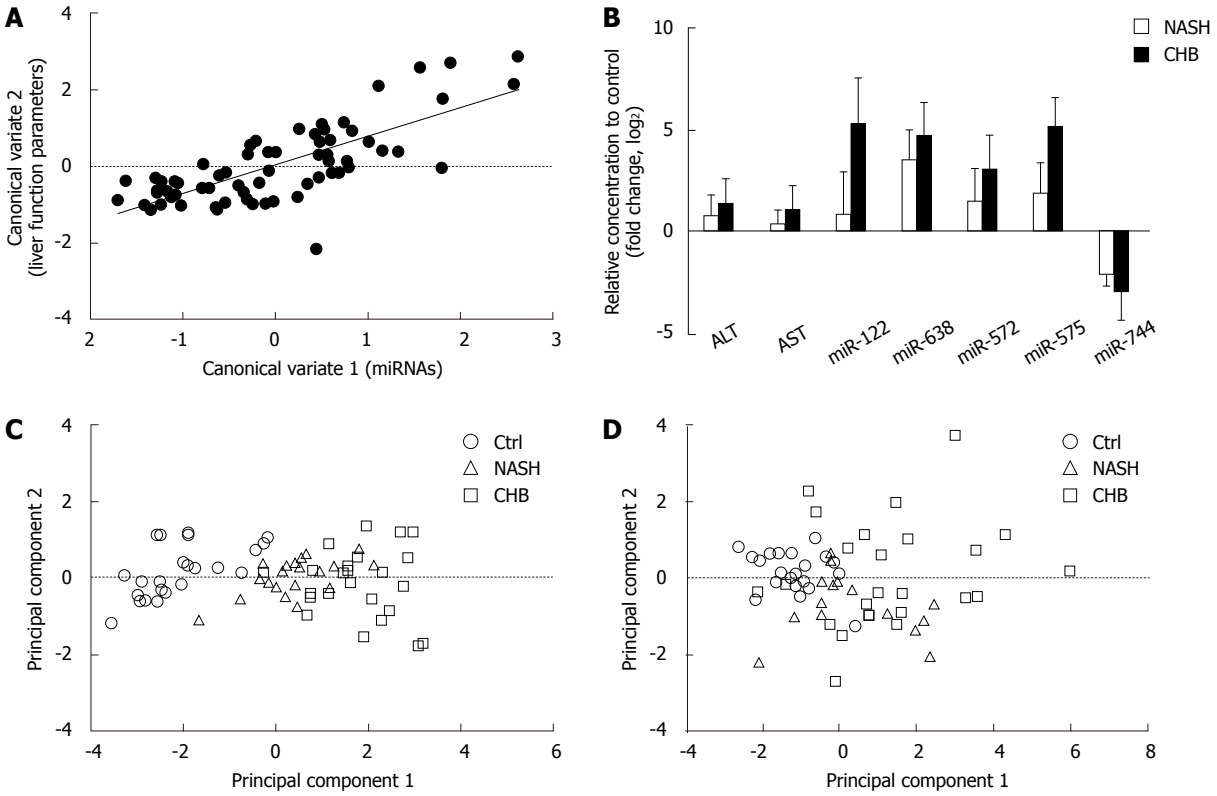

Figure 3 Aberrant levels of serum miR-122, -572, -575, -638 and -744 correlate with the liver pathological parameters. A: Canonical correlation analysis. The correlation between microRNA (miRNA) variables and liver function parameter variables were calculated by canonical correlation analysis. All the data were $\log_{10}$ transformed. Correlation coefficient $r = 0.74$ and $P < 1.00 \times 10^{-4}$. miRNAs: miR-122, -572, -575, -638 and -744; Liver function parameters: Alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltransferase (GGT), alkaline phosphatase (ALP), total bilirubin (TBIL) and bile acid; B: Comparison of serum miRNAs, ALT and AST in chronic hepatitis B (CHB) and nonalcohlic steatohepatitis (NASH). The comparison among the levels of ALT, AST, miR-122, -572, -575, -638 and -744 in serum samples collected from patients with NASH and CHB (indicated on x-axis). The relative change of ALT, AST and miRNA expression levels were expressed ratio in $\log_2$ compared with healthy control (Ctrl) (indicated on y-axis). The values of ALT, AST and miRNA fold change are the average of samples from CHB (n = 24), NASH (n = 20) and Ctrls (n = 24), and the SD is shown as an error bar; C, D: Scatter plot of principal components analysis. All the data were $\log_{10}$ transformed to carry out analysis. Scatter plot of first two principal component of miRNAs variables including miR-122, -572, -575, -638 and -744 (C), and liver function parameters including ALT, AST, GGT, ALP, TBIL and bile acid (D) in CHB (n = 24), NASH (n = 20) and Ctrls (n = 24).

biomarkers for monitoring the progress of certain diseases[8-17]. This possibility is supported by several recent reports which demonstrate the potential of miR-9 as a novel non-invasive molecular marker for traumatic spinal cord injury[10], miR-208a for early detection of acute myocardial infarction[11], miR-146a/223 for sepsis[12], miR571 and miR-652 for liver cirrhosis-induced alcoholic hepatitis or hepatitis C[33] and a plasma miRNA panel (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-801) for HBV-related HCC[34]. In this study, we further validated that the levels of a subset of miRNAs possess the value as novel and sensitive biomarkers for predicting liver diseases.

miR-122 was previously reported as a liver-specific miRNA[35]. In rodents, liver injury induced by alcohol or chemicals leads to the increased level of plasma miR-122 which occurs earlier than the increase in commonly-used marker ALT[9,36]. Moreover, the level of plasma miR-122 exhibits an excellent correlation with the necroinflammatory activity of HBV[23] and HCV infection[37]. In addition to miR-122, the levels of miR-575, -572, -638 and -744 in serum were also altered in patients with CHB or NASH compared with the Ctrls (Figure 1 and Table 2). Importantly, the alteration of these miRNAs correlated well with well-established liver functional parameters (Figure 3 and Table 3). Our study supports a notion that serum miRNA profile may be used to envisage the occurrence of liver injury caused by CHB and NASH.

CHB and NASH have different histological features in necro-inflammation and fibrosis[18-20]. In this study, the median levels of ALT and AST were significantly higher in CHB than in NASH (ALT: 82.6 U/L *vs* 51.7 U/L, $P < 0.05$; AST: 62.6 U/L *vs* 31.55 U/L, $P < 0.05$) (Table 1). Patients with CHB generally exhibited more severe liver damage than those with NASH, thus explaining the detection of higher levels of serum miR-122, -638 and -572, but lower level of miR-744 in CHB than those in NASH (Figure 1). Current laboratory testing with the established liver function parameters do not reliably predict the type and severity of liver injury[4,5]. It is supported by our observation that scatter plot of principal components analysis with liver function parameters was unable to distinguish CHB from NASH (Figure 3D). However, the changes in the levels of these five miRNAs were significantly greater than those in the values of ALT or AST in the CHB and NASH patients (Figure 3B). Scatter

plot of principal components analysis with the levels of serum miRNAs clearly distinguished CHB, NASH and Ctrls (Figure 3C). In hepatitis, hepatocytes die through the mechanisms of both apoptosis (programmed cell death) and necrosis. Hepatocytes presumably synthesize less AST and ALT in the process of apoptosis[38]. In contrast, either apoptosis or necrosis of hepatocytes will release cellular miRNAs directly into the circulating system. This may explain the reason why the sensitivity of serum miRNAs was superior to ALT or AST in diagnosing liver damages. Although investigations with a larger number of samples may be necessary to fully validate our findings, our results do suggest that the alteration of serum miRNA profile may be a more precise molecular biomarker for predicting the seriousness of liver injury.

This study demonstrated that miR-122, -638, -572 and -575 were presented at higher levels while miR-744 was at lower levels in the sera of patients with CHB and NASH. The levels of these miRNAs were not only correlated with liver pathological parameters, but also were more precise indicators for the type and severity of liver diseases than commonly-used markers such as ALT and AST. In conclusion, analyzing the alteration of serum miR-122, -638, -572, -575 and -744 levels may represent a powerful strategy to diagnose liver injury caused by liver inflammation.

ACKNOWLEDGMENTS

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COMMENTS

Background

microRNAs (miRNAs) are small, non-protein coding transcripts involved in many cellular and physiological mechanisms. Recently, a new class of miRNA called "circulating miRNAs" was found in cell-free body fluids such as serum and urine. Circulating miRNAs have been shown to be very stable, specific, and sensitive biomarkers. In this paper, the authors investigated miRNAs with altered levels in the serum of patients with chronic hepatitis B (CHB).

Research frontiers

Currently, the most commonly used markers of liver injury are the enzymatic activities of alanine aminotransferase and aspartate aminotransferase in blood; however, these markers are devoid of sufficient sensitivity and specificity to diagnose virus-induced liver damages. Therefore, assessing the severity of hepatitis B virus-induced damages and monitoring the progression of CHB are major clinical challenges. Circulating miRNA as a biomarker is a new frontier in diagnostics. The goal of this study was to investigate whether the circulating miRNAs can be used as molecular biomarkers to monitor the pathological development of CHB.

Innovations and breakthroughs

Through comparison of serum miRNA expression profiles among CHB, nonalcohlic steatohepatitis (NASH) patients and healthy donors, the authors found that the expression of miR-122, -572, -575, -638 and -744 was deregulated in both CHB and NASH patients. The authors further showed that the expression of these five miRNAs was significantly correlated with pathological parameters of liver. The authors concluded that these five miRNAs may serve as potential biomarkers for CHB and NASH-caused liver injury.

Applications

The study results suggest that serum levels of miR-122, -572, -575, -638 and -744 are deregulated in patients with CHB or NASH. The levels of these miRNAs may serve as potential biomarkers for liver injury caused by CHB and NASH.

Peer review

The authors compared and analyzed the miRNAs profile of CHB, NASH and healthy control. They found that the level of some miRNAs varied in patients with different clinical diagnosis. For instance, the levels of miR-122, -572, -575, -638 and -744 were significantly correlated with the liver function parameters and they concluded that serum levels of these miRNAs may serve as potential biomarkers for liver injury caused by CHB and NASH. The study is quite interesting and useful for clinical practices, although the results have to be validated by using a larger number of samples.

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ORIGINAL ARTICLE

Inhalation of hydrogen gas reduces liver injury during major hepatotectomy in swine

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Abstract

AIM: To study the effect of H_2 gas on liver injury in massive hepatectomy using the Intermittent Pringle maneuver in swine.

METHODS: Male Bama pigs (n = 14) treated with ketamine hydrochloride and Sumianxin Ⅱ as induction drugs followed by inhalation anesthesia with 2% isoflurane, underwent 70% hepatotectomy with loss of bleeding less than 50 mL, and with hepatic pedicle occlusion for 20 min, were divided into two groups: Hydrogen-group (n = 7), the pigs with inhalation of 2% hydrogen by the tracheal intubation during major hepatotectomy; Contrast-group (n = 7), underwent 70% hepatotectomy without inhalation of hydrogen. Hemodynamic changes and plasma concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), hyaluronic acid (HA), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and malondialdehyde (MDA) in liver tissue were measured at pre-operation, post-hepatotectomy (PH) 1 h and 3 h. The apoptosis and proliferating cell nuclear antigen (PCNA) expression in liver remnant were evaluated at PH 3 h. Then we compared the two groups by these marks to evaluate the effect of the hydrogen in the liver injury during major hepatotectomy with the Pringle Maneuver in the swine.

RESULTS: There were no significant differences in body weight, blood loss and removal liver weight between the two groups. There was no significant difference in changes of portal vein pressure between two groups at pre-operation, PH 30 min, but in hydrogen gas treated-group it slightly decrease and lower than its in Contrast-group at PH 3 h, although there were no significant difference (P = 0.655). ALT and AST in Hydrogen-group was significantly lower comparing to Contrast-group (P = 0.036, P = 0.011, *vs* P = 0.032, P = 0.013) at PH 1 h and 3 h, although the two groups all increased. The MDA level increased between the two group at PH 1 h and 3 h. In the hydrogen gas treated-group, the MDA level was not significantly significant at pre-operation and significantly low at PH 1 h and 3 h comparing to Contrast-group (P = 0.0005, P = 0.0004). In Hydrogen-group, the HA level was also significantly low to Contrast-group (P = 0.0005, P = 0.0005) although the two groups all increased at PH 1 h and 3 h. The expression of cluster of differentiation molecule 31 molecules Hydrogen-group was low to Contrast-group. However, PCNA index (%) was not statistically significant between the two groups (P = 0.802). Microphotometric evaluation of apoptotic index (AI) in terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling-stained tissue after hepatotectomy for 3h, the AI% level in the hydrogen was significantly low to Contrast-group (P = 0.012). There were no significant difference between Hydrogen-group and Contrast-group at pre-operation (P = 0.653, P = 0.423), but after massive hepatotectomy, the TNF-α and IL-6 levels increase, and its in Hydrogen-group was significantly low compared with Contrast-group (P = 0.022, P = 0.013, *vs* P = 0.016, P = 0.012), respectively. Hydrogen-gas inhalation reduce levels of these markers and relieved morphological liver injury and apoptosis.

CONCLUSION: H_2 gas attenuates markedly ischemia and portal hyperperfusion injury in pigs with massive hepatotectomy, possibly by the reduction of inflammation and oxidative stress, maybe a potential agent for treatment in clinic.

Key words: Massive hepatotectomy; Hydrogen gas; Anti-oxidant; Hyperperfusion; Malondialdehyde; Oxidative stress

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INTRODUCTION

In hepatotectomy, it is often needed to occlude the portal inflow in order to reducing bleeding, causing liver ischemic injury, the Pringle maneuver, interrupts the blood flow to the liver, produces profound hepatic ischemia and intestinal congestion, it has been used clinically during hepatectomy[1-3]. However, ischemia-reperfusion injury (I/R-I) resulting from the Pringle maneuver is one of the pathogenetic factors involved in postoperative liver dysfunction and hepatic failure, especially when the liver is steatotic and cirrhotic[3-5]. The risk of post-operative liver failure (PLF) or "small of size" syndrome (SFSS) is the central problem in the field of liver resection[3,6]. Oxidative stress is regarded as a major contributor to the development of various hepatic disorders including acute hepatic failure, hepatic fibrosis, and hepatic cancer[7-9]. Protective effect of H_2 gas on liver ischemia reperfusion (I/R) injury and toxic liver injury in rodents has been demonstrated. Previously through ameliorating oxidative stress, H_2 becomes an important potential antioxygen spices agent in clinic[10-12]. However, all experiments about H_2 gas focus on small animal[11,13], and lack of the study in big animal which provide a much more clinically relevant means of investigating the pathophysiology of a disease process. Protective effect of H_2 in big animal can provid more treatment options that can be more readily applied in the human setting. In this study, we investigated firstly the effect of H_2 gas on liver remnant injury in major hepatectomy using the Pringle maneuver in swine, and its feasibility in clinic.

MATERIALS AND METHODS

Animals and husbandry

Fourteen pigs male Bama miniature pigs (15-20 kg) were obtained from the Pig and Poultry Production Institute (Guangxi Zhuang Autonomous Region, China). The swine were raised from a closed herd and kept under strict quarantine protocol. The study was approved by the Hospital Clinic Committee on Ethics in Animal Experimentation. All animals in this study were treated humanely and in accordance with institutional and national guide lines for ethical animal.

Surgical technique

An upper midline incision with right or bilateral subcostal extensions (inverse "L" shape or Mercedesb incision) was performed. The subtotal hepatotectomy with loss of bleeding less than 50 mL, and with hepatic pedicle occlusion for 20 min were performed according to the previous introduce[13]. A 16-gauge catheter was inserted into the main portal vein *via* the gastroduodenal vein to measure the portal vein pressure (PVP).

Study group

Hydrogen-group (n = 7) inhaled with 2% and H_2 98% oxygen supplied through trachea cannula, gas inhalation started once trachea cannula accomplished, ontrast-group (n = 7), only inhale oxygen though tracheal tube. In the two groups, the intraoperative PVP and flow were respectively monitored at the proctectomy, 1 h and 3 h after finishing the hepatectomy.

Serum sample analysis

Blood samples were obtained before laparotomy, posthepatectomy 60 min and 180 min. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), hyaluronic acid (HA), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) were evaluated. Serum AST, ALT and HA were measured using standard clinical methods for automated analysis (Model 7170; Hitachi Inc, Tokyo, Japan). Plasma TNF-α, IL-6 levels were examined by enzyme-linked immunosorbent assay (ELISA) using a commercial porcine TNF-α/TNFSF2 immunoassay kit (Shanghai Yi Hua Scientifi c, Inc. China). Serum HA levels reflect sinusoidal endothelial damage. HA was measured by a radiometric assay with the Pharmacia HA test (Shanghai Yi Hua Scientific, Inc. China) in prereperfusion and postreperfusion serum samples.

Histological examination

Tissue samples were obtained at 180 min post-hepatotectomy (PH) and were divided into two parts. One was immediately cut into cubes 1 mm and fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 mol/L sodium cacodylate-HCl buffer, pH 7.4) at 4 ℃, prior to sectioning for transmission electron microscopy. Another was fixed with 10% formalin for 24 h and embedded in paraffin. Three-micrometer-thick sections were stained with he-

Table 1 The characteristics of the experiment (mean ± SD)

	Hydrogen-group	Contrast-group	*P* value
BW (kg)	19.2 ± 2.7	18.8 ± 3.1	NS
RLW (g)	302 ± 21	296 ± 23	NS
ELB (mL)	43 ± 12	53 ± 16	NS
OT (h)	2.9 ± 0.3	3.3 ± 0.3	NS

RLW: Removal liver weight; ELB: Estimated loss of bleeding; BW: Body weight; OT: Operating time; NS: Not significant.

matoxylin and eosin and analyzed by the *in situ* terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method using an apoptosis *in situ* detection kit (Shanghai Yi Hua Scientifi c, Inc. China) according to the manufacturer's instructions[14]. The percentage of TUNEL-positive cells of total cell (apoptosis index, AI) nuclei in 10 high power fields were calculated for the 2 groups then compared. Proliferating cell nuclear antigen (PCNA) is a stable cell cycle nuclear. PCNA protein expression was detected by immunostaining using monoclonal anti-PCN-antibody (Jingmei Biotech Co. Ltd, Shenzhen, China). Data were expressed as the percentage of PCNA-stained hepatocytes per total number of hepatocytes (PCNA index). The mean numbers of PCNA-stained hepatocytes per 10 high power fields were calculated for the 2 groups, divided by the total cell number and then compared.

Malondialdehyde assay in liver tissue

Tissue samples were obtained at pre-hepatotectomy, 60 min, 180 min after hepatectomy for hepatic malondialdehyde (MDA) measurement. Hepatic MDA levels were determined using a agents were purchased from the Nanjing Jiangcheng Bioengineering Institute (Nanjing, China), measured according to the manufacturer's instructions. MDA levels were normalized against protein (pmol/mg).

Serum TNF-α and IL-6 measurement

Serum TNF-α and IL-6 measurement reagents were purchased from the Nanjing Jiangcheng Bioengineering Institute (Nanjing, China). TNF-α and IL-6 ELISA kits (Shanghai Yi Hua Scientifi c Inc., China). TNF-α and IL-6 were measured according to the manufacturer's instructions.

Statistical analysis

Values of parameters are presented as mean ± SD. Statistical significance was determined by Student's *t*-test. Fisher's exact test was used for comparison of adhesions. $P < 0.05$ was considered significant.

RESULTS

Characteristics of the experiment

There were no significant difference between two groups in body weight, removal liver weight, estimated loss of bleeding and operating time (Table 1).

PVP

There was no significant difference in changes of PVP between two groups at pre-operation, PH 30 min (Figure 1). The PVP in hydrogen gas treated-group and moderately increased beyond that measured at laparotomy. The PVP in Contrast-group continue to rise at 3 h of posthepatotectomy, but in hydrogen gas treated-group it slightly decrease and lower than its in Contrast-group, although there no statistical significant differernce ($P = 0.06$).

Hepatocellular injury

The preoperative and serial postoperative measurements of serum ALT and AST, are shown in Figure 2, on which significant differences are noted. There were no significant difference between two groups at pre-operation. After hepatotectomy ALT and AST increased in all of the animals and its in hydrogen gas treated-group was significantly lower comparing to Contrast-group ($P = 0.036$, $P = 0.011$).

Malondialdehyde assay in liver tissue

The serial change of hepatic MDA level in two groups was shown in Figure 3A. Baseline of hepatic MDA between two groups were no significant difference ($P = 0.747$). One hour after massive hepatotectomy, the MDA concentration increased in all the swines. In hydrogen gas treated-group, H_2 gas significantly decreased levels of hepatic MDA, a marker of oxidative stress, the MDA level was significantly low to Contrast-group ($P = 0.0005$, $P = 0.0004$).

Serum HA

The serial change of serum HA level in two groups were showed in Figure 3B. Baseline of HA between two groups were no significant difference ($P = 0.488$). One hour after subtotal hepatotectomy, the HA concentration in serum increased in all the pigs. In Hydrogen-group, the HA level was significantly low to Contrast-group ($P = 0.0051$, $P = 0.0052$).

DISCUSSION

The risk of PLF is the central problem in the field of liver resection[2,3]. This is principally due to the PLF or SFSS, an excessive and destructive portal flow through a remnant liver that is too small, which becomes functionally insufficient[3,4,15], the intraoperative injury including ischemia and inflammatory response is another important pathogenic factors involved in postoperative liver dysfunction and hepatic failure. In studies of extended hepatectomy in dogs, severe damage to the sinusoidal endothelial cells (SECs) of the remnant liver 3 h after the operation was one of the main factors responsible for the high mortality rates[16,17]. Therefore, to reducing the intraoperative injury sometimes is determinant to prevention the PLF or SFSS, when the intraoperative damage is irreversible. It is well known oxidative stress is a major

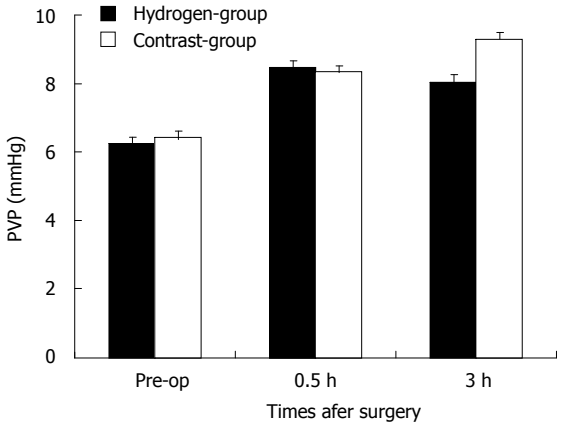

Figure 1 Serial changes of portal vein pressure in two groups. A bar graph shows the mean ± SD of portal vein pressure (PVP) (mmHg) in the two groups. Each group is represented by the mean of 7 swines. There was no significant difference in changes of PVP between two groups at the pre-operation (pre-op), 0.5 h and 3 h.

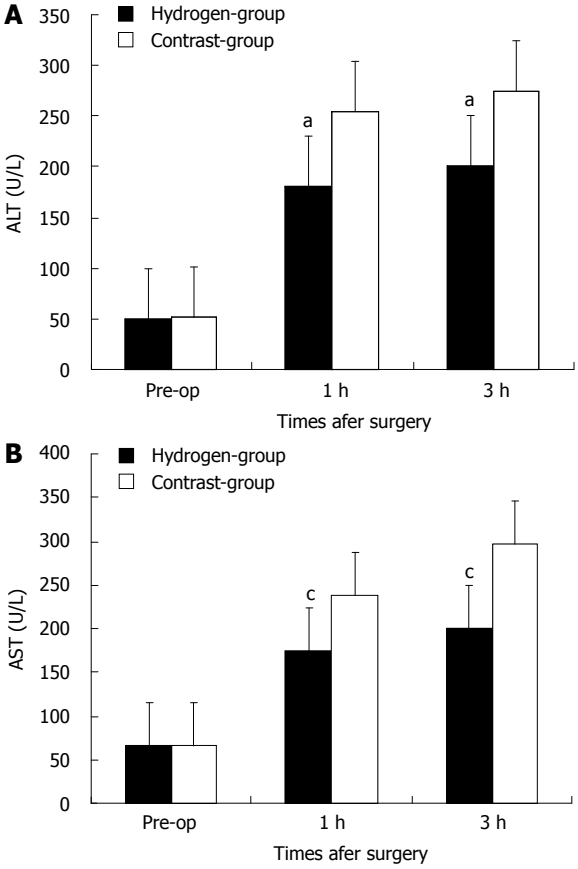

Figure 2 Change of serum alanine aminotransferase level and serum aspartate aminotransferase level in two groups. Each group is represented by the mean of 7 swines. A, B: In hydrogen gas treated-group, the alanine aminotransferase (ALT) (A) and aspartate aminotransferase (AST) (B) levels were significantly lower to Contrast-group. $^{a}P < 0.05$ *vs* ALT level in Contrast-group; $^{c}P < 0.05$ *vs* AST level in Contrast-group. Pre-op: Pre-operation.

contributor to the development of various hepatic disorders including acute hepatic failure, at present, there are no effective agents to alleviate the oxidative stress during clinically operation. However, molecular H_2, has recently been defined as a novel antioxidant, which selectively quenches detrimental the reactive oxygen species (ROS), while maintaining metabolic oxidation reduction reaction and other less potent ROS[10,11,18], indicating it is promising strategies to alleviate intraoperative injury.

In massive hepatotectomy, the intrahepatic vascular space in the remnant liver experiences a drastic reduction, and this leads to portal congestion and hemodynamic instability[4,5,9], the ischemia attenuate the instability, increases the metabolic burden, mitochondria produce more oxygen radicals. During the hepatic inflow occlusion, the intestinal congestion causing the damage of intestine barrier function and the increase of endotoxin absorbtion or bacterial translocation, however, the function of reticuloendothelial system decrease due to the removing of most of liver masss, which contained a lot of phagocyte[6,9,17]. Each individual Kupffer cell in a small-remnant is exposed to higher amounts of endotoxin than that in a whole liver, and triggers Kupffer cells to release a large quantity of free radicals. Lipid peroxidation, which plays a significant role in oxidative damage[18,19], was measured indirectly by assessing the increases in the levels of a lipid peroxidation product, MDA[19-21]. The MDA level is widely used as an indicator of free radical-mediated lipid peroxidation injury. In the present study, serum levels of MDA in contra-group increased rapidly during hepatotectomy (Figure 3A). The observed increase of liver MDA levels was an indicator of lipid peroxidation, which also verified the oxidative damage in the liver tissue in this animal model. While H_2 inhalation inhibited this increase significantly, inhalation of H_2 gas dramatically decreased MDA levels almost to the normal level.

In the Contrast-group and Hydrogen-group, there were significant endothelial denudation in the medium-sized portal vein branches, sinusoidal dilation, hydropic changes of hepatocytes and hemorrhage into perivenular connective tissue, which extended into the hepatic parenchyma (Figure 4B), and there was no intraparenchymal hemorrhage present in H_2 group (Figure 4A). Transmission electron microscopic photographs of the sinusoid was shown (Figure 4C and D). In the Hydrogen-group, the SECs (arrows) and hepatocytes were well preserved, and the structure of the endothelial lining can also be perceived (Figure 4C), in contrast, the sinusoidal endothelial lining was partially destroyed and detached into the sinusoidal space with enlargement of the Disse's spaces (asterisks). Cluster of differentiation molecule 31 (CD31) immunostaining was notable for destruction of the endothelial lining among animals in Contrast-group (Figure 4F) and, in contrast to mild sinusoidal microarchitecture injury in Hydrogen-group (Figure 4E).

Many studies had demonstrated that the high shear-stress or hyperperfusion, due to small liver remnant could cause the sinusoidal endothelial injury, and hepatocyte injury, swelling degeneration of hepatocytes[22,23]. In Contrast-group, the portal overflow damage the 30% liver remnant underwent 20 min ischemia, causing the endothelial denudation, sinusoidal dilation, hydropic changes of hepatocytes and hemorrhage into perivenular con-

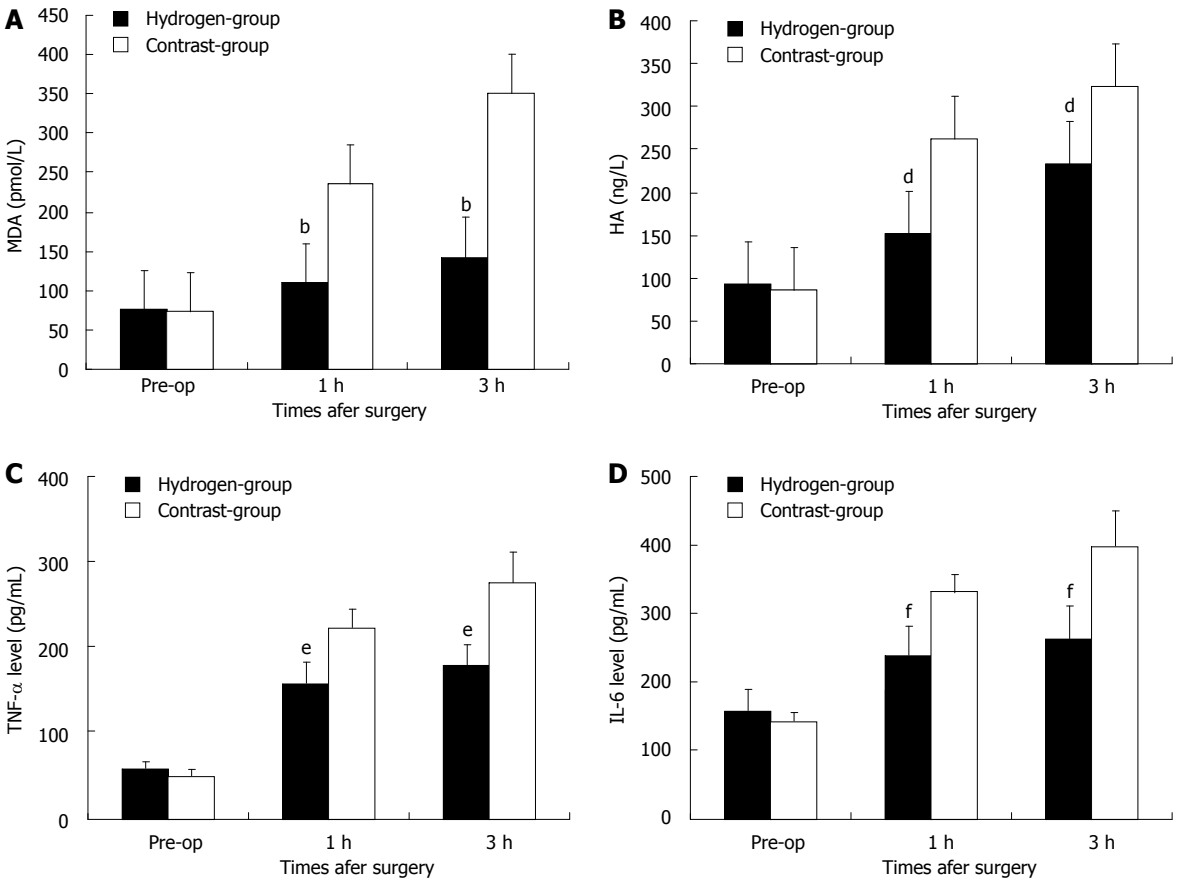

Figure 3 Changes of hepatic malondialdehyde, hyaluronic acid, tumer necrosis factor-α and interleukin-6 levels in two groups. A bar graph shows the mean ± SD of hepatic malondialdehyde (MDA) (A), hyaluronic acid (HA) (B), tumer necrosis factor (TNF)-α (C) and interleukin (IL)-6 (D) level in two groups. Each group is represented by the mean of 7 swines. A, B: In hydrogen gas treated-group, the MDA (A) and HA (B) levels were significantly lower to Contrast-group; C, D: In hydrogen gas treated-group, the TNF-α (C) and IL-6 (D) levels were significantly lower to Contrast-group. Pre-op: Pre-operation. $^{b}P < 0.01$ *vs* MDA level in Contrast-group; $^{d}P < 0.01$ *vs* HA level in Contrast-group; $^{e}P < 0.01$ *vs* TNF-α level in Contrast-group; $^{f}P < 0.01$ *vs* IL-6 level in Contrast-group.

nective tissue, in contrast, the H_2 inhalation alleviated the hyper reperfusion, make the rise in PVP low to Contrast-group, attenuate markedly these injuries (Figure 4A and B). It also identified by the measurement of HA level. HA is synthesized by mesenchymal cells and eliminated in the hepatic sinusoidal endothelium; increased serum HA levels reflect sinusoidal endothelial damage[24,25]. In the present study, an elevation in serum HA level,caused by liver hyperfusion is also significantly low to Contrast-group, indicating the effect of H_2 on hyperfusion injury in the hepatic sinusoidal endothelial (Figure 3B). CD31 immunoglobulin helps maintain endothelial stability by interdigitating with other CD31 molecules at the extracellular border of adjacent cells[26]. The study also showed that hydrogen-inhalation decreased the expression of CD31 molecules (Figure 4E and F), it means the H_2 can reduce the injury of hyperperfusion,and was further demonstrated by the observation of transmission electron microscope examination (Figure 4C and D).

Normal liver has vigorous regenerative potential, portal hyperperfusion is likely to be an important physiologic trigger that stimulates liver regeneration[27,28]. The strength of the regenerative stimulus is proportional to the increase in portal blood flow, as previous shown in experimental animals[29]. The results in the study revealed also there were no significantly difference between the two group at PH 3 h (Figure 5). This means the liver regeneration in the early stage was determined to the portal shear stress, although H_2 decrease free radicals injury. On the other hand, apoptotic cell death is an important contributor to the organ failure common to ALF, even for etiologies thought traditionally to involve mainly hepatocyte necrosis[25,26]. The free radicals may exert a strong cytotoxic effect, and played an important role in inducing apoptosis in the postoperative liver insufficient. The study demonstrated the AI in the hydrogen gas treated-group significantly decrease comparing to the Contrast-group (Figure 6A), this probably attribute to the protection of the H_2 against injury of ROS. Therefore, H_2 play an important role in deceasing the injury of SFSS with decreasing the apoptosis of hepatocyte, without increasing the regeneration.

Even though free radical scavengers have been demonstrated to reduce liver I/R damage[29,30], this is the first observation that the H_2 decreases PVP or hyperperfusion injury in an animal model, which is determinant factor to PLF or SFSS. We observed that H_2 inhalation reduced not only morphological injury, but also serum ALT, AST,

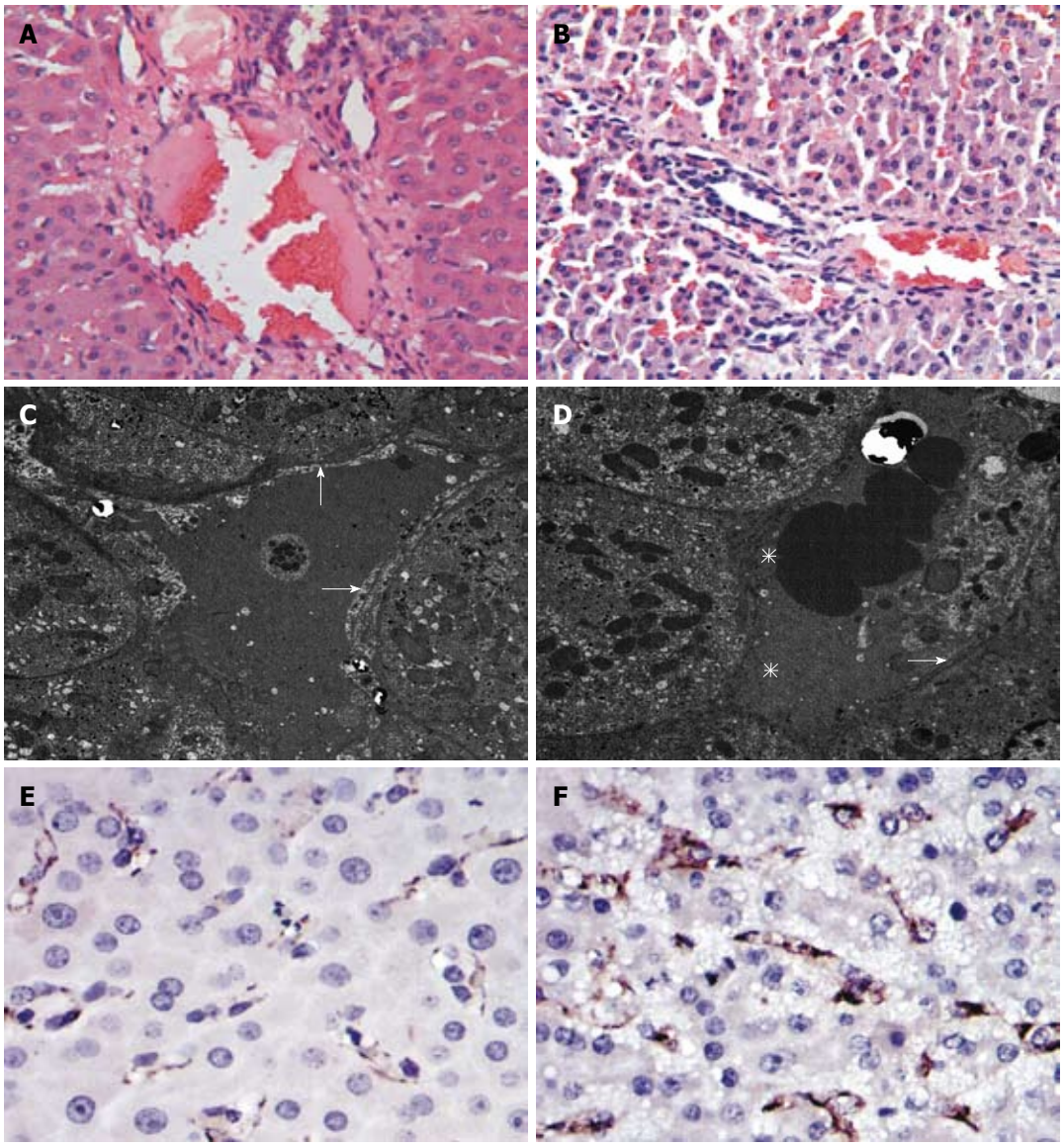

Figure 4 Hematoxylin and eosin, transmission electron microscopic photographs and cluster of differentiation molecule 31 immunohistochemical staining of tissue samples taken 3 h after hepatotectomy. A: Hematoxylin and eosin (HE) staining of the Contrast-group; B: HE staining of the hydrogen gas treated-group; C, D: Transmission electron microscopic photographs of the sinusoid, arrows indicatethe sinusoidal endothelial, asterisks indicate the enlargement of the Disse's spaces; E: Cluster of differentiation molecule 31 (CD31) immunostaining of the hydrogen gas treated-group; F: CD31 immunostaining of the Contrast-group.

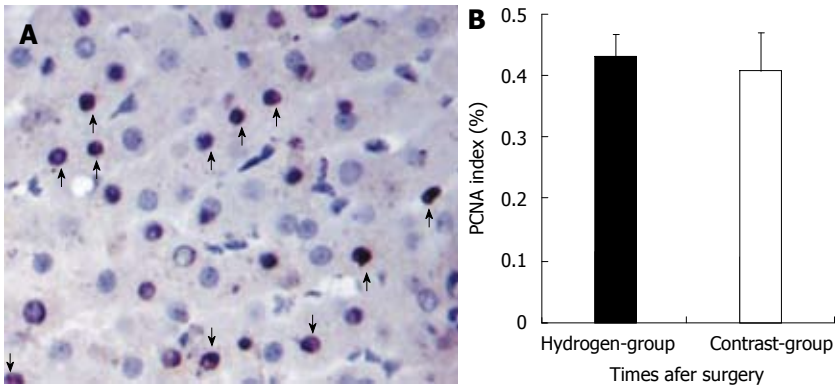

Figure 5 Proliferating cell nuclear antigen immunostaining in liver and the percentage of proliferating cell nuclear antigen stained in two groups. A: Proliferating cell nuclear antigen (PCNA) staining in liver remnant (arrows, positive cell: × 400); B: Microphotometric evaluation in PCNA stained tissue after hepatotectomy for 3 h between two groups. A bar graph shows the mean ± SD of PCNA stained level (%) in two groups. Each group is represented by the mean of 7 swines.

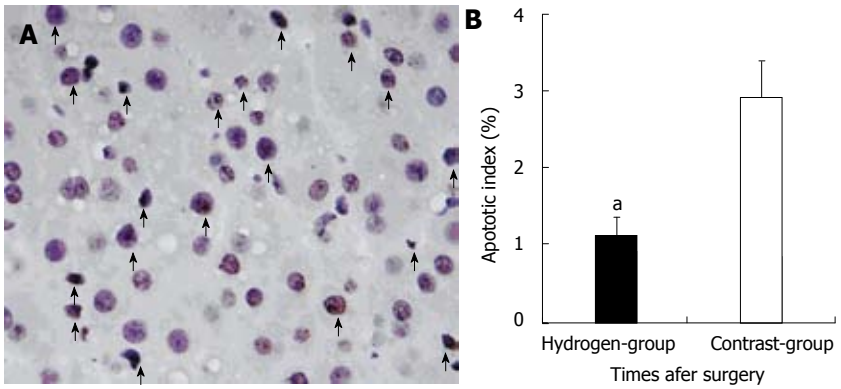

Figure 6 Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling staining after hepatectomy and protective effect of H_2 against liver apoptotic cell death in two groups. A: Revealed many terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL)-positive cells (arrows, identified morphologically by dark brown staining nuclei) in the liver remnant × 400; B: Microphotometric evaluation of apoptotic index (AI) in TUNEL-stained tissue after hepatotectomy for 3 h. A bar graph shows the mean ± SD of AI level (%) in two groups. Each group is represented by the mean of 7 swines. In hydrogen gas treated-group, the AI level was significantly low to Contrast-group ($^aP < 0.05$ *vs* Contrast-group).

IL-1, TNF-α (Figures 2 and 3C, D). We especially investigated the effect of hydrogen on oxidative stress PVP, and injury/regeneration in this liver hypefusion injury model. The use of gas inhalation to treat diseases has become increasingly popular. There are three endogenous gas include nitric oxide, carbon monoxide and H_2 sulfate. The increased production of these gases under stress conditions may reflect the active involvement of these gases in the protective response[31-33]. However, the inherent toxicity of these gases must be investigated for gas inhalation to be considered an effective therapeutic strategy. H_2 is not produced endogenously in mammalian cells since the hydrogenase activity responsible for the formation of H_2 gas has not been identified.

In conclusion, intraoperative H_2 inhalation in massive hepatotectomy was feasible and can protected the liver injury from hyperperfusion, by reduction of inflammation and oxidative stress, liver remnant apoptosis or necrosis, although it didn't increase the regeneration. However, the exact mechanism and signalling pathway involved in the protection role of H_2 in the small liver remnant injury need to be studied in the future. It is required to fully exploit inhalation of H_2 gas as a therapeutic strategy.

COMMENTS

Background

Hydrogen selectively reduce levels of hydroxyl 1 radicals and alleviates acute oxidative stress in many animal models. But most of these study were used in small animal models and lack of the study in big animal which provide a much more clinically relevant means of investigating the pathophysiology of a disease process. In this study, the authors investigated firstly the effect of H_2 gas on small liver remnant injury or "small of size" syndrome (SFSS) after massive hepatectomy, and its feasibility in clinic.

Research frontiers

It is well known oxidative stress is a major contributor to the development of various hepatic disorders including acute hepatic failure, at present, there are no effective agents to alleviate the oxidative stress during clinically operation. However, molecular H_2, has recently been defined as a novel antioxidant, which selectively quenches detrimental the reactive oxygen species (ROS), while maintaining metabolic oxidation reduction reaction and other less potent ROS, indicating it is promising strategies to alleviate intraoperative injury. Oxidative stress is regarded as a major contributor to the development of various hepatic disorders including acute hepatic failure, hepatic fibrosis, and hepatic cancer. Through ameliorating oxidative stress, H_2 becomes an important potential anti-oxygen spices agent in clinic.

Innovations and breakthroughs

Even though free radical scavengers have been demonstrated to reduce liver ischemia reperfusion damage, this is the first observation that the H_2 decreases portal vein pressure or hyperperfusion injury in an animal model, which is determinant factor to post-operative liver failure or SFSS. It was also firstly demonstrated the feasibility of intraoperative inhalation in big animals. As a kind of gas, intraoperative inhalation was convenient and safe.

Applications

The present study demonstrated firstly the protective effect of H_2 gas on liver ischemia-reperfusion injury (I/R-I), toxic liver injury, and portal hyperperfusion injury in swine, that the physiology is similar to human, indicating intraoperative H_2 gas inhalation will be a treatment modality as potential anti-inflammation response agent in clinic .

Peer review

In this study, many biochemical markers mostly investigated in the study of hepatic I/R-I were evaluated and showed that inhaled hydrogen gas attenuated the I/R-I. This study gives a new insight into the pivotal role of hydrogen gas toward the hepatic I/R-I in clinical settings because hydrogen gas was used in swine model.

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ORIGINAL ARTICLE

Repair of bile duct defect with degradable stent and autologous tissue in a porcine model

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Author contributions: Cai XJ designed the study; Liang YL, Yu YC, Wang WJ, Liu K, Ying JB carried out the study; Wang YF provided analytic tools and analyzed the data; and Liang YL and Wang YF wrote the paper.
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Abstract

AIM: To introduce and evaluate a new method to repair bile duct defect with a degradable stent and autologous tissues.

METHODS: Eight Ba-Ma mini-pigs were used in this study. Experimental models with common bile duct (CBD) defect (0.5-1.0 cm segment of CBD resected) were established and then CBD was reconstructed by duct to duct anastomosis with a novel degradable stent made of poly [sebacic acid-co-(1,3-propanediol)-co-(1,2-propanediol)]. In addition, a vascularized greater omentum was placed around the stent and both ends of CBD. Cholangiography *via* gall bladder was performed for each pig at postoperative months 1 and 3 to rule out stent translocation and bile duct stricture. Complete blood count was examined pre- and post-operatively to estimate the inflammatory reaction. Liver enzymes and serum bilirubin were examined pre- and post-operatively to evaluate the liver function. Five pigs were sacrificed at month 3 to evaluate the healing of anastomosis. The other three pigs were raised for one year for long-term observation.

RESULTS: All the animals underwent surgery successfully. There was no intraoperative mortality and no bile leakage during the observation period. The white blood cell counts were only slightly increased on day 14 and month 3 postoperatively compared with that before operation, the difference was not statistically significant (P = 0.652). The plasma level of alanine aminotransferase on day 14 and month 3 postoperatively was also not significantly elevated compared with that before operation (P = 0.810). Nevertheless, the plasma level of γ-glutamyl transferase was increased after operation in both groups (P = 0.004), especially 2 wk after operation. The level of serum total bilirubin after operation was not significantly elevated compared with that before operation (P = 0.227), so did the serum direct bilirubin (P = 0.759). By cholangiography *via* gall bladder, we found that the stent maintained its integrity of shape and was still *in situ* at month 1, and it disappeared completely at month 3. No severe CBD dilation and stricture were observed at both months 1 and 3. No pig died during the 3-mo postoperative observation period. No sign of necrosis, bile duct stricture, bile leakage or abdominal abscess was found at reoperation at month 3 postoperatively. Pigs had neither fragments of stent nor stones formed in the CBD. Collagen deposit was observed in the anastomosis by hematoxylin and eosin (HE) and Masson's trichrome stains. No severe cholestasis was observed in liver parenchyma by HE staining. Intestinal obstruction was found in a pig 4 mo after operation, and no bile leakage, bile duct stricture or biliary obstruction were observed in laparotomy. No sign of bile duct stricture or bile leakage was observed in the other two pigs.

CONCLUSION: The novel method for repairing bile

duct defect yielded a good short-term effect without postoperative bile duct stricture. However, the long-term effect should be further studied.

Key words: Degradable stent; Bile duct defect; Biliary reconstruction; Autologous tissue; Omentum

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INTRODUCTION

Laparoscopic cholecystectomy (LC) has been the gold standard procedure for benign gall bladder diseases. Unfortunately, the widespread application of LC has led to a concurrent rise in the incidence of major bile duct injuries (BDI) ranging from 0.3% to 0.65%[1-5]. Occurrence of BDI results in difficult reconstruction, even for experienced hepatobiliary surgeons, and a prolonged hospital stay and a high risk of long-term complications. Roux-en-Y hepaticojejunostomy has been the most commonly used approach for biliary reconstruction, especially in cases of duct transection injury[1,6-9]. But its long-term outcome is still far from satisfied due to the high incidence of reflux cholangitis, choledocholithiasis, anastomotic stenosis caused by scar contracture[10-12] and even canceration[13-15]. In recent years, primary duct-to-duct reconstruction has been used in living-donor liver transplantation and has gained good effects[16-18]. It preserves the function of Oddi's sphincter, which provides a barrier to prevent any reflux into the bile duct[13]. However, major drawbacks, including early ischemic necrosis, leakage, and late anastomotic stricture, cannot be overcome so far. In this research, we created a novel method for bile duct defect repair, and proved its feasibility and safety.

MATERIALS AND METHODS

Animals

Eight experimental Ba-Ma mini-pigs of either sex, weighing 15-20 kg, were provided by Shanghai Multi-Bio-Sci-Tech Co., Ltd., China (license No: SCXK 2005-0002). The animals were housed one per cage at the Experimental Animal Center at Zhejiang University. They were allowed to be accustomed to the laboratory environment for more than one week before the start of the experiment. All animals had free access to water and standard food until the day before surgery. The study was approved by the Ethics Committee of Zhejiang University.

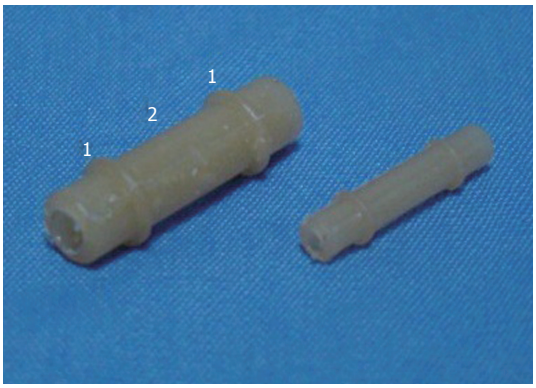

Figure 1 Biodegradable stents of different sizes. 1: Hump rings; 2: Main spindle, 10 mm in length between two hump rings.

Stents

The stent (Figure 1) is made of a novel biodegradable elastomer which is manufactured by the Institute of Polymer Science of Zhejiang University. The elastomer is synthesized with 1,3-propanediol, 1,2-propanediol, and sebacic acid, and then shaped into hollow tube by injecting molding, with a hump ring in both ends. The stent used in the research is 6 mm in external diameter, 1 mm in thickness, and 4 mm in inner diameter. The distance between two hump rings is 10 mm. The stent can completely degrade into carbon dioxide and water in about 3 mo when submerged in fresh human bile *in vitro*. The biocompatible quality of the novel elastomer was approved by the State Food and Drug Administration of China (No. G20090993).

Experimental design

A total of eight Ba-Ma mini-pigs (15-20 kg) were included in this study. The pigs were fasted for 24 h before surgery. Experimental models with common bile duct (CBD) defect (0.5-1.0 cm segment of CBD resected) were established and CBD was reconstructed by duct to duct anastomosis with the novel biodegradable stent. In addition, a vascularized greater omentum was placed around the stent and both ends of CBD. The incidence of jaundice and bile leakage was evaluated. Five pigs were subjected to the examination of complete blood count before operation and on day 14 and month 3 after operation to estimate the inflammatory reaction. Alanine aminotransferase (ALT), γ-glutamyl transferase (γ-GT), serum total bilirubin (TBIL) and direct bilirubin test were also examined pre- and post-operatively to evaluate the liver function. Cholangiography was performed for each pig on months 1 and 3 postoperatively to rule out stent translocation and bile duct stricture. The five pigs were reoperated on to observe anastomosis three months after operation and were sacrificed immediately after that. The

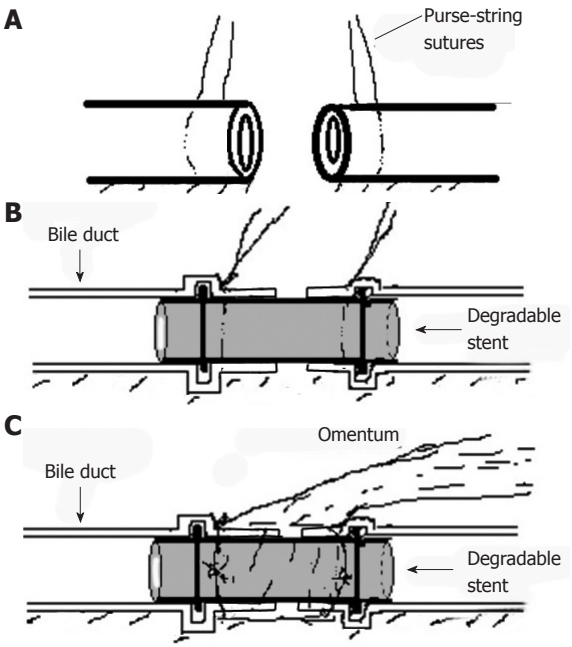

Figure 2 Schematic diagram of repairing bile duct defect with degradable stent and omentum. A: Purse-string sutures with 4-0 Vicryl were made on both ends of bile duct; B: The stent was inserted into both ends of bile duct; C: A vascularized greater omentum was placed around the stent and both ends of common bile duct.

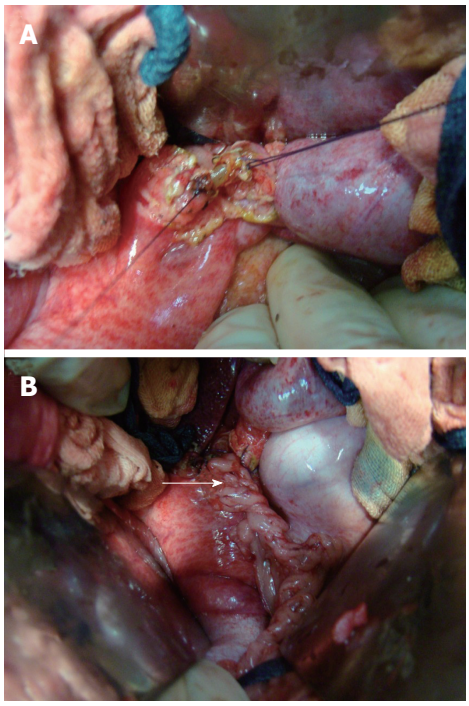

Figure 3 Surgical procedure. A: Stent is placed into common bile duct and two purse-string sutures are tied; B: A layer of large omentum is covered around the stent and two ends of bile duct (arrow).

peritoneal cavity was observed for signs of bile leakage and stricture. The anastomosis was evaluated pathologically, including hematoxylin and eosin (HE) stain and Masson trichrome stain. Liver tissue slides with HE staining were also observed. The other three pigs were raised for one year for long-term observation.

Procedure

Pigs were anesthetized by intramuscular injection of pentobarbital sodium solution (20 mg/mL) with 0.1 mL/kg. Laparotomy was performed *via* a midline incision. Hepatic hilar was dissected firstly to free the CBD. Then a 0.5-1.0 cm segment of CBD under junction of cystic duct was resected. The CBD was reconstructed by duct to duct anastomosis with a stent: purse-string sutures with 4-0 Vicryl (Ethicon, Somerville, NJ, United States) were made on both ends of bile duct (Figure 2A). The stent was inserted into the both ends of bile duct as shown in Figure 2B and 3A. Two ends of bile duct were slightly closed and the purse-string sutures were tied to fix both ends of bile duct on the stent to reconstruct the CBD. A vascularized greater omentum was placed around the stent and both ends of CBD (Figure 2C and 3B).

All pigs were given free access to water, but without food for 24 h postoperatively. They were given half of their normal diet on postoperative day 2. Normal diet was resumed on postoperative day 3.

Statistical analysis

For statistical analysis, the Kruskal-Wallis test was used. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using the SPSS statistical software package (Version 13.0, SPSS Inc, Chicago, IL).

RESULTS

Surgery was successful in all the cases. There was neither intraoperative death nor bile leakage detected during the observation period. The white blood cell counts were only slightly increased on day 14 and month 3 postoperatively compared with that before operation, the difference was not statistically significant. The plasma level of ALT on day 14 and month 3 postoperatively was also not significantly elevated compared with that before operation. Nevertheless, the plasma level of γ-GT was increased after operation in both groups, especially 2 wk after operation. The plasma level of TBIL after operation was not significantly increased compared with that before operation, so did the plasma level of serum direct bilirubin (Table 1).

By cholangiography *via* gall bladder, we found that the stent maintained its integrity of shape and was still *in situ* on month 1, and it disappeared completely on month 3. No severe CBD dilation and stricture were observed both on months 1 and 3 (Figure 4).

No pig died during the 3-mo postoperative observation period. No sign of necrosis, bile duct stricture, bile leakage or abdominal abscess was found when the animals were reoperated on month 3 postoperatively, and none of the pigs had fragment of stents, and stones formed in the CBD (Figure 5).

Histologically, collagen deposit and bile duct glands proliferation were observed in the anastomosis by hema-

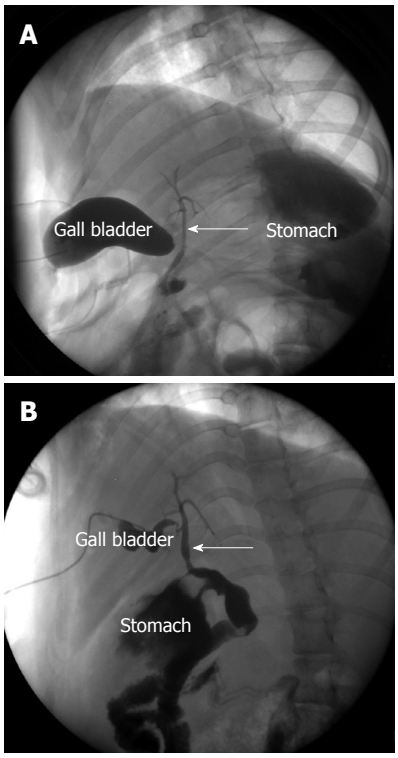

Figure 4 Cholangiography through gall bladder. A: One month after operation, the shape of the stent (arrow) could be visualized in common bile duct; B: Three months after operation, the stent disappeared.

Table 1 Laboratory values

	Before operation	After day 14	Operation month 3	*P* value
WBC	16.62	18.5	18.34	0.652
ALT	51.2	57.2	54.4	0.810
γ-GT	50.2	125.8	72.8	0.004
DBIl	1.1	1.78	1.44	0.227
TBIl	1.76	2.2	2.06	0.759

WBC: White blood cell; ALT: Alanine aminotransferase; γ-GT: γ-glutamic peptidase; DBIl: Direct bilirubin; TBIL: Total bilirubin.

toxylin and eosin and Masson's trichrome stains (Figure 6). No severe cholestasis was found in liver parenchyma by HE staining.

In long-term observation, a pig had intestinal obstruction 4 mo after operation, but no bile leakage, bile duct stricture or biliary obstruction were observed in laparotomy. There was no sign of bile duct stricture or bile leakage in the other two pigs.

DISCUSSION

Bile duct injury is a major complication in biliary surgery such as LC. It can be classified by several classification systems according to the injured site, e.g., Bismuth classification[19] and Strasberg classification[20]. In some cases, bile duct was partially resected and reconstruction with end to end suture was almost impossible for the high incidence of bile leakage and postoperative anastomosis stricture[21,22].

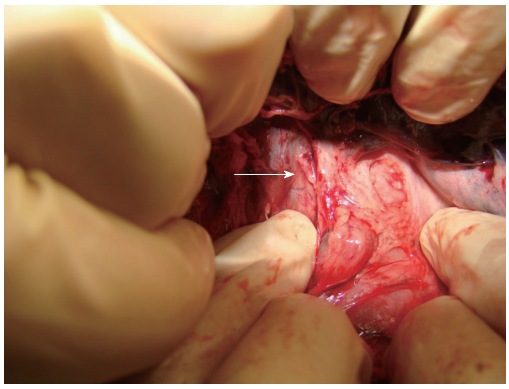

Figure 5 The common bile duct (arrow) three months after operation.

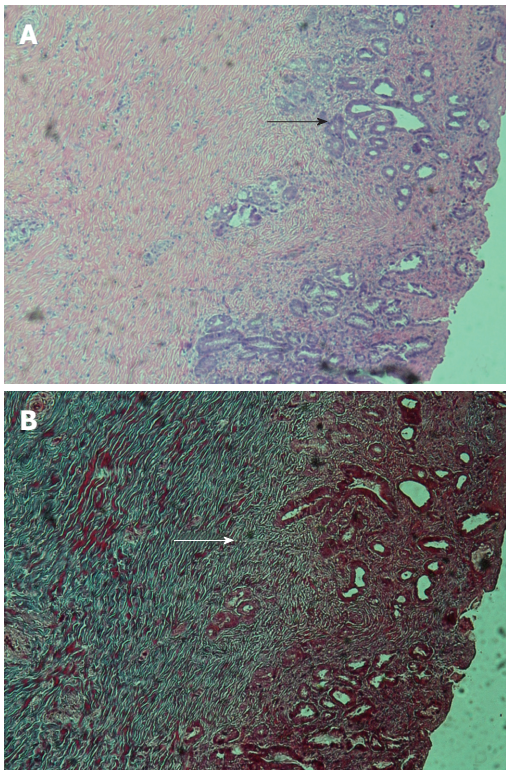

Figure 6 Histology of anastomosis. A: Hematoxylin and eosin stain × 100, black arrow indicates proliferation of bile duct glands; B: Masson's trichrome stain × 100, white arrow indicates deposited collagen fibers.

To keep the biliary continuity and integrality of function is an essential principle for biliary reconstruction. A Roux-en-Y hepaticojejunostomy has been the most commonly used procedure for CBD defect. However, the function of Oddi's sphincter was lost in patients undergoing this procedure, leading to a high incidence of postoperative reflux cholangitis[13,23]. In this research, we aimed to create a new method to repair bile duct defect without sacrificing the function of Oddi's sphincter.

In this procedure, the stent played an important role. The stent was made of degradable material named poly [sebacic acid-co-(1,3-propanediol)-co-(1,2-propanediol)]

(PSPP)[24-26]. Degradable stent had a number of advantages, especially in eliminating the need for stent removal[27,28]. The PSPP belongs to polyester elastomer and possesses a good biocompatibility which is essential for medical applications. The stent connected the two ends of bile duct to maintain the continuity of biliary structure, and it was also used as an inner stent to prevent anastomosis restenoses[22,29,30]. Its degradation time in bile duct is set between 2 and 3 mo, conforming to the healing process of bile duct.

According to the previous reports, the defect of bile duct could be replaced by autologous veins and stents (both silicone stents and biodegradable stents) and they concluded that construction of bile duct appeared to take place by tissue migration[31-34]. Blood supply is important for tissue migration and repair of bile duct defect[35], so vascularized large omentum may be a better autologous tissue compared with veins for temporary repair before bile duct tissue migration was completed. With the assistance of the degradable stent and omentum, we do not need to free much residual bile duct or to mobilize the duodenum to make sure that the anastomosis is tension-free and properly vascularized[36].

Taking into account that bile leakage and bile duct stricture were the two major complications, postoperative cholangiography through gall bladder was repeated to rule out these complications, and liver function was also examined repeatedly to rule out the complication of biliary stricture. The postoperative level of bilirubin and ALT was not significantly elevated compared with that before operation. No obvious bile duct dilatation or stenosis was observed in any pigs by cholangiography. As a result, there was no sign of bile leakage or biliary stricture.

Histologically, collagen deposit and bile duct gland proliferation were observed in the anastomosis three months after operation by HE and Masson's trichrome stains. The neonatal biliary tissue had completely replaced the omentum. Liver parenchyma was also observed by HE stain to rule out cholestasis, and positive result was found. Those results proved that the method is feasible and the biliary tissue can migrate along the surface of stent.

The stent degraded in 3 mo in this research, and the presence of stricture after degradation of the stent is essential to the success of this method, so three pigs were raised for one year to observe its long-term effect. One pig was excluded from long-term observation due to the complication of intestinal obstruction occurring in month 4 after operation and no sign of biliary stricture was found in laparotomy.

This is also a simple method. Only two purse-string sutures are needed without end to end suture and dissection of vein. Vascularized large omentum is easy to mobilize and cover around defect and anastomosis. In addition, two sizes of the stent are available for different situations; the stent would be easily placed into the bile duct if a proper size is selected.

These results suggested that it is a feasible method for repairing bile duct defect, however, the long-term effect (more than one year) should be further observed.

COMMENTS

Background

By the early 1990s, laparoscopic cholecystectomy (LC) had replaced open cholecystectomy as the gold standard procedure for benign gall bladder diseases. Nevertheless, the widespread application of LC has led to a concurrent rise in the incidence of major bile duct injuries. The management of major bile duct injury is a surgical challenge even for experienced hepatobiliary surgeons at tertiary referral centers. Hepaticojejunostomy and duct to duct anastomosis are two commonly used methods for repairing bile duct injury. Despite their advantages, both methods have their critical defects which are difficult to overcome.

Research frontiers

Bile duct injury is a severe complication encountered by almost every hepatobiliary surgeon. The management of major bile duct injury has become a hotspot in clinical studies. With the development of tissue engineering, several researches on biodegradable materials for biliary duct have been conducted.

Innovations and breakthroughs

In this research, the authors created a new method for repairing the bile duct defect with satisfactory effect in a three-month observation period. A novel anastomotic stent and omentum are involved in the new method. By this method, a larger proportion of patients can undergo end-to-end anastomosis and avoid the need for a hepaticojejunostomy. It is also of importance to preserve the function of Oddi's sphincter.

Applications

The study results suggest that repairing bile duct defect by this new method yielded a good short-term effect without postoperative bile duct stricture. It might be applicable in clinical settings in the near future.

Terminology

Elastomer is a class of polymers with a good elasticity. Poly [sebacic acid-co-(1,3-propanediol)-co-(1,2-propanediol)] belongs to polyester elastomer which is considered to have a good biocompatibility.

Peer review

The authors studied the efficacy of the new degradable stent for the bile duct repair in pig experiment. And they concluded that it is a feasible method for repairing bile duct defect. The idea and research of this study are very interesting and applicable for clinical settings in the near future.

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BRIEF ARTICLE

Clinical outcome and predictors of survival after TIPS insertion in patients with liver cirrhosis

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Abstract

AIM: To determine the clinical outcome and predictors of survival after transjugular intrahepatic portosystemic stent shunt (TIPS) implantation in cirrhotic patients.

METHODS: Eighty-one patients with liver cirrhosis and consequential portal hypertension had TIPS implantation (bare metal) for either refractory ascites (RA) (*n* = 27) or variceal bleeding (VB) (*n* = 54). Endpoints for the study were: technical success, stent occlusion and stent stenosis, rebleeding, RA and mortality. Clinical records of patients were collected and analysed. Baseline characteristics [e.g., age, sex, CHILD score and the model for end-stage liver disease score (MELD score), underlying disease] were retrieved. The Kaplan-Meier method was employed to calculate survival from the time of TIPS implantation and comparisons were made by log rank test. A multivariate analysis of factors influencing survival was carried out using the Cox proportional hazards regression model. Results were expressed as medians and ranges. Comparisons between groups were performed by using the Mann-Whitney *U*-test and the χ^2 test as appropriate.

RESULTS: No difference could be seen in terms of age, sex, underlying disease or degree of portal pressure gradient (PPG) reduction between the ascites and the bleeding group. The PPG significantly decreased from 23.4 ± 5.3 mmHg (VB) *vs* 22.1 ± 5.5 mmHg (RA) before TIPS to 11.8 ± 4.0 *vs* 11.7 ± 4.2 after TIPS implantation (P = 0.001 within each group). There was a tendency towards more patients with stage CHILD A in the bleeding group compared to the ascites group (24 *vs* 6, P = 0.052). The median survival for the ascites group was 29 mo compared to > 60 mo for the bleeding group (P = 0.009). The number of radiological controls for stent patency was 6.3 for bleeders and 3.8 for ascites patients (P = 0.029). Kaplan-Meier calculation indicated that stent occlusion at first control (P = 0.027), ascites prior to TIPS implantation (P = 0.009), CHILD stage (P = 0.013), MELD score (P = 0.001) and those patients not having undergone liver transplantation (P = 0.024) were significant predictors of survival. In the Cox regression model, stent occlusion (P = 0.022), RA (P = 0.043), CHILD stage (P = 0.015) and MELD score (P = 0.004) turned out to be independent prognostic factors of survival. The anticoagulation management (P = 0.097), the porto-systemic pressure gradient (P

= 0.460) and rebleeding episodes (P = 0.765) had no significant effect on the overall survival.

CONCLUSION: RA, stent occlusion, initial CHILD stage and MELD score are independent predictors of survival in patients with TIPS, speaking for a close follow-up in these circumstances.

Key words: Transjugular intrahepatic portosystemic stent shunt; Liver cirrhosis; Ascites; Gastrointestinal hemorrhage; Treatment outcome

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INTRODUCTION

Portal hypertension is a common problem in gastroenterology and the treatment of its complications is still a challenging task. Major complications of liver cirrhosis and portal hypertension include variceal bleeding (VB) and refractory ascites (RA)[1]. Despite a wide range of therapeutic modalities, including medical and surgical treatments, there is ongoing debate about the most effective treatment algorithm for the complications of portal hypertension[2-5].

At the end of the 1980's a new nonsurgical procedure was developed to enable decompression of the portal circulation *via* expandable metal stents between hepatic veins and the intrahepatic portal vein system - the transjugular intrahepatic portosystemic shunt (TIPS)[6-8]. Since then, the method has been established and improved systematically, culminating in the actual guidelines of the American Association for the Study of Liver Diseases (AASLD)[9].

Most clinicians agree that TIPS has an excellent hemostatic effect in VB (95%), with low rebleeding rates (< 20%)[10]. When endoscopic hemostasis of esophageal varices fails, TIPS becomes the first-line treatment of choice, with an estimated technical success rate in the range of 93%-100%[11-13]. Due to the circulatory effects on portal hypertension, TIPS is also an interesting approach in cases of RA[14-18] and hepatorenal syndrome[19]. However, following TIPS higher rates of hepatic encephalopathy are observed in patients with cirrhosis and RA[11]. Additionally, TIPS insertion has been reported to be successful in patients with portal vein thrombosis[20,21], Budd-Chiari syndrome[22] and portal cavernoma[23].

The use of bare metal stents has been the gold standard in TIPS procedure[24], but the higher occlusion rate with consecutive bleeding complications has recently led to the development of covered metal stents with significantly lower occlusion rates after TIPS implantation[25-28].

In a retrospective single centre study, we evaluated the efficacy and safety of TIPS in the treatment of portal hypertension using a self-expanding bare metal mesh-wire stent. The major objectives of the present study were to observe stenosis and occlusion rates, occurrence of rebleeding and predictors of survival.

MATERIALS AND METHODS

Patients

This retrospective single center study was conducted at the tertiary referral center of Muenster University Hospital (Department of Medicine B). One hundred and one patients were initially scheduled for TIPS implantation. Eventually 81 patients with complications of portal hypertension were enrolled from 1998 until 2008. Twenty patients were excluded because TIPS insertion was technically not feasible. The indication for TIPS treatment included acute or recurrent VB and RA.

Objectives of the study

Endpoints for the study analysis were: technical success (completed TIPS insertion, lowering of the portosystemic pressure gradient), rates of stent occlusion and stent stenosis, rebleeding, RA and mortality. Clinical records of patients were collected and carefully analysed. Baseline characteristics (e.g., age, sex) were retrieved as shown in Table 1.

Definitions

According to Bureau *et al*[29], the following definitions were used:

Stent dysfunction: > 50% reduction of the lumen of the stent at angiography with an increase of the portosystemic pressure gradient of more than 50% of the initial post-interventional value.

Recurrent VB: Recurrent VB that did not respond to the usual pharmacological and endoscopic therapy[30].

RA: Ascites that did not respond to conservative (low-salt diet) and pharmacological (diuretics) treatment or lack of treatment options because of treatment-induced complications[31].

Transjugular intrahepatic portosystemic stent procedure

All TIPS procedures were conducted in strong collaboration with an interventional radiologist and gastroenterologist at our hospital using standard techniques[32]. Through a transjugular venous approach, the right hepatic vein was catheterized. An intrahepatic branch of the portal vein was punctured. Before dilation of the liver parenchyma

Table 1 Baseline characteristics

Variables	Bleeding	Ascites	*P* value
Patients	54	27	
Age (yr)			0.497
mean ± SD	61.7 ± 10.4	63.3 ± 10.8	
Range	38-79	46-84	
Sex (male/female)	33/21	15/12	0.634
CHILD score			
A	24	6	0.052
B	28	18	NS
C	2	3	NS
MELD score	9.4 ± 4.9	13.7 ± 5.2	< 0.001
Underlying disease			
Chronic viral hepatitis B/C	7/1	2/1	0.949
Alcohol abuse	38	21	NS
Autoimmune hepatitis	2	0	NC
PSC/PBC	3	0	NC
Cryptogen	3	3	NS
Re-bleeding after TIPS	13	0	NC
PPG before TIPS (mmHg)	23.4 ± 5.3	22.1 ± 5.5	0.765
PPG after TIPS (mmHg)	11.8 ± 4.0	11.7 ± 4.2	0.883
Stent diameter[1] (mm)			
< 12/≥ 12	6/44	4/23	0.728
Anticoagulation after TIPS	31	9	0.042
LTX after TIPS	7	1	0.131
Median survival time (mo)	> 60	29	0.009
Number of radiological controls until evaluation	6.3 ± 4.8	3.8 ± 3.1	0.029
Time interval until first radiological control	9.3 ± 10.6	4.5 ± 5.6	0.133

[1]In four patients data acquisition of stent diameter not available. NS: Not significant; NC: Not calculated; LTX: Liver transplantation; TIPS: Transjugular intrahepatic portosystemic stent; PPG: Portal pressure gradient; MELD: Model for end-stage liver disease; PSC: Primary sclerosing cholangitis; PBC: Primary biliary cirrhosis.

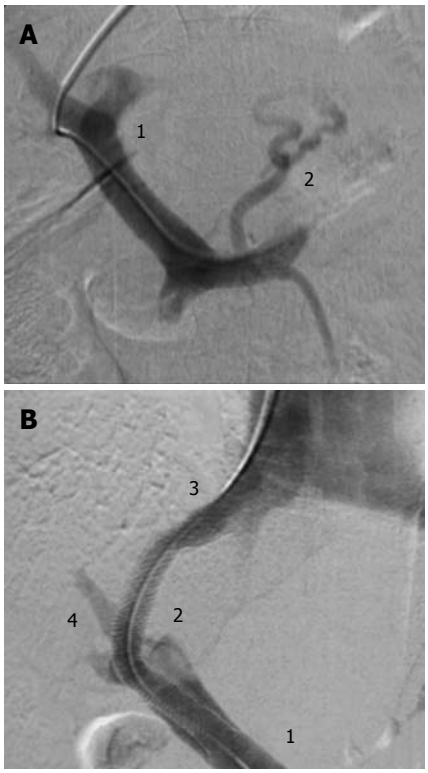

Figure 1 Fluoroscopic images showing transjugular intrahepatic portosystemic shunt placement procedure. A: Portogram after catheterisation of the portal vein, showing perfusion of the portal vein system (1) and oesophageal varices (2); B: Portogram after transjugular intrahepatic portosystemic stent placement. Contrast can be seen in the portal vein (1), through the shunt (2) flowing into the hepatic vein and inferior vena cava (3). Decompression of the portosystemic pressure can be seen in reduced contrast in the portal branch (4). The varices can no longer be identified in the fluoroscopic image.

both the portal pressure and the blood pressure of the right atrium were measured. Then the optimal stent length was defined using a special catheter with opaque markers. After the deployment of the bare metal stent the pressures of the portal vein and the right atrium were measured again. Pressures were measured using an Exadyn transducer set (Braun, Melsungen, Germany). The portal pressure gradient (PPG) resulted as the difference of the portal pressure minus the right atrium pressure (Figure 1). Postinterventional Doppler ultrasonography was carried out the day after TIPS insertion assessing stent patency. As presented by Sahagun *et al*[33] in 1997 shunt stenosis of bare metal stents can effectively be treated by interventional techniques to maintain patency. Stent stenosis due to endothelial growth usually occurs after 3 mo. It was therefore the policy of our institution to reevaluate each patient regularly with Doppler ultrasonography every 3 mo. Interventional angiography was performed every 12 mo or earlier when there was sonographic evidence of stenosis (fall of the initial increase of the portal blood velocity after stenting by > 50% according to Biecker *et al*[34]) or clinical features of recurrent portal hypertension (e.g., hepatic encephalopathy, worsening ascites, presence of high-risk varices at endoscopy or re-bleeding). A TIPS reintervention was performed, when a restenosis or occlusion was affirmed during the angiographic follow-up examination.

Model for end-stage liver disease score

To judge the clinical status of each cirrhotic patient, the model for end-stage liver disease score (MELD score) was calculated based on creatinine, bilirubin and clotting time.

The MELD score for each patient was computed according to the modified method of Wiesner *et al*[35]. This approach differs from the method originally published method by Malinchoc *et al*[36] in two ways: firstly, to avoid negative scores, laboratory serum creatinine levels that were less than 1 mg/dL were rounded off to 1. Preliminary studies in cohorts of non-transplantation candidates have implied that inclusion of the liver disease diagnosis variable does not increase the predictive value of the MELD score; secondly, as previously described by Wiesner *et al*[37,38], 6.43 points as a constant for liver disease aetiology was added to each patient's score to make the results comparable to the originally published studies. The following MELD equation was applied to calculate the severity score: 3.78 [Ln serum bilirubin (mg/dL)] + 11.20 (Ln international normalized ratio) + 9.57 [Ln serum creatinine (mg/dL)] + 6.43.

Table 2 Median survival times depending on various parameters

Parameter	Survival (mo)	95% CI (mo)	Tests	*P* value
Stent open	> 60	NC	Stent occluded *vs* open	0.027
Stent occluded at first control	50	36.6-63.4		
Ascites prior to TIPS	29	1.36-56.64	Ascites *vs* bleeding	0.009
Bleeding prior to TIPS	> 60	NC		
LTX after TIPS	> 60	NC	LTX *vs* no LTX	0.024
Stent diameter < 12 mm or ⩾ 12 mm	> 60	NC	Stent diameter < 12 mm *vs* ⩾ 12 mm	0.486
Anticoagulation	50	14.8–85.2	Anticoag *vs* no anticoag	0.060
No anticoagulation	> 60	NC		
PPG < 12 mmHg or ⩾ 12 mmHg after TIPS	> 60	NC	PPG < 12 mmHg *vs* ⩾ 12 mmHg	0.507
Age (yr)				
⩾ 65	51	33.4-68.6	Age < 65 yr *vs* ⩾ 65 yr	0.053
< 65	> 60	NC		
CHILD score				
A	48.9	40.6-57.2	CHILD A *vs* B	0.013
B	40.0	32.7-47.4		
C	15.0	1.4-28.6		
MELD score				
⩽ 10	52.2	46.3-58.0	MELD score ⩽ 10 *vs* > 10	0.001
> 10	35.3	26.9-43.6		

LTX: Liver transplantation; TIPS: Transjugular intrahepatic portosystemic stent; PPG: Portal pressure gradient; MELD: Model for end-stage liver disease; 95% CI: 95% confidence interval; NC: Not calculable.

Statistical analysis

Data were analyzed using SPSS 17.0 (Chicago, IL, United States). Results are expressed as medians and ranges. Comparisons between groups were performed by using the Mann-Whitney *U*-test and the χ^2 test as appropriate. $P < 0.05$ was considered statistically significant.

For screening of risk factors, univariate analysis was performed. The Kaplan-Meier method was employed to calculate survival from the time of TIPS implantation and comparisons were made by log rank test. A multivariate analysis of factors influencing survival was carried out using the Cox proportional hazards regression model.

RESULTS

Patient characteristics

In the study period (1998-2008), a total of 81 patients were admitted to the study with a mean age of 62.2 ± 10.5 years (range: 38-84 years). According to the indication for TIPS implantation, the patient cohort was subdivided into two groups: VB (group A) and RA (group B). The baseline characteristics of the study population are given in Table 1.

The aetiology of cirrhosis was related to chronic viral hepatitis B or C, alcohol abuse, autoimmune hepatitis and primary sclerosing cholangitis/primary biliary cirrhosis. The mean age in the VB and the RA group showed no statistical difference (61.7 years *vs* 63.3 years, $P = 0.497$). Likewise, the male/female ratio in both groups was comparable, with a slight trend to male patients. The severity of liver disease was calculated according to the CHILD scoring system[39]. Overall, 37% of patients with CHILD A, 57% with B and 6% with CHILD C were enrolled in this study. The MELD score in the RA group was significantly higher compared to the VB group (13.7 ± 5.2 *vs* 9.4 ± 4.9, $P = 0.001$).

TIPS shunt function and patient survival

The PPG significantly decreased from 23.4 ± 5.3 mmHg (VB) *vs* 22.1 ± 5.5 mmHg (RA) before TIPS to 11.8 ± 4.0 mmHg *vs* 11.7 ± 4.2 mmHg after TIPS implantation ($P = 0.001$ within each group). On the other hand, gradient reduction in the VB group did not statistically differ from that in the RA group. Referring to stent diameters there were no relevant differences between both groups. Anticoagulation therapy with enoxaparin at weight-calculated dose was applied for 12 wk after TIPS implantation in 50% of the patients. Thirty-one out of 54 patients in the bleeding group received subcutaneous anticoagulation therapy after TIPS, while only 9 out of 27 patients with RA were anticoagulated post-procedurally ($P = 0.042$). Neither the stent occlusion rate nor the rebleeding rate depended on the anticoagulation state ($P = 0.7$ and $P = 0.47$, respectively). In our patient cohort, the median patency rate of the TIPS shunt was 10 mo. The median survival time was > 60 mo in the VB group *vs* 29 mo in the RA group, showing a significant difference ($P = 0.009$). The number of radiological controls for stent patency was 6.3 ± 4.8 (VB) *vs* 3.8 ± 3.1 (RA) ($P = 0.029$). The mean time interval until the first radiological control was 9.3 ± 10.6 mo (VB) *vs* 4.5 ± 5.6 mo (RA) ($P = 0.133$).

Kaplan-Meier calculation indicated that the stent function (open *vs* occluded) at first control was a significant predictor of survival ($P = 0.027$) (Table 2 and Figure 2B). Furthermore, the median survival time was longer in patients with TIPS due to VB compared to that in patients with RA ($P = 0.009$) (Table 2 and Figure 2A). Seven patients in the VB group and one patient in the RA group underwent liver transplantation. As expected, in univariate analysis survival rates were significantly higher after liver transplantation ($P = 0.024$). The PPG after TIPS had no significant influence on median survival times in both groups (Table 2). Mortality was not significantly increased in patients aged > 65 years (Table

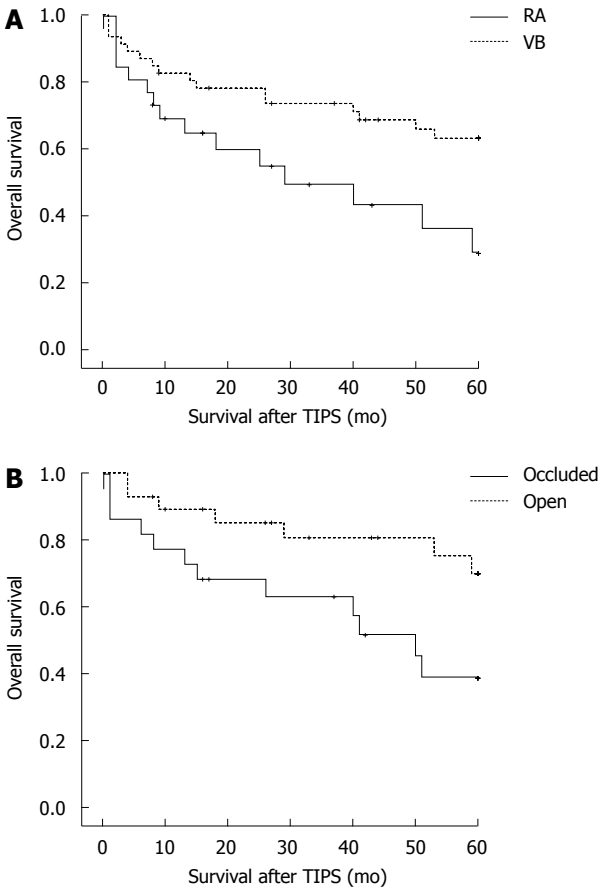

Figure 2 Kaplan-Meier survival analysis of patients after transjugular intrahepatic portosystemic shunt placement. A: In patients with initial ascites as indication for transjugular intrahepatic portosystemic stent (TIPS), survival is significantly shorter than that in patients with variceal bleeding [refractory ascites (RA) *vs* variceal bleeding (VB), log rank test $P = 0.009$]; B: In patients with occluded stent at first fluorographic control, survival is significantly shorter than that in patients with open stent (occluded *vs* open, log rank test $P = 0.027$).

2). For those patients having a MELD score greater than 10, the median survival was significantly shorter than for those with a MELD score less than or equal to 10 (35.3 mo *vs* 52.2 mo, $P = 0.001$).

In the Cox regression model, only stent occlusion at first control ($P = 0.022$), ascites prior to TIPS ($P = 0.043$), CHILD stage ($P = 0.015$) and MELD score ($P = 0.004$) were independent prognostic factors of survival. In contrast, anticoagulation management ($P = 0.097$), the porto-systemic pressure gradient ($P = 0.460$) and rebleeding episodes ($P = 0.765$) had no significant effect on the overall survival.

We further performed a subgroup analysis using the Kaplan-Meier method in terms of survival of the two groups considering the independent risk factors by Cox regression model analysis such as age, stent patency at first control, CHILD and MELD scores.

When survival was analyzed based on MELD scores (Figure 3A and B) we found that patients with VB had a statistically improved survival over those with RA (MELD score < 10 *vs* $\geqslant 10$, log rank $P = 0.001$).

Stratification by CHILD stages B and C or age > 65 years demonstrated that patients in the VB group had a significantly improved long-term survival compared with those in the RA group (log rank test $P = 0.021$ each) (Figure 3C and D).

Due to limited patient numbers the overall survival in patients with stent occlusion at first control did not differ significantly in both groups (Figure 3E, log rank test $P = 0.289$).

DISCUSSION

Since its introduction in the 1980s, the TIPS procedure has played a major role in the management of portal hypertension[9,24,40-43]. In the present study, shunt insertion was completed successfully in 81 patients (80% of patients scheduled). The baseline characteristics show the heterogeneous patient population at our hospital, the distribution of underlying diseases is typical for western countries[44,45] (Table 1).

Until recently, bare metal stents were the treatment of choice for establishing the TIPS tract. In contrast to the actual AASLD guidelines[9], in the United States[46] about 20% of all TIPS procedures still use uncovered TIPS stents.

Even though covered TIPS stents require fewer reinterventions, after a 12-mo-follow-up, the total procedure-related expenses were higher with covered TIPS stents due to their higher initial cost[43]. Further, a study by Bureau *et al*[25] in 2007 could not detect any survival benefit of covered *vs* uncovered stents. For these reasons we used non-coated TIPS stents during the study period of 1998 until 2008. Since this study was initiated at our institution, polytetrafluoroethylene-covered stents are now widely used, with the recent literature showing a significant improvement of primary patency up to 90% within 12 mo of application[25,28,47].

In agreement with Membreno *et al*[48], we show that in patients with TIPS due to VB, the overall long-term survival is significantly better than that in patients with TIPS due to RA (> 60 mo *vs* 29 mo, $P = 0.009$) (Figure 2A).

In the VB and RA groups of our study, the degree of reduction of the PPG following TIPS implantation was almost identical and there was no significant correlation with stent diameters. According to the literature, an adequate decompression of portosystemic hypertension can be achieved by 50% reduction of the initial pressure[49]. Other series describe a 20% reduction as sufficient and the PPG should be decreased and maintained under 12 mmHg[50]. In our study, the PPG was lowered post-procedurally at a recommended threshold of approximately 12 mmHg[24] (VB 11.8 ± 4.0 mmHg *vs* RA 11.7 ± 4.2 mmHg). Biecker *et al*[34] demonstrated in their study with 118 cirrhotic patients, that the initial decrease in the PPG after TIPS is a predictor of the rebleeding risk, but not of survival. Our study was not able, however, to confirm these findings.

In our patient cohort, the Cox multivariate regression analysis identified stent occlusion at first control as an

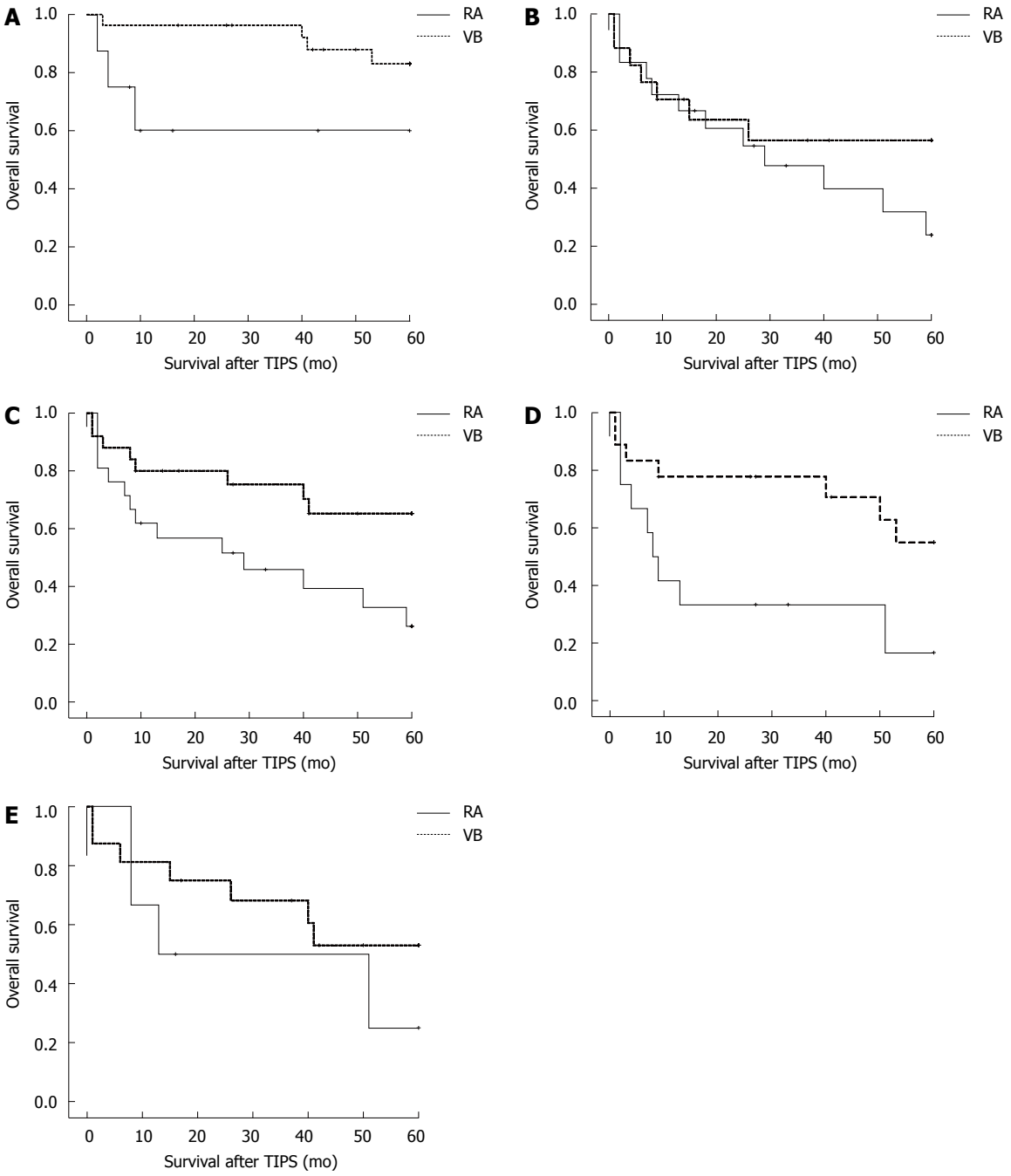

Figure 3 Kaplan-Meier survival analysis of patients after transjugular intrahepatic portosystemic shunt placement. A: Subgroup analysis with patients having a model for end-stage liver disease score (MELD) < 10: Significant difference in overall survival relating to indication [refractory ascites (RA) *vs* variceal bleeding (VB) group, log rank test $P = 0.031$]; B: Subgroup analysis with patients having a MELD score > 10: No significant difference in overall survival relating to indication (RA *vs* VB group, log rank test $P = 0.274$); C: Subgroup analysis with patients with CHILD B or C cirrhosis: Significant difference in overall survival relating to indication (RA *vs* VB group, log rank test $P = 0.021$); D: Subgroup analysis with patients age > 65 years: Significant difference in overall survival relating to indication (RA *vs* VB group, log rank test $P = 0.021$); E: Subgroup analysis with stent occlusion at first control: No significant difference in overall survival relating to indication (RA *vs* VB group, log rank test $P = 0.289$).

independent predictor of survival regardless of the indication for TIPS (Figure 2B and Figure 3E). Therefore, regular monitoring of the TIPS patients is highly recommended to provide early intervention when stenosis occurs[51]. In our institution, after successful TIPS insertion the first controls are conducted within 3 mo. Based on the results of the first interventional control (angiography), the following examinations are scheduled. Routinely colour Doppler ultrasound is used as a non-invasive device for monitoring the TIPS function.

Unsurprisingly, CHILD stage was an independent prognostic factor of survival ($P = 0.015$), probably due to the fact that the CHILD scoring system is a validated tool for assessing prognosis[39,52]. When survival was analyzed based on CHILD B or C, we found that patients with VB had a statistically improved survival over those

with RA (Figure 3C). Similar findings could be observed for patients being older than 65 years or having a MELD score < 10 leading to a significant overall survival relating to the indication for TIPS as displayed in Figure 3A, B and D.

These observations are consistent with those by Membreno *et al*[48]. The retrospective design and the use of uncovered stents as well as the relatively small sample size may introduce a certain bias. Nevertheless, our retrospective study emphasises several clinical aspects of portal hypertension in liver cirrhosis to be considered in conjunction with TIPS treatment.

In conclusion, TIPS is an established and safe nonsurgical method to decompress portal hypertension and to avoid its sequelae. RA prior to TIPS and stent occlusion at first control are independent predictors of survival in patients with bare metal TIPS shunts. This observation militates in favour of close follow-ups for patients with TIPS due to RA.

COMMENTS

Background

Liver cirrhosis is a common problem in gastroenterology. Various medical and interventional treatment options have been developed to manage the complications of portal hypertension. Minimal invasive placement of a transjugular intrahepatic portosystemic shunt (TIPS) is now widely used to lower the portosystemic pressure gradient.

Research frontiers

The authors undertook a retrospective study to work out the clinical outcome and predictors of survival after TIPS insertion with bare metal stents.

Innovations and breakthroughs

Refractory ascites (RA), stent occlusion at first control, initial CHILD stage and model for end-stage liver disease score were identified as independent predictors of survival in cirrhotic patients after TIPS implantation.

Applications

By understanding, which risk factors can influence survival in cirrhotic patients scheduled for TIPS insertion, the authors contribute to a better knowledge of this common clinical scenario. This may lead to a better risk stratification for the indication of TIPS insertion.

Terminology

The TIPS procedure decompresses the portosystemic pressure by establishing a "short cut" between the portal vein and the caval venous system.

Peer review

This is a retrospective study with the major objective to observe the role of stenosis and occlusion rates of uncovered stents on the survival of cirrhotic patients with TIPS inserted for variceal bleeding or RA.

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BRIEF ARTICLE

Effectiveness of infliximab after adalimumab failure in Crohn's disease

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Abstract

AIM: To evaluate the effectiveness of infliximab as a second-line therapy in Crohn's disease patients after adalimumab failure.

METHODS: A historical cohort study in a community-based gastroenterology practice evaluated Crohn's disease patients treated with infliximab (induction plus maintenance) after adalimumab failure. Patients were identified using a large Spanish database (ENEIDA).

RESULTS: We included 15 Crohn's disease patients who received infliximab after adalimumab failure. Five patients discontinued adalimumab due to loss of response, 3 due to adverse events and 7 due to partial response. After infliximab therapy was started, all patients who had interrupted adalimumab due to loss of efficacy regained response. All patients who discontinued adalimumab due to adverse events responded to infliximab and maintained this response; one of these patients had an uneventful course on infliximab, but 2 developed adverse events. None of the 7 patients who interrupted adalimumab due to partial response reached remission with infliximab.

CONCLUSION: Switching from adalimumab to infliximab may be useful in patients who develop adverse effects or loss of response, however, the benefit of infliximab in primary nonresponders was not established.

Key words: Adalimumab; Biologics; Crohn's disease; Infliximab; Switch

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INTRODUCTION

Crohn's disease (CD) is a chronic, relapsing, transmural inflammation of the gastrointestinal tract that affects mainly young patients, and results in a considerably decreased quality of life[1]. There is no medical or surgical cure for CD, and the goal of the existing therapeutic modalities is to induce and maintain remission[2]. Since tumor necrosis factor (TNF)-α has a pivotal role in the pathogenesis of CD[3], the introduction of antibodies against TNF-α has created new perspectives for the management of this disease[4,5].

Infliximab is a murine, chimeric, monoclonal immunoglobulin G1 antibody against TNF-α which was approved for CD treatment ten years ago. Infliximab is effective in inducing and maintaining remission in patients with moderate to severe CD and in patients who have failed conventional non-biologic therapy[6]. However, one-third of these patients do not respond to induction therapy. In addition, a subset of patients who initially respond to infliximab will discontinue therapy due to loss of response or intolerance[7,8].

Adalimumab is a recombinant fully human immunoglobulin G1 monoclonal antibody against TNF-α. Randomized controlled trials have demonstrated the efficacy of adalimumab for induction and maintenance of remission in patients naïve to anti-TNF therapy[9-11]. There are also several studies providing data on the efficacy of adalimumab after infliximab failure[12-16]. The CHARM trial[9] evaluated adalimumab as a maintenance therapy in patients after infliximab failure as part of a subgroup analysis. The GAIN study[16], a randomized placebo-controlled trial, demonstrated that adalimumab therapy is effective in inducing remission in patients with active CD who initially responded to infliximab, but then lost response or became intolerant to the drug.

Since adalimumab was approved in 2007 for use in CD, it can be prescribed as a first-line therapy, and similar to infliximab, one-third of patients do not respond to this drug and a proportion of patients can lose efficacy or become intolerant over time[12,17,18]. In clinical practice, patients who do not respond to infliximab, who lose response, or become intolerant may be prescribed adalimumab in an attempt to regain response, but to the best of our knowledge, there are no published data on the effectiveness of infliximab after adalimumab failure. Therefore, the aim of this study was to evaluate the effectiveness and safety of infliximab after adalimumab failure in CD patients.

MATERIALS AND METHODS

Study subjects

Patients who received infliximab for CD after adalimumab failure at a community-based gastroenterology practice were evaluated in a historical cohort study. They were identified using a large Spanish database (ENEIDA), promoted by the Spanish Working Group in Crohn's and Colitis (GETECCU), including patients with inflammatory bowel disease. The database prospectively records the use, effectiveness and adverse events of immunomodulators and anti-TNF therapy. The database at the time of the study included 10752 patients, of whom 5467 had CD. Patients were excluded from the study if adalimumab or infliximab was initiated for treatment of a disease other than CD.

Data collection

Data collected included: sex, age, smoking status, age at diagnosis, location of disease, disease behavior (inflammatory, stenosing or fistulizing), perianal disease, concurrent use of immunomodulators, indication of anti-TNF therapy, data regarding adalimumab therapy (start date for adalimumab therapy, initial response, loss of response, date of loss of response, dose escalation of adalimumab after loss of response, response to escalated dose, loss of response to escalated dose, date of loss of response to escalated dose, adverse events with standard and with escalated treatment), reasons for discontinuation of adalimumab therapy, data regarding infliximab therapy (start date for infliximab therapy, initial response, loss of response, date of loss of response, dose escalation of infliximab after loss of response, response to escalated dose, loss of response to escalated dose, date of loss of response to escalated dose, adverse events with standard and with escalated treatment) and reasons for discontinuation of infliximab treatment. Individual charts were reviewed to obtain all data.

Definitions

Dose escalation: Dose escalation of adalimumab was defined as a decrease in the interval of administration from every-other-week to every-week. In the case of infliximab, dose escalation was defined either as an increase in infliximab dose, e.g., from 5 to 10 mg/kg, or a decrease in infliximab infusion interval, e.g., from every 8-wk to every 4-wk, or both a dose increase and an interval decrease.

Evaluation of response: For luminal disease, response to adalimumab and infliximab was evaluated using the Harvey-Bradshaw index (HBI)[19] four weeks after the first dose. Partial response was defined as a decrease in the HBI of more than 3 points. Remission was defined as a HBI below or equal to 4 without steroids. In perianal CD, complete response was defined as closure of all fistulas and partial response as a 50% or more reduction in the number of draining fistulas.

Loss of efficacy: Loss of efficacy was defined as impairment in patient's symptoms coupled with endoscopic, radiographic, and/or serologic (elevated C-reactive protein) evidence of inflammation that made the physician escalate the dose of treatment or change to other drug.

Disease behavior and location: Disease behavior was categorized based on the Montreal classification as: (1) inflammatory or CD without fistulizing or stricturing complications; (2) stricturing disease was defined as the presence of clinical symptoms of partial or complete obstruction with fixed narrowing and/or narrowing with proximal dilatation; and (3) fistulizing, which included the presence of enteric fistulas, intraabdominal abscesses, or bowel perforation. The location of disease was established by identifying macroscopic evidence of CD in any part of the gastrointestinal tract. Possible categories of disease location included the ileum, colon, ileum and colon, upper gastrointestinal tract, and perianal/perineal area.

Concomitant immunomodulators: Concomitant immunosuppressive treatment was considered if a patient had been on immunomodulators for at least the first 6 mo after starting the anti-TNF therapy.

Smoking history: Smoking was defined as the consumption of at least 1 cigarette daily for a period of at least 3 mo prior to study entry.

Statistical analysis

mean ± SD was calculated for continuous variables. Percentages and 95% confidence intervals were provided for categorical variables.

RESULTS

We included 15 CD patients who received infliximab after adalimumab failure. The main characteristics of the study population are summarized in Table 1. Mean time from diagnosis to adalimumab treatment was 69 mo and the median time of adalimumab treatment was 5 mo (range: 2-19 mo). Four patients had stricturing CD. Two of these patients discontinued treatment with adalimumab due to partial response, one patient had an initial partial response but lost this response, and the other patient was in remission but experienced adverse events which led to the interruption of adalimumab. The two patients who had a partial response achieved a partial response after switching to infliximab and the patient who lost the partial response achieved a partial response with infliximab. The patient who had adverse events tolerated infliximab without secondary effects. Four patients received immunosuppressants concomitantly with adalimumab and seven patients were on immunosuppressants previously and maintained this therapy when they started adalimumab.

Adalimumab discontinuation due to loss of efficacy

Five patients discontinued adalimumab due to loss of efficacy. Three were women, 3 had CD of the ileum, 4 showed inflammatory behavior and one perianal disease. Two of these patients had extraintestinal manifestations and 2 were on concomitant immunomodulators (azathioprine).

Median time to loss of efficacy of adalimumab was 6.2 mo (range: 3-9 mo). After loss of efficacy, the dose of adalimumab was escalated in 3 of these patients, but only 1 responded to this treatment strategy (Table 2).

All patients who discontinued adalimumab due to loss of efficacy regained response after switching to infliximab (3 reached remission and 2 had a partial response) (Table 2).

Table 1 Characteristics of study patients *n* (%)

Gender (female %)	10 (67)
Median age (yr)	33
Time of evolution to adalimumab therapy (mo)	69
Location	
L1	7 (47)
L2	1 (6)
L3	7 (47)
Behavior (%)	
Inflammatory	10 (67)
Stricturing	4 (27)
Fistulizing	1 (6)
Perianal disease	4 (27)
Smoking habit	3 (21)
Previous surgical resection	7 (47)
Concomitant immunosuppressants	11 (71)
Reason for discontinuation of adalimumab	
Partial response	7 (47)
Loss of efficacy	5 (33)
Adverse events	3 (20)

L1: Ileum; L2: Colon; L3: Ileum and colon.

Adalimumab discontinuation due to lack of response

Seven patients discontinued adalimumab due to partial response. Four were women, 5 had CD of the ileocolic region, 6 showed inflammatory behavior and 2 perianal disease. The dose of adalimumab was escalated in 3 of these patients with the aim of reaching remission, but without improvement in response (Table 2).

None of the 7 patients who interrupted adalimumab due to partial response reached remission with infliximab: 5 (71%) maintained partial response and 2 (29%) lost partial response (Table 2).

Adalimumab discontinuation due to adverse events

Three patients discontinued adalimumab due to adverse events: 1 had facial edema, 1 an injection site reaction and 1 had dizziness. All of these patients were female, 2 had CD of the ileum, 2 showed stricturing behavior, 1 perianal disease and 2 were on concomitant immunomodulators (azathioprine). Two of these patients were in remission and 1 had a partial response at the time of adalimumab discontinuation due to adverse events. All patients maintained the response they had with adalimumab after switching to infliximab (patients in remission maintained

Table 2 Responses to the switch from adalimumab to infliximab

Reason for ADA discontinuation	Indication for anti-TNF	Initial response to ADA	Final response to ADA	ADA escalation	Initial response to ADA escalation	Final response to ADA escalation	Initial response to IFX	AE with IFX
Loss of response	Perianal	Partial response	No response	No	NA	NA	Remission	No
Loss of response	Luminal	Remission	No response	No	NA	NA	Remission	No
Loss of response	Luminal	Partial response	No response	Yes	Partial response	No response	Remission	No
Loss of response	Luminal	Partial response	Partial response	Yes	No response	NA	Partial response	No
Loss of response	Luminal	Partial response	No response	Yes	No response	NA	Partial response	No
Partial response	Luminal	Partial response	Partial response	No	NA	NA	Partial response	No
Partial response	Perianal	Partial response	No response	Yes	Partial response	Partial response	Partial response	No
Partial response	Luminal	Partial response	Partial response	No	NA	NA	Partial response	No
Partial response	Luminal	Partial response	Partial response	No	NA	NA	Partial response	No
Partial response	Luminal	Partial response	Partial response	Yes	Partial response	Partial response	No response	No
Partial response	Luminal	Partial response	Partial response	Yes	Partial response	Partial response	No response	No
Partial response	Perianal	Partial response	Partial response	No	NA	NA	Partial response	No
Adverse events	Luminal	Remission	Remission	No	NA	NA	Remission	No
Adverse events	Perianal	Partial response	Partial response	No	NA	NA	Partial response	Yes
Adverse events	Luminal	Remission	Remission	No	NA	NA	Remission	Yes

ADA: Adalimumab; TNF: Tumour necrosis factor; IFX: Infliximab; NA: Not applicable; AE: Adverse events.

remission with infliximab and the single patient with partial response had a partial response to infliximab). One of these patients received infliximab uneventfully, 1 had a delayed hypersensitivity reaction controlled by premedication with steroids before infliximab infusion, and the third patient interrupted infliximab due to facial edema (the same adverse event that forced the discontinuation of adalimumab).

DISCUSSION

The treatment of CD has evolved over the past decade with the introduction of anti-TNF agents. However, some patients do not respond or show suboptimal response to these drugs. Furthermore, patients who respond initially may lose efficacy over time or develop adverse events, which sometimes forces them to discontinue treatment. In these different scenarios, switching from one anti-TNF-α to another could represent an option for CD patients who fail the first anti-TNF drug.

The findings of the present observational study suggest that the probability of achieving clinical response after switching from adalimumab to infliximab may be higher in patients who discontinue adalimumab due to loss of efficacy or adverse events; as compared to those switching due to primary failure with adalimumab. In fact, patients who did not reach remission with adalimumab had no response to infliximab, whereas a relatively high proportion of patients showed a satisfactory response after discontinuing adalimumab due to loss of response or adverse events.

These observations seem to be in agreement with some of the published reports focusing on the effectiveness of adalimumab after infliximab failure[11,12,16,17]. In this respect, we have evidence that after loss of efficacy or intolerance to infliximab, adalimumab can be effective. However, to the best of our knowledge, this is the first study evaluating the effectiveness of infliximab after adalimumab failure.

Both infliximab and adalimumab are monoclonal antibodies and can be recognized by the human immune system as foreign antigens which respond by creating their own antibodies to different sites on the molecule[7,20,21]. In the case of infliximab, the development of antibodies to infliximab and, as a consequence, low trough concentration of the drug, have been implicated as predisposing factors for infliximab treatment failure[21-24]. The presence of antibodies to infliximab has been associated with the development of hypersensitivity reactions (infusional or delayed), while low trough concentration of infliximab has shown a high correlation with loss of response to treatment[7,21,24,25].

Although fully human, adalimumab is not devoid of immunogenicity. Antibodies to adalimumab have been reported in 2.6%-38% of patients treated for CD and rheumatoid arthritis[11,26,27]. In patients with rheumatoid arthritis, antibodies to adalimumab have been associated with low adalimumab trough serum concentration and decreased clinical response[26]. Karmiris *et al*[20] in a recently published study on CD patients treated with adalimumab after infliximab failure, found that 9% of patients developed antibodies to adalimumab, and that patients who developed antibodies to adalimumab frequently had low trough serum concentrations. They also reported that adalimumab trough serum concentration was lower throughout the follow-up period in patients who had to discontinue treatment due to loss of efficacy[20]. These findings suggest that the role of immunogenicity in the loss of response and in the development of adverse events following adalimumab treatment may be similar to that previously described with infliximab.

There is a lack of data on the development of adverse events with an anti-TNF drug in the subgroup of patients that had discontinued other anti-TNF drugs due to this reason, as the published studies provide this information globally irrespective of the type of failure (loss of efficacy or adverse events)[7,11,16]. The occurrence of immunoallergic reactions has been related to the for-

mation of antibodies against the anti-TNF drug and, in this respect, Karmiris *et al*[20] found that the presence of antibodies to infliximab before initiation of adalimumab therapy was not associated with a higher incidence of antibodies against adalimumab. There are no data on the development of antibodies to infliximab in patients with previous antibodies to adalimumab, but we would expect that they were not increased based on the findings of the previously mentioned study[20]. In our study, among the 3 patients who discontinued adalimumab due to adverse events, 2 had adverse events with infliximab and 1 of them had to interrupt infliximab due to the same adverse event which had developed with adalimumab. Considering that antibodies to one anti-TNF drug do not seem to be increased in patients with antibodies to other anti-TNF drug, our findings could be explained by the existence of cross reactions between antibodies to adalimumab and infliximab molecules, but this hypothesis has not been proved.

Finally, we found no benefit after switching from adalimumab to infliximab in patients who discontinued treatment due to partial response to adalimumab. The lack of efficacy of the anti-TNF agent in these patients could be due to particular disease characteristics in which TNF-α does not play a pivotal role[23]. In the adjunctive catheter-directed thrombolysis trial performed in rheumatoid arthritis patients, the authors found that an incomplete response to anti-TNF therapy in the population included in the study was not related to an insufficient concentration of the drug or to the presence of antibodies against it, concluding that other pro-inflammatory molecules different from TNF-α could play a main role in these patients.

One limitation of our study is the small sample size. Although our results are original, since there are no published data on the efficacy and safety of infliximab after adalimumab failure in CD patients, studies with a larger sample size are needed to establish the benefit of switching to another anti-TNF agent after one anti-TNF agent has failed.

In conclusion, the results of the present study suggest that CD patients may be successfully treated with infliximab after adalimumab failure, specifically those withdrawing for loss of efficacy or adverse events. Conversely, in patients discontinuing adalimumab due to lack of response, the efficacy of infliximab was not established and other drugs with different targets might offer a greater chance of therapeutic success.

COMMENTS

Background

There is no medical or surgical cure for Crohn's disease (CD), and the goal of the existing therapeutic modalities is to induce and maintain remission. Infliximab and adalimumab are monoclonal antibodies against tumor necrosis factor (TNF-α). However, one third of patients can lose response or become intolerant to these drugs.

Research frontiers

Studies are being performed in order to identify which are the factors responsible of the loss of efficacy or intolerance to anti-TNF drugs.

Innovations and breakthroughs

In clinical practice, patients who do not respond to infliximab, who lose response or become intolerant may be prescribed adalimumab in an attempt to regain response. Data regarding the outcome of patients who received infliximab after adalimumab failure are scarce. This study assesses the effectiveness of infliximab after adalimumab failure in CD patients.

Applications

The results of the present study suggest that CD patients may be successfully treated with infliximab after adalimumab failure, specifically those withdrawing due to loss of efficacy or adverse events. Conversely, in patients discontinuing adalimumab due to lack of response, the efficacy of infliximab has not been established and other drugs with different targets might offer a greater chance of therapeutic success.

Peer review

This is a good study in which the authors valuated the effectiveness of infliximab as a second-line therapy in CD patients after adalimumab failure. The result is interesting and suggested that CD patients may be successfully treated with infliximab after adalimumab failure.

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BRIEF ARTICLE

Contrast-enhanced ultrasound evaluation of hepatic microvascular changes in liver diseases

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Abstract

AIM: To assess if software assisted-contrast-enhanced ultrasonography (CEUS) provides reproducible perfusion parameters of hepatic parenchyma in patients affected by chronic liver disease.

METHODS: Forty patients with chronic viral liver disease, with (n = 20) or without (n = 20) cirrhosis, and 10 healthy subjects underwent CEUS and video recordings of each examination were then analysed with Esaote's Qontrast software. CEUS dedicated software Qontrast was used to determine peak (the maximum signal intensity), time to peak (TTP), region of blood value (RBV) proportional to the area under the time-intensity curve, mean transit time (MTT) measured in seconds and region of blood flow (RBF).

RESULTS: Qontrast-assisted CEUS parameters displayed high inter-observer reproducibility (κ coefficients of 0.87 for MTT and 0.90 TTP). When the region of interest included a main hepatic vein, Qontrast-calculated TTP was significantly shorter in cirrhotic patients (*vs* non-cirrhotics and healthy subjects) (71.0 ± 11.3 s *vs* 82.4 ± 15.6 s, 86.3 ± 20.3 s, $P < 0.05$). MTTs in the patients with liver cirrhosis were significantly shorter than those of controls (111.9 ± 22.0 s *vs* 139.4 ± 39.8 s, $P < 0.05$), but there was no significant difference between the cirrhotic and non-cirrhotic groups (111.9 ± 22.0 s *vs* 110.3 ± 14.6 s). Peak enhancement in the patients with liver cirrhosis was also higher than that observed in controls (23.9 ± 5.9 *vs* 18.9 ± 7.1, P = 0.05). There were no significant intergroup differences in the RBVs and RBFs.

CONCLUSION: Qontrast-assisted CEUS revealed reproducible differences in liver perfusion parameters during the development of hepatic fibrogenesis.

Key words: Contrast enhanced ultrasound; Cirrhosis; Hepatitis; Liver perfusion; Hepatic microcirculation

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INTRODUCTION

Changes in hepatic perfusion are a key feature of cirrhosis[1]. Capillarization of the sinusoids with loss of

endothelial fenestration and increased tone of activated hepatic stellate cells (HSCs) increase the mechanical resistance to portal blood flow and augment hepatic vascular tone, leading to portal hypertension[2]. Alterations involving the microvascular bed of the liver are already evident during the precirrhotic stages of hepatic fibrogenesis. The ongoing liver damage induces the overexpression of angiogenic growth factors (such as vascular endothelial growth factor or platelet-derived growth factor)[3], which promote the persistence of inflammatory changes and fibrogenic tissue repair, facilitating the development of shunts between branches of the portal vein, the hepatic veins, and the hepatic artery within the newly formed fibrotic septa[2,4].

Computed tomography (CT) and magnetic resonance imaging (MRI) have both been used for quantitative and qualitative assessment of parenchymal perfusion in cirrhotic livers. CT studies revealed increased hepatic arterial flow and decreased mean transit times (MTT), as compared with values observed in control subjects[5]. In contrast, MRI with low molecular weight contrast material revealed increases in the MTT[6]. Use of these methods in clinical practice is limited, however, by the intensive post-processing required to obtain perfusion data, high costs, and the lack of standardized examination protocols[7].

B-mode ultrasonography with Doppler study of hepatic vessels is often the first-line imaging study for the work-up of patients with diffuse liver disease. The Doppler technique is used mainly to measure flow in macroscopic vessels and is not strictly related to the microcirculation of the liver[8]. Contrast-enhanced ultrasonography (CEUS) is performed after intravenous administration of a suspension of gas-filled microbubbles, which remain entirely within the intravascular space and thus act as a blood pool tracer. CEUS studies of perfusion in the liver parenchyma have focused mainly on the measurement of contrast-medium transit times (from the portal vein to the hepatic veins), which have proved to be significantly shorter in patients with cirrhosis (compared with non-cirrhotic patients with chronic liver disease)[9-11]. CEUS has also documented increased regional perfusion of the hepatic parenchyma in cirrhotic patients (compared with healthy subjects), and this increase displayed correlation with the degree of liver failure[12].

A major shortcoming of CEUS is its user-dependency. Qontrast™ (Esaote S.p.a., Florence, Italy) is a post-processing computational tool, which can be used with CEUS to obtain objective, quantitative parameters of microvascular damage in various organs, including the liver.

We examined patients with chronic liver disease using CEUS with Qontrast analysis of hepatic parenchymal perfusion. The aims of this study were to assess the reliability and reproducibility of software assisted-CEUS studies of hepatic parenchymal perfusion and to evaluate if the analysis of the parameters obtained could help to understand vascular changes developing during liver fibrogenesis.

MATERIALS AND METHODS

The study protocol, which conformed to the guidelines outlined in the 1975 Declaration of Helsinki, was pre-approved by the institutional ethics committee. Written informed consent was obtained from all participants.

Study populations

Participants were enrolled between March 2007 and December 2010. They included 10 control patients with no liver disease (as documented by self-reported history and blood chemistry data obtained during screening) and 40 patients consecutively seen by our staff for chronic viral liver disease. At the time of enrollment, all of these patients had been positive for hepatitis B surface antigen and/or anti-hepatitis C virus antibodies for at least 6 mo.

All participants underwent complete physical examinations, laboratory tests, and standard B-mode abdominal ultrasonography. Candidates were excluded if they presented any of the following: (1) sonographic evidence of focal liver lesions; (2) history of alcohol consumption of $\geqslant$ 20 g/d; and (3) current use of medication known to affect the intra- or extra-hepatic circulation.

The patients with chronic liver disease included 20 patients in whom the absence of cirrhosis had been confirmed by liver biopsy (non-cirrhotic group) and 20 others (cirrhotic group) with cirrhosis diagnosed by liver biopsy or on the basis of commonly accepted clinical criteria. The latter included a history of chronic liver disease together with portal hypertension manifested by two or more of the following: (1) endoscopic evidence of esophageal or gastric varices and/or portal hypertensive gastropathy; (2) hypersplenism (reflected by a white blood cell count of $< 3500/mm^3$ and/or a platelet count of $< 100\,000\ /mm^3$); (3) sonographic evidence of ascites; and (4) a hepatic venous pressure gradient (HVPG) of $\geqslant$ 12 mmHg[13,14]. The severity of the cirrhosis was rated with the Child-Pugh[15] and model for end-stage liver disease (MELD)[16] systems.

The liver biopsies used for patient classification had all been performed under sonographic guidance 1-3 mo prior to enrollment (mean: 1.8 mo; median: 1 mo). For the purposes of this study, all slides were independently reviewed by a single experienced liver pathologist, who was blinded to the patient's clinical data and the results of his/her hepatic ultrasound examination. This examiner rated the presence of inflammation and fibrosis in each case with the Metavir scoring system[17].

Ultrasound and CEUS analysis

All sonographic examinations were carried out by an experienced radiologist using a 3.5-MHz convex array transducer and a General Electric Logiq 9 scanner (Milwaukee, WI, United States) equipped with software for contrast imaging and color and power Doppler. Patients were examined after a fast of 6 h. After a standard B-mode scan, the second phase of the examination started, during which we evaluated perfusion within the hepatic parenchyma using a sonographic contrast agent composed of sulfur hexafluoride-filled microbubbles (Sonovue®, Bracco Spa, Milan, Italy). The probe was placed over the middle hepatic vein (at least 3 cm from the confluence)

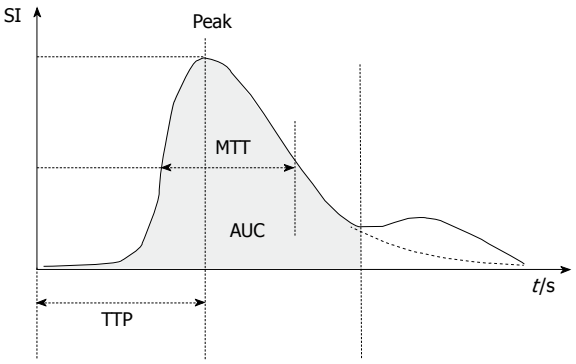

Figure 1 Main parameters extrapolated by Qontrast analysis of the time-intensity curve obtained by liver perfusion with an ultrasound contrast agent. SI: Signal intensity; TTP: Time to peak; MTT: Mean transit time; AUC: Area under the curve.

and surrounding tissue, where heartbeat artifacts were negligible. If an acceptable signal could not be obtained from this vein due to abdominal gas, the scan was made over the left or right hepatic vein. Tissue enhancement of these areas was recorded in a digital video format from 20 s before (baseline signal) to 130 s after the injection of a 2.5 mL bolus of Sonovue. Sonovue was injected manually into the antecubital vein at the rate of 1 mL/s, and the line was flushed with a 2.5-mL bolus of normal saline delivered at the same rate. Patients were instructed to breath gently during the procedure to minimize movement-related artifacts. Vital signs were monitored for 1 h after the examination, and patients were interviewed by phone 48 h after discharge to identify any adverse effects.

Video recordings were then analysed with the Qontrast software, which performs a full-map parametric analysis of perfusion within a selected set of frames in a specific region of interest (ROI). The loop of images is automatically processed after the tissue region and perfusion period have been defined; translational movements of the selected area can also be corrected. The area is then automatically aligned over all frames, and perfusion is analysed for points that continuously identify the moving tissue. Signal brightness is analysed separately at each point, and the optimal fitting curve is evaluated for each point.

In each patient, we evaluated two different ROIs. The first was a 25 cm^2 area of parenchyma that included one of the main hepatic veins and surrounding tissue; the second was a smaller area (5 cm^2) that included no major vessels. For each ROI, time-enhancement intensity curves were plotted with the Qontrast software, and the following parameters were generated (Figure 1): peak signal intensity (in dB) reached during the transit of the Sonovue bolus; time to peak (TTP) intensity, measured in seconds; regional blood volume (RBV), which is proportional to the area under the time-intensity curve; MTT (measured in seconds); and regional blood flow (RBF), which is the ratio of the RBV to MTT.

Intraobserver and interobserver agreement

We evaluated interobserver and intraobserver variation in measurements of the MTT and TTP during CEUS. For the former assessment, two observers (one experienced physician the other one in training) independently and blindly reviewed the video recording of each examination. For the latter analysis, each video was re-examined by one of the observers (still blind) 2-3 mo after the original review. Concordant measurements were those that differed by no more than ± 1 s.

Table 1 Clinical and biochemical characteristics of the three study groups (mean ± SD)

Group characteristics	Controls *n* = 10	Patients with chronic liver disease	
		Non-cirrhotic *n* = 20	Cirrhotic *n* = 20
Age (yr)	48.5 ± 14.4	48.9 ± 12.4	57.7 ± 11.4
Males, *n* (%)	4 (40)	12 (60)[c]	17 (85)[c]
Viral etiology (HBV/HCV)	0/0	2/18	4/16
Necroinflammatory score			
A0	-	3	0
A1	-	10	0
A2	-	7	3
A3	-	0	1
Fibrosis score			
F0	-	5	0
F1	-	6	0
F2	-	4	0
F3	-	5	0
F4	-	0	4
ALT (IU/L)	22.3 ± 5.2	113.8 ± 89.1[c]	76.9 ± 59.2[c]
Bilirubin (mg/dL)	0.6 ± 0.1	0.7 ± 0.4	1.5 ± 0.9[a]
Albumin (g/dL)	3.7 ± 0.3	4.1 ± 0.4	3 ± 0.8[a]
INR	1.1 ± 0.1	1 ± 0.1	1.2 ± 0.2[e]

[a]$P < 0.05$ *vs* other groups; [c]$P < 0.05$ *vs* control group; [e]$P < 0.05$ *vs* non-cirrhotic group. ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HCV: Hepatitis C virus; INR: International normalized ratio; A0: No activity; A1: Mild activity; A2: Moderate activity; A3: Severe activity; F0: No fibrosis; F1: Portal fibrosis without septa; F2: Portal fibrosis with few septa; F3: Numerous septa without cirrhosis; F4: Cirrhosis.

Statistical analysis

Data were expressed as group mean ± SD. Differences between the three groups (cirrhotic patients, non-cirrhotic patients, and controls) were evaluated with the Kruskall-Wallis test. A post-hoc *t* test was then used to evaluate differences between each group of participants. Differences in the proportion of male or female patients were assessed with the χ^2 test. Kappa statistics were used to assess interobserver and intraobserver agreement in the calculation of the Qontrast parameters[18,19].

McGraw-Hill Primer Statistical Software (2nd edition, 1986) and MedCalc Statistical Software (version 11.6, 2011, Mariakerke, Belgium) were used for all analyses. Statistical significance was defined as $P < 0.05$.

RESULTS

The characteristics of the control group and the patients with chronic liver disease are shown in Table 1. Most of the patients with cirrhosis [diagnosed by liver biopsy (*n* =

Table 2 Contrast-enhanced ultrasonography parameters calculated with Qontrast software in the three study groups (mean ± SD)

Perfusion parameter	Controls		Non-cirrhotic patients		Cirrhotic patients	
	ROI with HV	ROI without HV	ROI with HV	ROI without HV	ROI with HV	ROI without HV
TTP (s)	86.3 ± 20.3	86.8 ± 24.9	82.4 ± 15.6	78.2 ± 12.0	71.0 ± 11.3[a]	73.7 ± 17.8
Peak (%)	18.9 ± 7.1	18.5 ± 7.8	25.9 ± 7.8[c]	23.1 ± 8.9	23.9 ± 5.9[c]	25.5 ± 6.6
RBV	2828.7 ± 1720	2827.4 ± 1642.1	3402.3 ± 1515.1	2734.9 ± 1327.5	2809.3 ± 1111.6	3031.9 ± 1000.2
RBF	21.4 ± 9.5	21.4 ± 10.5	29.3 ± 10.5	25.6 ± 11.1	27.2 ± 7.9	28.8 ± 8.1
MTT (s)	139.4 ± 39.8	128.4 ± 37.8	110.3 ± 14.6[c]	105.8 ± 17.1[c]	111.9 ± 22.0[c]	104.7 ± 24.4[c]

MTT: Mean transit time; RBV: Regional blood volume; RBF: Regional blood flow; TTP: Time to peak; ROI: Region of interest; HV: Hepatic vein. [a]$P < 0.05$ *vs* other groups; [c]$P < 0.05$ *vs* control group; [c]$P = 0.05$ *vs* control group.

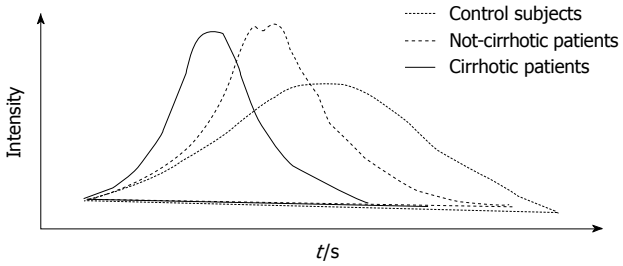

Figure 2 Schematic representation of time-intensity curves observed in the three study populations.

4) or on the basis of clinical criteria ($n = 16$)] were Child-Pugh class A ($n = 11$), but classes B and C were also represented (5 and 4 patients, respectively). The mean MELD score for the cirrhotic subgroup was 10.3 ± 3.9. Eleven of these patients had ascites, 2 had signs of hepatic encephalopathy, esophageal varices were found in 11, and HVPG more than 12 mmHg was evident in 3 patients. There were no significant age differences between the three groups, but males were significantly more common in the subgroups with chronic liver disease.

Ultrasound and CEUS studies were successfully completed for all participants, and no adverse effects were observed during or after the procedure (as documented by phone interviews with patients).

Qontrast analysis of contrast-enhanced ultrasound of liver parenchyma

The perfusion parameters generated by Qontrast analysis of the two ROIs examined is shown in Table 2: the first containing a hepatic vein and surrounding tissue, the second containing parenchyma alone, with no major vessels. In both cases, the MTTs in the patients with liver diseases were significantly shorter than those of controls, but there was no significant difference between the cirrhotic and non-cirrhotic groups. Analysis of the ROIs containing a hepatic vein (middle in 46 cases, right in 3, left in 1) revealed that TTPs in cirrhotic patients were significantly shorter than those of controls and of non-cirrhotic patients ($P < 0.05$). Similar findings emerged when we analyzed data obtained for the smaller ROIs although the differences in this case were not statistically significant. Peak enhancement in the patients with liver disease was also higher than that observed in controls, but again, this difference was significant only when the area analyzed included a main hepatic vein ($P < 0.05$). There were no significant intergroup differences in the RBVs, regardless of which ROI was considered. As for the RBF, the values observed in the chronic liver disease groups were appreciably (but not significantly) increased over those of controls, probably as a result of the shorter MTTs in these patients. This difference was also seen when the hepatic vein was not included in the ROI.

Observer agreement

Intraobserver agreement was calculated for MTT and TTP. Full agreement was considered when the two different analyses differed no more than ± 1 s. According to this finding, the agreement in reviewing Qontrast analysis for MTT and TTP by the same examiner was considered almost perfect: κ coefficient for MTT of 0.84 [95% confident interval (CI): 0.796-0.902] and for TTP of 0.92 (95% CI: 0.892-0.946). More interesting, the interobserver agreement for MTT and TTP by two examiners was found also to be almost perfect: κ coefficients for MTT of 0.87 (95% CI: 0.826-0.916) and for TTP of 0.90 (95% CI: 0.867-0.935).

DISCUSSION

CEUS represents the natural continuation of standard B mode ultrasound and Doppler studies, which are widely used for the diagnosis and follow-up of chronic liver disease. Compared with CT and MRI, ultrasound offers important advantages in terms of availability, safety, repeatability, and costs. We found CEUS of liver parenchyma to be safe and effective since we obtained a good quality digital video from each examination without any adverse effect observed. One of its main shortcomings is that it is highly operator dependent. We found Qontrast-assisted CEUS analysis of parenchymal perfusion to be highly reproducible. Intraobserver agreement was excellent (κ coefficient of 0.92) and, more interesting, interobserver agreement was almost perfect (κ coefficient: 0.90). Post-processing analysis of digitally recorded CEUS findings may thus be useful for standardizing this approach and improving its reproducibility.

When analysis was restricted to ROIs containing no major vessels, the time-intensity curves in the healthy control group were generally flat with a late enhancement peak (86.8 ± 24.9 s) and long MTT (range: 91.4-192.1 s),

findings that probably reflect an extensive vascular bed characterized by slow, continuous flow (Figure 2). By contrast, in patients with liver disease peak enhancement was higher and tended to occur earlier, and this pattern was more evident as the severity of the liver disease increased. From a quantitative point of view, this trend was reflected by MTTs in cirrhotic patients that were significantly shorter than those of the patients without liver disease. The steeper curve is an expression of faster, more concentrated flow of a volume of blood similar to that found in a healthy liver (the total hepatic blood volume is no different from that observed in the control group, as demonstrated by the RBVs). This picture (i.e., shorter MTTs associated with the same RBV) is fully compatible with sinusoid capillarization and increased activated HSC tone, which occur during the progression of chronic liver disease and can lead to high-velocity blood flow through the liver. When one of the major hepatic veins was included in the ROI, the TTP also decreased significantly across the three groups (controls > non-cirrhotics > cirrhotics). This picture could reflect the development of intrahepatic shunts that permit portal veins and branches of hepatic artery to cross cirrhotic areas, leading directly or indirectly into the central venous compartment[20].

Qontrast analysis indeed revealed more substantial differences between our patient subgroups when one of the hepatic veins was included in the ROI. The significant TTP shortening observed under these conditions is probably related in large part to the increasing presence of intra-hepatic shunts between branches of portal and/or hepatic artery and the hepatic veins. This is also the basis of the shortened hepatic vein arrival times documented in our cirrhotic patients (and those of other studies)[10,11,20] and also in patients with malignant liver disease[21].

The main limitation of our study is the small number of patients examined. Definitive conclusions on value of Qontrast analysis of CEUS data in diagnosing cirrhosis will have to be based on studies in much larger populations. In any case, use of this software does appear to increase the reproducibility of CEUS findings, and this could be useful for standardizing CEUS protocols and enhancing the comparability of findings obtained by different groups (that is for example a major weakness of Doppler examinations[22]).

Our initial experience with Qontrast-assisted CEUS studies of liver perfusion revealed clear differences between cirrhotic and non-cirrhotic patients with chronic liver disease and may serve as an incentive for further investigations. The reproducibility of Qontrast-assisted CEUS (although some training and experience are essential for optimal results) and its broad availability make it suitable for repeat examinations. Liver perfusion by CEUS could thus represent a valuable "non fibrotic-non invasive" tool to evaluate liver disease severity and to monitor the progression of chronic liver diseases.

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COMMENTS

Background

Changes in hepatic perfusion are a key feature that parallels the process of liver fibrogenesis developing during chronic liver diseases. These changes could be used to evaluate liver disease severity during the clinical follow up of chronic liver diseases.

Research frontiers

Evaluation of liver disease severity by studies of perfusion in the liver parenchyma has been mainly performed by computer tomography or magnetic resonance imaging. These methods in clinical practice are limited, however, by the intensive post-processing required to obtain perfusion data, high costs, and the lack of standardized examination protocols. Contrast enhanced ultrasonography (CEUS) could represent the best way to assess liver perfusion due to its wide availability, low cost and safety of ultrasound.

Innovations and breakthroughs

Previous studies performed by CEUS to assess liver disease severity have been mainly focused on the measurement of contrast-medium transit times (mainly from the portal vein to the hepatic veins). A major shortcoming of CEUS is its user-dependency. In this study the authors performed a software-assisted CEUS: Qontrast™ (Esaote S.p.a., Florence, Italy) is a post-processing computational tool, which can be used with CEUS to obtain objective, quantitative parameters of microvascular damage in the liver. The authors demonstrated that software assisted CEUS studies of liver perfusion revealed clear differences between cirrhotic and non-cirrhotic patients with chronic liver disease. The reproducibility of Qontrast-assisted CEUS is quite high and could be useful for standardizing CEUS protocols and enhancing the comparability of findings obtained by different groups.

Applications

The authors' initial experience with Qontrast-assisted CEUS studies of liver perfusion may serve as an incentive for further investigations. It is believed that liver perfusion by CEUS could represent a valuable "non fibrotic-non invasive" tool to evaluate liver disease severity and to monitor the progression of chronic liver diseases.

Terminology

Liver perfusion: Alterations involving the microvascular bed of the liver are already evident during the pre-cirrhotic stages of hepatic fibrogenesis. Main features are capillarization of the sinusoids with loss of endothelial fenestration and increased tone of activated hepatic stellate cells leading to the increase of the mechanical resistance to portal blood flow and augment hepatic vascular tone. The development of shunts between branches of the portal vein, the hepatic veins, and the hepatic artery within the newly formed fibrotic septa are also a key event; CEUS: Performed after intravenous administration of a suspension of gas-filled microbubbles, which remain entirely within the intravascular space and thus act as a blood pool tracer. CEUS studies of perfusion can be applied to the liver to evaluate the times and the intensity of enhancement of liver parenchyma occurring after the contrast injection (i.e., the time-intensity curve of enhancement).

Peer review

This is a good pivotal study in which the authors found time to peak contrast enhancement was significantly shorter in cirrhotic patients and contrast transit time was significantly shorter in the patients with liver diseases than those of controls. These results indicate that this new non-invasive method of analyzing hepatic vein transit time is useful for the prediction of liver disease progression.

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BRIEF ARTICLE

High-definition colonoscopy with i-Scan: Better diagnosis for small polyps and flat adenomas

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Abstract

AIM: To investigate if high-definition (HD) colonoscope with i-Scan gave a higher detection rate of mucosal lesions *vs* standard white-light instruments.

METHODS: Data were collected from the computerized database of the endoscopy unit of our tertiary referral center. We retrospectively analyzed 1101 consecutive colonoscopies that were performed over 1 year with standard white-light (n = 849) or HD+ with i-Scan (n = 252) instruments by four endoscopists, in an outpatient setting. Colonoscopy records included patients' main details and family history for colorectal cancer, indication for colonoscopy (screening, diagnostic or surveillance), type of instrument used (standard white-light or HD+ plus i-Scan), name of endoscopist and bowel preparation. Records for each procedure included whether the cecum was reached or not and the reason for failure, complications during or immediately after the procedure, and number, size, location and characteristics of the lesions. Polyps or protruding lesions were defined as sessile or pedunculated, and nonprotruding lesions were defined according to Paris classification. For each lesion, histological diagnosis was recorded.

RESULTS: Eight hundred and forty-nine colonoscopies were carried with the standard white-light video colonoscope and 252 with the HD+ plus i-Scan video colonoscope. The four endoscopists did 264, 300, 276 and 261 procedures, respectively; 21.6%, 24.0%, 21.7% and 24.1% of them with the HD+ plus i-Scan technique. There were no significant differences between the four endoscopists in either the number of procedures done or the proportions of each imaging technique used. Both techniques detected one or more mucosal lesions in 522/1101 procedures (47.4%). The overall number of lesions recognized was 1266; 645 in the right colon and 621 in the left. A significantly higher number of colonoscopies recognized lesions in the HD+ plus i-Scan mode (171/252 = 67.9%) than with the standard white-light technique (408/849 = 48.1%) (P < 0.0001). HD+ with i-Scan colonoscopies identified more lesions than standard white-light imaging (459/252 and 807/849, P < 0.0001), in the right or left colon (mean ± SD, 1.62 ± 1.36 *vs* 1.33 ± 0.73, P < 0.003 and 1.55 ± 0.98 *vs* 1.17 ± 0.93, P = 0.033), more lesions < 10 mm (P < 0.0001) or nonprotruding (P < 0.022), and flat polyps (P = 0.04). The cumulative mean number of lesions per procedure detected by the four endoscopists was significantly higher with HD+ with i-Scan than with standard white-light imaging (1.82 ± 2.89 *vs* 0.95 ± 1.35, P < 0.0001).

CONCLUSION: HD imaging with i-Scan during the withdrawal phase of colonoscopy significantly increased the detection of colonic mucosal lesions, particularly small and nonprotruding polyps.

Key words: Colonoscopy; High-definition+ with i-Scan colonoscopy; White-light colonoscopy; Colonic polyps;

Nonprotruding lesions; Adenoma detection rate; Withdrawal time; Surface enhancement; Contrast enhancement; Tone enhancement

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INTRODUCTION

Screening colonoscopy is widely considered the gold standard for detection of colonic neoplasia and adenomatous lesions[1]; however, there are several reports of failure to detect small and flat neoplastic lesions[1-10], meaning that in these cases, colonoscopy does not provide adequate protection against colorectal cancer. This inadequacy results in up to 6% of new or missed cancer 3 years after colonoscopy[11,12]. In a recent study, colonoscopy in the preceding 10 years was associated with an overall 77% lower risk for colorectal cancer and approximately 50% lower risk for right-sided cancer[13].

Major factors affecting this polyp miss rate are the presence of blind segments in the colon, poor colon cleaning, and the fact that standard white light may be unable to recognize some small or flat lesions, which are particularly frequent in the right colon. The operator's experience and a longer withdrawal time, permitting closer observation, can only partly overcome these limitations. Even experienced endoscopists may miss up to 6% of adenomas larger than 1 cm and 30% of all adenomas[2,14,15].

Endoscopes have now been designed to improve mucosal visualization, with a wide angle of view and high-resolution, high-definition imaging. Despite these technical improvements, however, there is still debate about the value of high-definition colonoscopy in clinical practice. Out of 11 studies published so far evaluating the capacity of high-definition imaging to improve the lesion detection rate during colonoscopy, five have concluded that it gave no significant advantage over standard white-light colonoscopy[16-26]. A recent meta-analysis evaluating five studies involving 4422 patients and comparing high-definition *vs* standard white-light colonoscopy showed that there were marginal differences between the two imaging technologies for detection of colonic polyps and no advantages of high-definition in the detection of high-risk adenomas[27]. The introduction of instantaneous non-white-light imaging that mimics chromoendoscopy (Narrow-band, Olympus Ltd. and FICE, Fujinon Ltd.) makes it possible to enhance contrast and potentially to improve the detection of mucosal lesions; these filter techniques significantly raised the polyp detection rate in all but three of 13 studies to date[16,28-39]. However, two meta-analyses gave conflicting results[38,40].

A newly developed post-processing filter technology, the i-Scan (Pentax Ltd., Tokyo, Japan), combined and integrated into a high-definition processor (EPKi) that generates images above the high-definition television standard (HD+ resolution), highlights the mucosal surface and architecture by surface enhancement (SE), contrast enhancement (CE), and tone enhancement (TE) modes. So far, in all reports but one, retrospective, it permitted significantly better recognition and characterization of the mucosal lesions during colonoscopy[41-45]. In one recent study, narrow-band imaging and i-Scan significantly improved the polyp detection rate and showed similar efficacy[46].

However, most of the studies using these new post-processing filter techniques are based on prospective, controlled clinical trials in a limited number of patients, in which endoscopists are likely to do the colonoscopy more diligently than in routine practice, with adequate bowel preparation, so it is not clear whether the better polyp detection rates reported can be maintained in routine practice.

The aim of the present study was therefore to determine whether the routine use of colonoscopes equipped with high-definition combined with i-Scan technology (HD+ plus i-Scan) gave a higher rate of detection of overall mucosal lesions, particularly of flat adenomas, than standard white-light video colonoscopes, in a consecutive series of patients undergoing screening, diagnostic or surveillance colonoscopy by different endoscopists with similar expertise, in an outpatient clinical practice setting.

MATERIALS AND METHODS

Data for the study were collected from the computerized database of the endoscopy unit of our tertiary referral center. Colonoscopy records included patients' main details and family history for colorectal cancer, indication for colonoscopy (screening, diagnostic or surveillance), type of instrument used (standard white-light or HD+ plus i-Scan), name of endoscopist, and bowel preparation, defined on the basis of a modified Ottawa scale[47].

Records for each procedure included whether the cecum was reached or not and the reason for failure (inadequate cleaning, strictures, and pain during the procedure), complications during or immediately after the procedure, and number, location and characteristics of the lesions. Polyps or protruding lesions were defined as sessile (Ⅰs) or pedunculated (Ⅰp), and nonprotruding lesions as elevated (Ⅱa), flat (Ⅱb), and depressed (Ⅱc), according to Paris classification[48]. For each lesion, histological diagnosis was recorded. Size and location of the lesions were classified as follows: 0-5 mm, 6-10 mm, 11-15 mm, 16-20 mm, 21-30 mm, > 30 mm; right and left colon. Withdrawal time was recorded for all screening colonoscopies, being

these procedures the object of other studies. Images of each lesion were stored in the database. For each patient, pO_2, heart rate, and blood pressure were measured and recorded before, during and at the end of the procedure.

Data collection

Over a 1-year period, all consecutive screening, diagnostic and surveillance colonoscopies in outpatients done by four expert endoscopists, each of whom had done 200-400 colonoscopies/year for at least 15 years and at least 50 procedures with HD+ plus i-Scan definition equipped instruments were evaluated. The four endoscopists used the two endoscopy techniques in a random fashion, depending of the availability of the instruments. Colonoscopies in subjects younger than 18 years, with genetic-associated colon cancer risk conditions, acute gastrointestinal bleeding, or inflammatory bowel disease were excluded. Procedures with insufficient bowel cleansing, patients in whom residual stool could not be removed by endoluminal washing and suctioning, and patients in whom the cecum was not reached were also excluded.

All patients gave informed consent for the procedures, diagnostic or therapeutic, and for data management for scientific purposes. The retrospective, observational study was approved by the institutional ethics committee.

Examination technique

The bowel was prepared in all cases with polyethylene glycol: 4 L SELG (Promefarm S.r.l, Milan, Italy) or 3 L Moviprep (Norgine GmbH, Marburg, Germany), divided into two parts, were taken the day before the procedure. All patients received conscious sedation with midazolam (Ipnovel, Roche SPA, Basel, Switzerland) and fentanyl (Fentanest, Pfizer, New York, United States) or deep sedation with propofol (Diprivan, AstraZeneca, Zug, Switzerland); 20 mg Butylscopolamin (Buscopan, Boehringer Ingelheim Pharma GmbH, Ingelheim, Germany) were administered if necessary, unless contraindicated.

Standard white-light video colonoscopy was carried out with Pentax colonoscopes EC-3870FZK, EC 3885F, EC 3885L (Pentax Ltd., Tokyo, Japan) and an EPM 3500 or EPK 1000 processor. The colon was inspected during withdrawal of the instrument and lesions were identified and characterized with light imaging only. Magnification was not possible with these endoscopes.

HD+ plus i-Scan video colonoscopy was carried out with Pentax colonoscopes EC-3890FI and EC 3870FZK, using the EPKi processor. The i-Scan technology is a digital contrast method using a light filter that uses different software algorithms with real-time image mapping embedded in the EPKi processor. It enhances mucosal imaging by activating three distinct functions-one for SE mode, the second for CE mode, and the third for TE mode. For SE and CE, there are three enhancement levels (low, medium and high); TE mode can be specifically tailored for the esophagus, stomach, or colon. SE mode enhances the structure through recognition of the edges; compared to normal images, SE images do not differ in brightness and differ little in color, but allow easier recognition of minute glandular structures, which makes it simpler to check changes on the basis of structural differences. With CE mode, areas with lower luminance intensity than surrounding pixels are identified on the basis of pixel-wise luminance intensity data. Processing images with CE does not change the image brightness but enhances minute irregularities and depressed areas of the mucosal surface with a slight bluish-white stain. With TE mode, the RGB components of an ordinary endoscope image are broken down into their parts, and each one is then converted independently along the tone curve, followed by resynthesis of the three components to yield a reconstructed image[43].

The three modes are arranged in series, so two or more can be applied at one time. The modes of enhancement and their levels can be switched on a real-time basis, permitting efficient endoscopic observation.

In all cases, colonoscopy was done using the SE (low) + CE (low) modes; the TE mode for the colon was routinely activated during withdrawal of the instrument once the cecum had been reached, so the whole retrieval phase of the procedure was done using the i-Scan technique with TE mode set for the colon.

Statistical analysis

Data were analyzed using SPSS version 17.0 software (Chicago, IL, United States). Continuous data were described by mean and standard deviation or compared with the Mann-Whitney test. Statistical differences in categorical variables were analyzed using two-sided Fisher's exact tests or χ^2 tests, as appropriate. All differences were considered significant at two-sided P value < 0.05.

RESULTS

A total of 1101 colonoscopy records with images obtained by the four endoscopists were eligible for the study: 849 with the standard white-light video colonoscope and 252 with the HD+ plus i-Scan video colonoscope. The four endoscopists did 264, 300, 276 and 261 procedures, respectively; 21.6%, 24.0%, 21.7% and 24.1% of them with the HD+ plus i-Scan technique. The number of colonoscopies carried out for screening, diagnosis, and surveillance with standard white-light and HD+ plus i-Scan technology by the four endoscopists are reported in Table 1. There were no significant differences between the four endoscopists in either the number of procedures done or the proportions of each imaging technique used.

Both techniques detected one or more mucosal lesions in 522/1101 procedures (47.4%). The overall number of lesions recognized was 1266: 645 in the right colon and 621 in the left. A significantly higher number of colonoscopies recognized lesions in the HD+ plus i-Scan mode (171/252 = 67.9%) than with the standard white-light technique (408/849 = 48.1%) ($P < 0.0001$). The number of mucosal lesions recognized by the two imaging tech-

Table 1 Colonoscopies carried out for screening, diagnosis and surveillance *n* (%)

Indications	HD+ with i-Scan	Standard white light	Total
Screening	69 (23.9)	219 (76.1)	288
Diagnosis	156 (22.1)	552 (77.9)	708
Follow-up	27 (25.7)	78 (74.3)	105
Total	252	849	1101

HD: High-definition.

Table 2 High-definition+ with i-Scan and standard white-light colonoscopy detection rates of mucosal lesions

Indications	HD+ with i-Scan[1]	Standard white light[1]	*P* value
Screening	179/69 (2.59)	207/219 (0.94)	< 0.0001
Diagnosis	203/156 (1.3)	524/552 (0.94)	0.0105
Follow-up	77/27 (2.8)	76/78 (0.97)	< 0.0001
Total	459/252 (1.82)	807/849 (0.95)	< 0.0001

[1]Number of lesion/procedure (mean). HD: High-definition.

Table 3 Number and size of protruding and nonprotruding lesions found with high-definition+ with i-Scan and standard white-light colonoscopy

	HD+ with i-Scan	Standard white light	Total	*P* value
0-10 mm				< 0.0001
Protruding	341	636	977	
Nonprotruding	43	31	74	
11-20 mm				0.83
Protruding	30	67	97	
Nonprotruding	12	23	35	
21-30 mm				0.36
Protruding	9	8	17	
Nonprotruding	12	21	33	
> 30 mm				
Protruding	9	12	21	0.46
Nonprotruding	3	9	12	

HD: High-definition.

niques and the mean numbers detected by each procedure were significantly higher for HD+ plus i-Scan than with standard white light, for screening, diagnostic, and surveillance colonoscopies (Table 2). In both the right and left colon, HD+ plus i-Scan colonscopy recognized a larger mean number of lesions than standard white light (mean ± SD 1.62 ± 1.36 *vs* 1.33 ± 0.73, $P < 0.003$ and 1.55 ± 0.98 *vs* 1.17 ± 0.93, $P = 0.033$).

Overall, 154 nonprotruding lesions were identified and removed: 70 with the HD+ plus i-Scan mode (27.8%) and 84 with the standard white-light technique (9.9%). The HD+ plus i-Scan mode recognized a significantly higher number of nonprotruding lesions than the standard white-light technique ($P = 0.04$) (Figures 1 and 2).

The overall number and size of the lesions, protruding or nonprotruding, found with HD+ plus i-Scan and standard white light are shown in Table 3. The HD+ plus i-Scan technique identified a significantly larger number of lesions smaller than 10 mm, either protruding or nonprotruding, than standard white light ($P < 0.0001$); the difference was not significant for lesions measuring 11-20 mm, 21-30 mm, and > 30 mm. Colonoscopies performed with HD+ with i-Scan technique also identified a significantly larger number of overall lesions and nonprotruding lesions smaller than 10 mm than did standard white light ($P < 0.0001$ and $P < 0.022$, respectively), while the difference was not different for larger lesions, either protruding or nonprotruding. The differences were not significant considering screening, diagnostic, and surveillance colonoscopies.

Among the 154 nonprotruding lesions, histological report was available for 133 lesions, because in 21 cases, resected specimens were missed during colonoscopy (Table 4). Adenoma detection rate was significantly higher with HD+ plus i-Scan mode than with standard white light only for lesions smaller than 10 mm (32/35 *vs* 19/27, $P = 0.05$), while the difference was not significant for larger adenomas.

The number of procedures managed by the four endoscopists and the distribution of HD+ plus i-Scan and standard white-light colonoscopies, with the mean numbers of lesions found by each one. The lesion detection rates were very similar for all four. The cumulative mean number of lesions per procedure detected with the two techniques was significantly higher with the HD+ plus i-Scan than with standard white-light imaging (mean ± SD, 1.82 ± 2.89 *vs* 0.95 ± 1.35, $P < 0.0001$). In fact, each of the four endoscopists identified twice as many lesions with the HD+ plus i-Scan as with standard white-light imaging.

The overall withdrawal time, reported only for screening colonoscopies, did not significantly differ between procedures performed with the HD+ plus i-Scan and standard white light (8.4 ± 1.2 min *vs* 8.3 ± 1.4 min, respectively) (Table 5).

DISCUSSION

To date, only one study has evaluated the impact of the routine use of i-Scan with TE mode and HD+ imaging in the detection of mucosal lesion during the withdrawal phase of colonoscopy, compared to standard white-light imaging, in a large series of patients in clinical practice[45]. The study was retrospective and did not improve adenoma detection rate in a population with mixed risk for colorectal cancer.

In our retrospective study, with the HD+ plus i-Scan imaging routinely activated during the withdrawal phase of colonoscopy, once the cecum had been reached, a significantly larger number of examinations identified some mucosal lesion and adenomas, either protruding or flat, and there were also significant improvements in the overall detection rate of lesions and the mean number of lesions recognized for each colonoscopy, compared with standard white-light imaging. The rate was most markedly higher for lesions not bigger than 10 mm and nonprotruding ones. Although the rates of detection of

Table 4 Histological report of nonprotruding lesions

	0-10 mm		11-20 mm		21-30 mm		> 30 mm		Total
	HD + with i-Scan	Standard white light	HD + with i-Scan	Standard white light	HD + with i-Scan	Standard white light	HD + with i-Scan	Standard white light	
Missing	8	4	0	3	0	3	0	3	21
Hyperplastic	3	8	3	2	3	0	0	0	19
Serrated	29	16	6	18	9	18	0	0	96
LGIN	3	3	0	0	0	0	3	6	15
Adenocarcinoma	0	0	3	0	0	0	0	0	3
Total	43	31	12	23	12	21	3	9	154

HD: High-definition; LGIN: Low grade intraepithelial neoplasia.

Table 5 Procedures performed by the four endoscopists using the two techniques

Operator	Procedures	HD+ with i-Scan	Standard white light	Lesions	No. of lesions (mean number of lesions/procedure)		*P* value
					HD+ with i-Scan	Standard white light	
1	264	57	207	330	117 (2.05)	213 (1.02)	< 0.0001
2	300	72	228	375	132 (1.83)	243 (1.07)	0.089
3	276	60	216	294	114 (1.9)	180 (0.83)	< 0.0001
4	261	63	198	267	96 (1.52)	171 (0.86)	0.71
Total	1101	252	849	1266	459 (1.82)	807 (0.95)	< 0.0001

HD: High-definition.

lesions larger than 10 mm did not differ with the two imaging techniques, protruding and nonprotruding lesions smaller than 10 mm were recognized significantly more frequently using the HD+ plus i-Scan technology. In particular, HD+ plus i-Scan technology identified flat polyps smaller than 10 mm three times more than the white-light technique.

The cumulative mean number of lesions per colonoscopy recognized by the four colonoscopists was significantly higher with HD+ plus i-Scan than with standard white-light imaging, while the withdrawal time, when recorded, did not differ between the two techniques.

Only two studies published so far have assessed the combined use of HD+ plus i-Scan for colonoscopy; they have reported similar results in favor of this technique but they were obtained in a prospective trial setting and in a smaller number of selected patients[42,44].

Identifying more polyps by colonoscopy in clinical practice, including small (< 10 mm) and flat ones, may have an important impact for colorectal cancer prevention. The polyp miss rate is probably the main factor accounting for a persistent risk of colorectal cancer reported in 10%-24% of cases after screening colonoscopy[49].

A systematic review of six tandem colonoscopy studies using standard white-light imaging showed an overall polyp miss rate of 22%. The rate rose with smaller lesions, ranging from 2.1% for lesions bigger than 10 mm, to 13% for those between 5 and 10 mm, and up to 26% for those smaller than 5 mm[8]. A prospective multicenter study of back-to-back colonoscopies with white-light imaging reported 9% and 27% miss rates for adenomas > 5 mm and < 5 mm, respectively, and 11% for advanced adenomas[9]. This means that small and flat mucosal lesions, mostly in the right colon, are the ones that may frequently be missed during colonoscopy.

A limited number of studies have compared the efficacy of HD+ colonoscopy with standard white-light colonoscopy, and the findings are far from clear: four of the nine studies concluded that high-definition imaging gave no benefit compared to standard resolution[17,18,21,24]. The addition of electronic filters, such NBI and FICE, to the high-definition imaging did improve the polyp detection rate for small/flat lesions but some results were still disappointing for this end-point[16,30].

Even though there is a general belief that detecting and removing small lesions (1-5 mm) in the colon may not have any significant clinical impact, a number of studies have found that small lesions, mainly flat ones, may have unfavorable histology. One reported that small depressed colorectal lesions had up to a 40% chance of submucosal invasion[49]; two found that 3.9% and 16% of adenomas between 6 and 10 mm had high-grade dysplasia[50,51], and 0.5% of adenomas measuring 6-9 mm were actually cancer[51]. These data might explain the reported occurrence of colorectal cancer after negative screening colonoscopy and support the need for detecting and removing all protruding lesions of the colon, regardless of the size, and selecting the most appropriate techniques to ensure maximum recognition of lesions at colonoscopy.

HD+ plus i-Scan can also differentiate diminutive adenomas and hyperplastic polyps[52], and a recent study using a Markov simulation model suggested that a resect and discard strategy for very small polyps might improve the cost-effectiveness of colorectal cancer screening[53].

A potential limitation of the present study was its retrospective nature. However, data used for analysis, in-

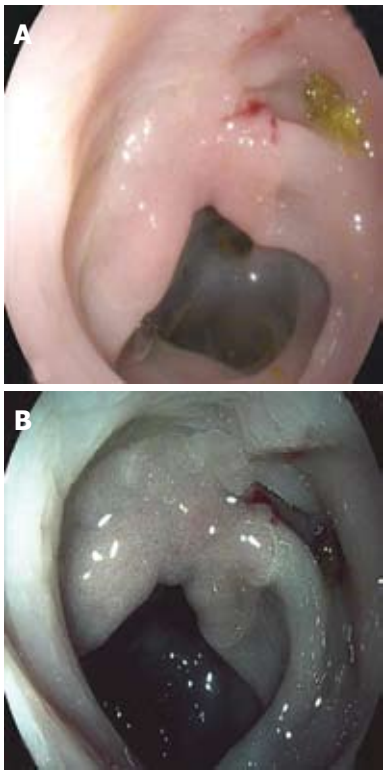

Figure 1 Flat lesion Ⅱb + Ⅱa on left colon examined by high-definition-white light and visualized with i-Scan. A: Flat lesion Ⅱb + Ⅱa of 25 mm × 25 mm on left colon examined by high-definition white light; B: Same lesion visualized with i-Scan.

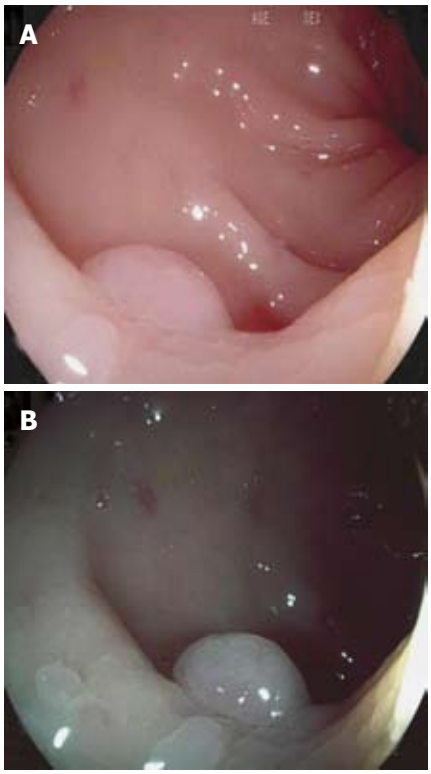

Figure 2 Flat lesion 0-Ⅱa visualized with high-definition white light and surface enhancement and visualized with i-Scan and digital chromoendoscopy. A: Flat lesion 0-Ⅱa visualized with high-definition white light and surface enhancement; B: Same lesion visualized with i-Scan and digital chromoendoscopy.

cluding the adequacy of bowel preparation, were detailed and were collected prospectively for each procedure and stored in a database. Only procedures that included all the data required for the study were considered. As with all nonrandomized trials, potential confounding variables cannot be entirely excluded; however, we examined a large number of colonoscopies and statistical analysis found highly significant differences. Although colonoscopies carried out for different purposes (screening, diagnostic and surveillance) may represent different settings, the differences reported from overall results were also confirmed in the three settings. On the other hand, the retrospective design has the advantage of providing information on the true yield of HD+ plus i-Scan imaging for detecting polyps during colonoscopy in current clinical practice. Prospective trials evaluating new imaging systems could allow the endoscopist to be more attentive during the procedures outside routine practice and very likely give greater accuracy for polyp detection, especially for flat and small lesions, but the good results are not necessarily directly transferable into routine clinical practice.

The lack of documentation of withdrawal time for all colonoscopies is another potential limitation of a retrospective study, compared with prospective ones, because withdrawal time plays an important role in adenoma detection, although here too data are conflicting. In this retrospective evaluation, we were able to assess reliably the withdrawal times only for screening colonoscopies without therapeutic interventions: withdrawal time was comparable by using the two imaging techniques. Besides the imaging technology, probably the endoscopist's technique and experience is perhaps more important than other factors, including withdrawal time, in detecting polyps by colonoscopy[54-56]. The endoscopists in this study were experts, with many colonoscopies behind them and on their current schedules, and adequate experience with HD+ plus i-Scan imaging in the year leading up to the study. In addition, we compared the numbers of colonic lesions recognized by the same endoscopist using the two techniques, thus applying similar expertise and technique, in a similar clinical setting, and found that the four endoscopists using HD+ plus i-Scan imaging detected cumulatively more lesions. Only one other study comparing the diagnostic yield for colonic polyps using standard white-light and HD+ colonoscopy followed a retrospective design, with an adequate number of unselected patients undergoing colonoscopy in routine practice. The findings confirmed the greater accuracy for detecting polyps of HD imaging compared with white light (42.2% *vs* 37.8%)[20]. In our hands, 67.8% and 27.8% of colonoscopies with HD+ plus i-Scan recognized some mucosal lesions and flat small polyps (< 10 mm), respectively, compared to 48.1% and 9.9% for standard white-light imaging. HD+ plus i-Scan thus gave an approximately 30% higher diagnostic yield for mucosal lesions of the colon and increased by three times the diagnostic accuracy for flat polyps smaller than 10 mm.

In conclusion, this retrospective study on a large se-

ries of consecutive outpatients undergoing colonoscopy in different settings by four expert endoscopists showed that the routine addition of i-Scan to HD imaging during the entire withdrawal phase of colonoscopy, once the cecum had been reached, significantly increased the diagnostic yield for detection of mucosal lesions of the colon, particularly small and nonprotruding ones, without affecting the withdrawal time. In colon cancer screening, the routine use of HD+ plus i-Scan can recognize more mucosal lesions without the need to prolong the withdrawal time to allow for closer inspection, as suggested in other studies, and could probably enable less-skilled endoscopists to achieve performances comparable to those of experienced ones in detecting colonic polyps.

COMMENTS

Background

Screening colonoscopy is widely considered the gold standard for detection of colonic neoplasia and adenomatous lesions, however, there are several reports of failure to detect small and flat neoplastic lesions, meaning that in these cases, colonoscopy does not provide adequate protection against colorectal cancer. Besides the operator's experience, withdrawal time, quality of colon cleansing, presence of blind segments in the colon, and quality of imaging provided by endoscopes play an important role in lesion detection. Standard white-light imaging may be unable to recognize some small or flat lesions, which are particularly frequent in the right colon, and it may affect the polyp miss rate during routine colonoscopy. High-definition (HD) imaging and filter technologies have been applied to colonoscopies to improve detection of lesions, but results are conflicting.

Research frontiers

Endoscopes have now been designed to improve mucosal visualization, with a wide angle of view, filter-aided techniques that can enhance characterization of mucosal morphology and surface architecture, and high-resolution/high-definition imaging that can improve endoscopic recognition of mucosal lesions. In this study, the authors demonstrated that the routine use of HD+ plus i-Scan recognized more mucosal lesions without the need to prolong the withdrawal time to allow closer inspection.

Innovations and breakthroughs

Recent studies have analyzed the capacity of high-definition imaging to improve the lesion detection rate during colonoscopy with conflicting results. The value of high-definition colonoscopy in clinical practice is still debated. In this study, the authors showed that the routine addition of i-Scan to HD+ imaging during the entire withdrawal phase of the colonoscopy significantly increased the diagnostic yield for detection of mucosal lesions of the colon, particularly small and nonprotruding ones, without affecting the withdrawal time, and could probably enable less-skilled endoscopists to achieve performances comparable to those of experienced ones in detecting mucosal lesions.

Applications

This study may encourage the utilization of advanced imaging technologies to reduce polyp miss rate and improve colonoscopy performance in the prevention of colorectal cancer.

Terminology

The i-Scan technology is a digital contrast method employing a light filter that uses different software algorithms with real-time image mapping embedded in the Pentax EPKi processor. i-Scan enhances mucosal imaging by activating three distinct functions: one for surface enhancement (SE), the second for contrast enhancement (CE), and the third for tone enhancement (TE), allowing a better recognition and characterization of the mucosal lesions during colonoscopy. SE mode enhances the structure through recognition of the edges, compared to normal images, and allows easier recognition of minute glandular structures which makes it simpler to identify changes on the basis of structural differences. CE mode enhances minute irregularities and depressed areas of the mucosal surface with a slight bluish-white stain. In TE mode, the RGB components of an ordinary endoscope image are broken down into their parts, and each one is then converted independently along the tone curve, followed by resynthesis of the three components to yield a reconstructed image.

Peer review

The authors examined the role of HD+ i-Scan *vs* white-light colonoscopy on polyp detection rates. The research is a significant addition to the literature on the use of contrast technology in improving the quality of colonoscopy in detecting polyps. The results of the study will encourage those regularly involved in performing colonoscopy to consider a lower threshold in utilizing these techniques to improve polyp detection rates. The research novelty is in the fact that the study was conducted in a real clinical practice environment and could be considered to have greater clinical applicability.

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BRIEF ARTICLE

Graft-versus-host disease after liver transplantation: A comprehensive literature review

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Abstract

AIM: To determine the factors affecting mortality in patients who developed graft-versus-host disease (GvHD) after liver transplantation (LT).

METHODS: We performed a review of studies of GvHD following LT published in the English literature and accessed the PubMed, Medline, EBSCO, EMBASE, and Google Scholar databases. Using relevant search phrases, 88 articles were identified. Of these, 61 articles containing most of the study parameters were considered eligible for the study. Risk factors were first examined using a univariate Kaplan-Meier model, and variables with a significant association ($P < 0.05$) were then subjected to multivariate analyses using a Cox proportional-hazards model.

RESULTS: The 61 articles reported 87 patients, 58 male and 29 female, mean age, 40.4 ± 15.5 years (range: 8 mo to 74 years), who met the inclusion criteria for the present study. Deaths occurred in 59 (67.8%) patients, whereas 28 (32.2%) survived after a mean follow-up period of 280.8 ± 316.2 d (range: 27-2285 d). Among the most frequent symptoms were rash (94.2%), fever (66.6%), diarrhea (54%), and pancytopenia (54%). The average time period between LT and first symptom onset was 60.6 ± 190.1 d (range: 2-1865 d). The Kaplan-Meier analysis revealed that pancytopenia (42.8% *vs* 59.3%, $P = 0.03$), diarrhea (39.2% *vs* 61.0%, $P = 0.04$), age difference between the recipient and the donor (14.6 ± 3.1 years *vs* 22.6 ± 2.7 years, $P < 0.0001$), and time from first symptom occurrence to diagnosis or treatment (13.3 ± 2.6 mo *vs* 15.0 ± 2.3 mo, $P < 0.0001$) were significant factors affecting mortality, whereas age, sex, presence of rash and fever, use of immunosuppressive agents, acute rejection before GvHD, etiological causes, time of onset, and donor type were not associated with mortality risk. The Cox proportional-hazards model, determined that an age difference between the recipient and donor was an independent risk factor ($P = 0.03$; hazard ratio, 7.395, 95% confidence interval, 1.2-46.7).

CONCLUSION: This study showed that an age difference between the recipient and donor is an independent risk factor for mortality in patients who develop GvHD after LT.

Key words: Liver transplantation; Graft-versus-host disease; Immunosuppression; Rash; Pancytopenia; Diarrhea; Chimerism; Age factors

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INTRODUCTION

Graft-versus-host disease (GvHD) results from the reac-

tion of donor immunocompetent cells against tissues of an immunosuppressed host[1-5]. GvHD is a well-known complication in patients who undergo allogeneic bone-marrow transplantation. However, few reports of GvHD after solid-organ transplantation include liver transplantation (LT)[6-9]. The reported incidence of this complication varies from 0.1% to 2%, with a mortality rate of > 75%; GvHD usually occurs between the second and sixth week after LT[5,10,11]. The clinical manifestations of GvHD following LT include fever, rash, diarrhea, and hematocytopenia, but the basic function of the transplanted liver is not affected[2,9,12,13]. The diagnosis of GvHD following LT can be difficult, as many of the clinical signs can be caused by drug reactions or viral infections including cytomegalovirus (CMV)[9]. Although a sizable number of modalities have been used to manage this disease, the most effective combination has not been determined.

MATERIALS AND METHODS

The primary purpose of this study was to examine the existing literature on GvHD following LT. Thus, we conducted a thorough literature search regarding GvHD developing after LT using the PubMed, Medline, EBSCO, EMBASE, and Google Scholar databases from November 2011 to March 1988, when Burdick *et al*[14] presented the first study on GvHD following LT. The keywords we used for the search were "graft-versus-host disease," "graft-versus-host disease after liver transplantation," "graft-versus-host disease following liver transplantation," and "graft-versus-host disease and solid-organ transplantation." The reference lists of all articles introduced as reviews were checked to attain a wider search range. The search identified 88 article titles. More detailed information was requested through contact with the corresponding authors and/or the related journal editors for studies in which insufficient data were provided or in which full texts could not be accessed. Twenty-seven full-text articles were excluded from the study because the authors could not be reached, a case presentation was duplicated, or only a literature review was provided that did not include sufficient information for comparison with other studies. A total of 61 articles containing most of the parameters mentioned below were considered eligible for the study. One of the two cases presented by Schuchmann *et al*[15] was excluded because it was presented in another study. The study by Knox *et al*[16] was excluded because only pulmonary GvHD developed following LT. In the 61 studies for which full texts could be accessed, the following data were evaluated: age, sex, donor age, age difference between the recipient and the donor, blood group compatibility (identical or not), donor type (living/cadaveric), use of primary immunosuppressive medications (tacrolimus, cyclosporine, or azathiopurine), primary hepatic disease, time of onset (postoperative day), first manifestations (rash, fever, pancytopenia, thrombocytopenia, leukopenia, and diarrhea), time interval elapsed between the first manifestation and the diagnosis and/or treatment (d), re-transplantation, mortality, and follow-up. The aim of this literature search was to identify factors affecting the occurrence of mortality in post-transplantation GvHD. Thus, the patients were divided into a mortality group (n = 59) and a survival group (n = 28). Accordingly, symptoms such as fever, rash, and diarrhea were collected under the title of "first symptoms" after ruling out other possible causes. Similarly, pancytopenia, thrombocytopenia, or leukopenia that developed before the confirmation of the GvHD diagnosis were all collected under the title of "pancytopenia." Symptoms or hematological disorders developing after commencement of treatment were left out of the former classifications. The time period between development of the first symptom associated with the disease and the transplantation was termed "time of onset."

Statistical analysis

SPSS version 13.0 (SPSS, Inc., Chicago, IL, United States) was used for the statistical analysis. Data are presented as mean ± SD for continuous variables and as frequencies for categorical variables. The statistical significance of differences between groups was examined using Pearson's χ^2 test for categorical variables and the Student t-test for continuous variables. Risk factors for outcomes were first examined using a univariate Kaplan-Meier model, and variables with a significant association ($P < 0.05$) were then subjected to multivariate analyses using a Cox proportional-hazards model. All statistical tests were two-sided with a significance level of 0.05.

RESULTS

We retrospectively evaluated 61 studies that included 87 patients, 58 male and 29 female, with age range of 8 mo to 74 years (mean, 40.4 ± 15.5 years). There were 59 (67.8%) deaths, while 28 (32.2%) survived at a mean follow-up of 280.8 ± 316.2 d (range: 27-2285 d). In the Kaplan-Meier model, parameters such as pancytopenia (P = 0.03), diarrhea (P = 0.04), age difference between the recipient and the donor ($P < 0.0001$), and the time elapsed between development of the first symptoms and the diagnosis or treatment ($P < 0.0001$) were significant risk factors for mortality. The results of multivariate Cox proportional-hazards model analysis revealed that age difference was an independent and strong risk factor (P = 0.03; hazard ratio, 7.395, 95% confidence interval, 1.2-46.7). Kaplan-Meier mortality curves for patients with and without diarrhea and pancytopenia are presented in Figure 1. Demographic and statistical data for the mortality and survival groups is provided in Tables 1 and 2. The distribution of both groups by time since the first description of GvHD after LT is depicted in Figure 2. We noted that mortality rates peaked in some years and that cases in the survival group pursued a more stable course compared with those in the mortality group.

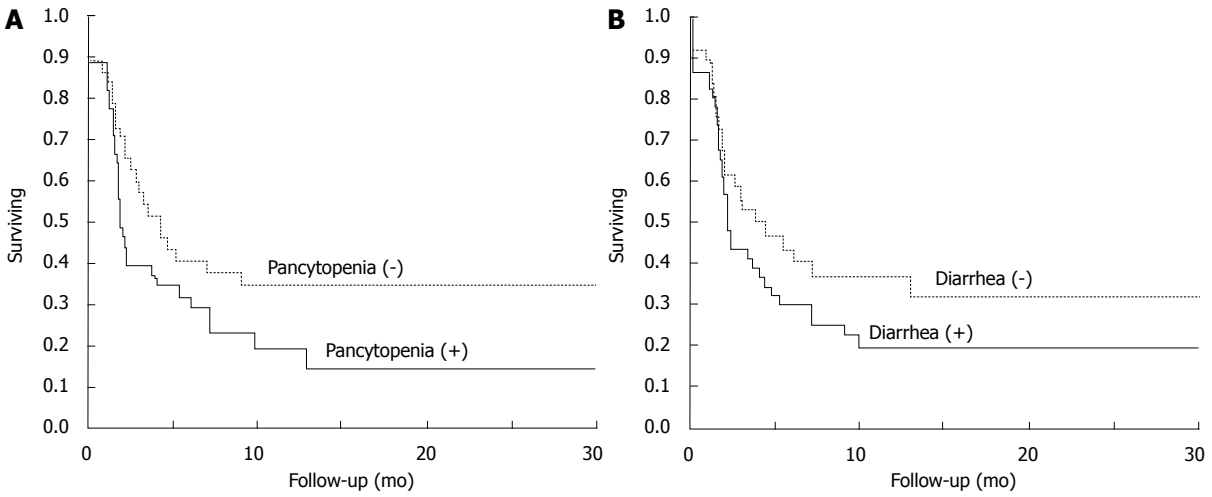

Figure 1 Kaplan-Meier survival curves for patients with and without pancytopenia and diarrhea. A: Pancytopenia; B: Diarrhea.

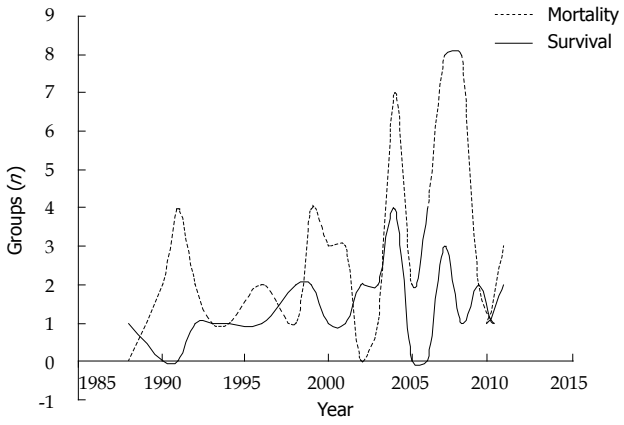

Figure 2 Distribution of survival and mortality groups by time of first description of graft-versus-host disease after liver transplantation.

DISCUSSION

Description, pathogenesis, and incidence of GvHD after liver transplantation

GvHD was first described by Billingham in 1966 as a reaction of the donor's immunocompetent cells against the recipient's cellular antigens[17,18]. The development of GvHD implies the fulfillment of three prerequisites: (1) a source of immunocompetent lymphocytes; (2) histocompatible antigenic differences between donor and host; and (3) inability of the host to reject donor lymphocytes[10,19-21]. This reaction occurs in as many as 80% of patients after bone-marrow transplantation. It has also been infrequently reported after transfusion of blood products or after solid-organ transplantation, such as pancreas-spleen, heart-lung, and liver[6,14,17,22,23]. The development of GvHD after solid-organ transplantation was first defined in 1984 by Starzl *et al*[24] in a patient undergoing a combined pancreas and splenic transplantation operation. GvHD developing after LT was first defined by Burdick *et al*[14] in 1988.

Although the exact mechanisms are still unclear, the three basic prerequisites mentioned above are also applicable to GvHD after LT. An estimated 10^9-10^{10} donor lymphocytes remain in the portal tracts and the parenchyma of a donor liver graft after flushing with cold preservative solution[18,21,25-27]. These T-cells are detectable in the peripheral blood and organs of patients during the first weeks after LT[18,20,25,26,28]. The donor lymphocytes colonize the recipient, recognize the host tissue antigens as foreign, and react against the host tissue. In other words, if the "balance of power" between the donor and recipient immune systems favors the donor, donor lymphocytes may be activated, leading to GvHD.

Although the exact incidence of GvHD following LT remains to be determined, various studies have cited rates of 0.1%-2%[27,29-32]. Yuksekkaya *et al*[11] reported that the incidence of GvHD was as high as 22.2% in patients whose donors were mismatched on at least one human leukocyte antigen (HLA) A and B antigens[11,33]. In our examination of 15 articles, we found that GvHD was evident in only 62 (0.06%) of 9492 patients undergoing LT, which was similar to frequencies reported previously[32-36].

Classification of GvHD after liver transplantation

GvHD has been reported after solid-organ transplantation with humoral and cellular presentations. The humoral type, also known as graft-versus-host hemolysis, is characterized by hemolysis and fever and occurs in patients transplanted with ABO-incompatible or nonidentical grafts. The cellular type of GvHD occurs when immunocompetent donor lymphocytes originating from the transplanted liver undergo activation and clonal expansion, allowing them to mount a destructive cellular immune response against recipient tissues. The response is directed against the major histocompatibility complex and often results in severe multisystem disease with a high mortality rate[19,37-40].

GvHD responses can be classified as acute or chronic, depending on the timing and character of alloimmune activity[11,41,42]. Acute GvHD comprises all manifestations that occur during the first 100 d after transplantation, and chronic GvHD includes all manifestations that oc-

Table 1 Comparison of variables between surviving and dead patients *n* (%)

Characteristics	Surviving (*n* = 28)	Dead (*n* = 59)	Total (*n* = 87)	Univariate analysis	Multivariate analysis
Age, yr	38.7 ± 22.7	40.4 ± 15.5	40.4 ± 15.5	0.1	
Sex				0.8	
Male	20 (71.4)	38 (64.4)	58 (66.6)		
Female	8 (28.6)	21 (35.6)	29 (33.4)		
Rash				0.8	
Present	27 (96.4)	55 (93.2)	82 (94.2)		
Absent	1 (3.6)	4 (6.8)	5 (5.8)		
Fever				0.1	
Present	17 (60.7)	41 (69.5)	58 (66.7)		
Absent	11 (39.3)	18 (30.5)	29 (33.3)		
Pancytopenia				0.03	0.6
Present	12 (42.9)	35 (59.3)	47 (54)		
Absent	16 (57.1)	24 (40.7)	40 (46)		
Diarrhea				0.04	0.1
Present	11 (39.3)	36 (61)	47 (54)		
Absent	17 (60.7)	23 (39)	40 (46)		
Acute rejection before GvHD				0.4	
Yes	7 (25)	8 (13.6)	15 (17.3)		
No	21 (75)	51 (86.4)	72 (82.7)		
Immunsuppressive agent				0.5	
Tacrolimus	15 (53.6)	25 (42.4)	40 (46)		
Cyclosporine	10 (35.7)	20 (33.9)	30 (34.5)		
Azathiopurine	0 (0)	1 (1.7)	1 (1.1)		
Un-noted	3 (10.7)	13 (22)	16 (18.4)		
Re-transplantation				0.4	
Yes	2 (92.9)	2 (3.4)	4 (4.6)		
No	26 (7.1)	57 (96.6)	83 (95.4)		
Time of onset (POD), d	109 ± 64	38 ± 5	60.6 ± 190.11	0.4	
Etiology				0.3	
Donor type				0.2	
Cadaveric	16 (57.1)	16 (27.1)	32 (36.8)		
Living	2 (7.2)	8 (13.6)	10 (11.5)		
Un-noted	10 (35.7)	35 (59.3)	45 (51.7)		
Age difference between recipient and donor, yr	14.6 ± 3.1	22.6 ± 2.7	19.8 ± 13.2	< 0.0001	0.03[1]
Blood group				0.4	
Identical	16 (57.1)	30 (51)	46 (52.9)		
Non-identical	3 (10.7)	3 (5)	6 (6.9)		
Un-noted	9 (32.1)	26 (44)	35 (40.2)		
Time between symptoms and diagnosis or first treatment, mo	13.3 ± 2.6	15.0 ± 2.3	14.3 ± 14.3	< 0.0001	0.1

[1]Hazard ratio: 7.3, 95% confidence interval: 1.2-46.7. GvHD: Graft-versus-host disease; POD: Post-operative day.

cur after 100 d[11,42-44]. However, multiple findings suggest that this may no longer be a suitably useful distinction. Acute GvHD lesions may be found after 100 d, whereas chronic GvHD lesions sometimes appear before 100 d. Acute GvHD histological findings can be found in biopsies performed after day 100, and lichenoid findings can be found in biopsies performed before day 100[42,44]. The number of days after transplant is an insufficient criterion to distinguish acute from chronic GvHD. Good clinical and pathological descriptions are needed. Chronic GvHD can occur as a progression of acute GvHD, as a recurrence following a disease-free interval, or without a history of acute GvHD. Each of these forms accounts for approximately one-third of cases.

Risk factors for development of GvHD

The causes of GvHD following organ transplantation have not been clarified, but several risk factors have been implicated, including close HLA matching between the recipient and donor[18,27,45], blood transfusion prior to transplantation[11], immunosupressive treatment before transplantation[11,27], glucose intolerance[35], rejection before GvHD[4], autoimmune hepatitis[35], alcoholic liver disease[35], hepatocellular carcinoma (HCC)[27,35], re-transplantation[27], a large age discrepancy between donor (younger) and recipient (older)[25], recipient age > 65 years[18,27,45,46], and multiorgan transplantation[5,21,33,35,40]. Only two studies offered an evidence-based risk analysis with regard to the development of GvHD after LT. In a study by Smith *et al*[33], risk factors included recipient age ≥ 65 years, recipient-donor age difference ≥ 40 years, and close matching of the HLA types of the donor to those of the recipient. Chan *et al*[35] documented glucose intolerance, autoimmune hepatitis, alcoholic liver disease, HCC, and various combinations of these but not such parameters as age, sex, ischemia duration, HLA mismatch, or age dif-

Table 2 Distribution of patients according to underlying liver disease

Etiology	Surviving (*n* = 28)	Dead (*n* = 59)	Total (*n* = 87)
ALD	4	7	11
ALD + HCC	1	3	4
ALD + HCV	0	3	3
HBV	1	3	4
HBV + HCC	3	6	9
HBV + HDV	1	0	1
HCV	0	7	7
HCV + HCC	1	0	1
HCC	1	3	4
PBC	1	5	6
PSC	1	3	4
PSC + HCC	0	2	2
Biliary atresia	4	2	6
Hemangioma	0	1	1
Cryptogenic	4	5	9
Acute failure	3	4	7
Autoimmune hepatitis	1	1	2
HAV	1	0	1
Policystic disease	0	1	1
Laennec's cirrhosis	1	0	1
Wilson disease	0	1	1
Langerhans' cell histiocytosis	0	1	1
NRH	0	1	1

ALD: Alcoholic liver disease; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; PBC: Primary biliary chirosis; PSC: Primary sclerosing cholangitis; NRH: Nodular regenerative hyperplasia; HAV: Hepatitis A virus; HBV: Hepatitis B virus.

ferences as risk factors. Chan *et al*[35] argued that most of the risk factors they identified permitted patients to lapse into an immunosuppressive state, suggesting an inclination toward development of the disease before the LT operation. Our literature search showed that the most frequently encountered liver diseases in affected patients were HCC (23%) and alcoholic liver disease (20.7%). The suggestions by Chan *et al*[35] support our findings, but we lack confirming evidence.

Clinical presentation of GvHD

The clinical presentation of GvHD following LT includes skin rash, fever, diarrhea and hematocytopenia[5,47]. Characteristically, the transplanted liver is not a target of GvHD after LT because both graft liver and immunocompetent cells responsible for GvHD are of donor origin[25,28,30]. The most frequently appearing symptoms in our search were rash (94.2%), fever (66.6%), diarrhea (54%), and pancytopenia (54%). Among these symptoms, pancytopenia (P = 0.03) and diarrhea (P = 0.04) were confirmed by univariate analysis to be risk factors affecting mortality. These results indicate that intestinal and bone-marrow involvement may give rise to severe complications.

The clinical symptoms of GvHD usually become apparent between 1 and 8 wk after LT, often after an initial uneventful recovery from surgery and discharge from the hospital[40,48]. Our literature review revealed that the first symptoms appear 60.6 ± 190.1 d (range: 2-1865 d) after the LT operation. Although the time interval was shorter in the non-surviving group, it was not among the risk factors for death (P = 0.4). Despite this result, we believe that the mortality in cases complicated with GvHD within the first month is much higher.

Diagnosis of GvHD

A diagnosis of GvHD after LT is based on the presence of clinical manifestations, a demonstration of chimerism, and histopathological evidence[29,42,47,49]. As the clinical presentation of GvHD is inconsistent, a high degree of suspicion is necessary to pursue a diagnosis. Any or all clinical symptoms mentioned above may be seen during the initial presentation of GvHD. A skin biopsy showing epidermal dyskeratosis with epithelial cell necrosis is highly suggestive but not pathognomonic for GvHD[29,39,40,48]. Chimerism can be established by various methods that examine the presence of donor cells in the recipient's peripheral blood or various tissues[41,50]. These methods include serological HLA typing of peripheral blood, restriction fragment length polymorphism[28,29,51-53], and fluorescent *in situ* hybridization (FISH), which have been used to demonstrate chimerism in recipients with suspected GvHD after LT[28,34,48]. Chimerism at the tissue level has been shown by polymerase chain reaction, short tandem repeat analysis, and FISH techniques in the skin and bone marrow of patients with GvHD after LT[46,48,52,54,55]. Peripheral blood chimerism appears transiently in the majority of patients during the early postoperative period after LT, particularly in the first week, and rapidly declines by the third to fourth week post-transplant[39,45]. For this reason, chimerism may not be evident in the peripheral blood of patients with late-onset GvHD[32,56].

Differential diagnosis and clinical significance

The differential diagnosis of GvHD after LT is frequently delayed because early symptoms are often non-specific. The differential diagnosis consists of (1) drug-induced skin reactions, including toxic epidermal necrolysis and mycophenolate mofetil toxicity; (2) viral exanthemas; (3) infectious enteritis, including CMV infection and *Clostridium difficile* colitis, and (4) organ rejection[13,41,57-59]. Many of the clinical signs of GvHD may also be seen with CMV infection. The presence of CMV in a patient with GvHD may complicate the appropriate diagnosis and delay treatment. A significant association between acute GvHD and CMV after transplant has been documented and may be related to pancytopenia resulting from bone-marrow depletion by attacking donor lymphocytes[9,11,34,39].

A rapid differential diagnosis and early implementation of treatment for GvHD following LT are two factors that affect survival. In contrast, studies showing that early treatment was not effective in the ultimate outcome have also been published[18,40]. Taylor *et al*[40] based their opinions on a literature search. They reported that early implementation of treatment did not produce a statistically significant difference in mortality. We found that the time interval between the appearance of first symptoms

and definitive diagnosis and/or treatment, which ranged from 1 to 65 d, was a statistically significant predictor of death ($P < 0.0001$).

Treatment of GvHD after liver transplantation

The evidence base for selecting the most appropriate therapy for established GvHD after LT is very limited; thus, treatment is largely empirical, although the extensive literature on managing acute GvHD after stem cell transplantation provides guidance[21,25,27,40]. A number of treatment modalities have been proposed based on the known pathophysiological mechanism of GvHD. However, as most of the treatment modalities are implemented in combinations, the optimal combination has not yet been identified. Moreover, some patients respond well to a decrease in the intensity of immunosuppressive treatment[38,55], or to replacement with another immunosuppressive agent[38,39,60,61], but good outcomes have also been reported using incremental doses of immunosuppressive drugs[52,57,58,62]. On the other hand, the literature has also reported the development of acute rejection in patients whose immunosuppressive drug dosage was decreased or the relevant medication was ceased; hence, switching to another medication may seem more reasonable than changing the dosage of the main immunosuppressive agent[31]. Each patient should be evaluated individually.

Among the most frequently administered treatment modalities for GvHD after LT mentioned in the literature are corticosteroid treatment[9,25,46,56], decrease/cessation/increase in or replacement of the immunosuppressive medication[22,26,52,55,62], and the use of antibodies directly targeting T lymphocytes, monoclonal antibodies targeting various receptors on the surfaces of lymphocytes, intravenous immunoglobulin[31,42,58,63,64] as an immune support, and antimicrobial treatments appropriate to suppress the infection[10,18].

Most of the experience regarding corticosteroid use in treating GvHD is based on the practices of hematopoietic stem cell transplantation[4]. The lympholytic and immunosuppressive effects of steroids, in addition to their potent anti-inflammatory characteristics, have provided justification for their widespread administration[4]. In our literature search, steroid treatment was instituted in 61 of 87 patients in whom GvHD developed after LT, whereas other treatment modalities were preferred in 21, and the remaining 5 patients were monitored for symptoms[19,38,65]. Death occurred in 43 patients on steroid treatment. Immunosuppressive treatment was re-administered upon development of acute rejection in two patients in whom the main immunosuppressive treatment was replaced by steroid treatment. Etanercept (Enbrel) therapy was commenced in one patient due to a failed response to steroid treatment, and a reduction in cyclosporine, and this approach yielded a successful outcome[45]. Most of the patients who experienced complications or a suboptimal response to treatment were administered various monoclonal antibodies or antagonist agents to T-lymphocytes[65-69]. The most commonly used drugs were the following: daclizumab (Zenapax)[21,29,32,68] and basiliximab (Simulect)[4,5,11,28,41], which bind to the CD25 subunit of interleukin (IL)-2 receptors on the surface of T-lymphocytes; muromonab (OKT3)[7,23,47,53], which binds to CD3 receptors on the surface of T-lymphocytes; alemtuzumab (Campath-1H)[50], which binds to CD52 receptors on the surface of mature lymphocytes; infliximab (Remicade)[30,32], which was developed against tumor necrosis factor-alpha; denileukin diftitox (Ontak)[39], which was developed by conjugation with diphtheria toxin for use against the IL-2 receptors on the surface of T-lymphocytes; and rituximab (Mabthera)[5], which binds to CD20 receptors on the surface of B lymphocytes. In addition to these agents, anti-thymocyte globulin (ATG)[26,28,66,67,69], effective directly on T-lymphocytes, and anti-lymphocyte globulin (ALG)[6,19,22] were also frequently utilized during treatment. In our literature analysis, we found that ATG, basiliximab, muromonab, ALG, daclizumab, infliximab, alemtuzumab, and rituximab and denileukin diftitox were administered in 25, 11, 7, 5, 4, 2, 1, 1, and 1 of the patients, respectively. Of these 57 patients, 13 were placed on monoclonal antibodies and/or T-lymphocyte antagonists as a first treatment modality, whereas steroids, immunosuppressive agents, and various combinations thereof were administered in 44 patients. Mortality rates did not differ among treatment conditions but were quite high in all treatment modalities, indicating that the most appropriate treatment modality has yet to be developed.

Prognosis of GvHD after liver transplantation

The prognosis for GvHD that develops after LT is rather poor, and mortality rates mentioned in the literature range from 75% to 91.6%[9,27,28,32,58]. The mortality rate observed in our literature analysis (67.8%) was lower than that reported in studies cited above. Nearly all patients died of multiple organ dysfunction syndrome, sepsis, or gastrointestinal bleeding despite significant antimicrobial and hematologic support. The only study evaluating mortality in GvHD after LT was a literature search conducted by Taylor *et al*[40] that included 51 cases. According to that study, rash and fever were identified as risk factors for mortality. We obtained different results (Table 1), which suggest that bone marrow (pancytopenia) and intestinal (diarrhea) involvement had a severe effect on mortality. However, the retrospective nature of this study, exclusion of some studies due to inadequate data, failure to obtain sufficient data regarding an HLA match, and the absence of a standardized treatment protocol were limiting factors. Such high rates of mortality despite any type of aggressive treatment revive the issue of protective precautions prior to LT.

Prevention of GvHD after liver transplantation

Preventing GvHD among patients undergoing LT is an important issue. Depletion of T-lymphocytes from the liver before transplantation would eliminate the risk of GvHD. This could be achieved, at least in principal, by

treating the cadaveric donor with ALG or by modifying the donor liver *ex vivo* by irradiation or perfusion with lytic monoclonal antibodies directed against a lymphocyte cell-surface protein[7,8]. However, whether these approaches can be justified is debatable, given the low incidence of GvHD after LT[40,47]. The donor's immunoactive cells can be removed by sufficient perfusion of the graft by carefully removing perihepatic lymph nodes or through graft radiation[31]. Based on our LT experience, perfusion of grafts from living or cadaveric donors with University of Wisconsin (Viaspan) or histidine-tryptophan-ketoglutarate (Custodiol) solution, followed by lactated Ringer's solution at 4 ℃, has proved a fairy efficacious method to remove donor-related lymphocytes from graft material. Some authors believe that transfusion-associated GvHD can be prevented by irradiating blood products and avoiding the use of related donors. Therefore, limiting the application of blood products and using washed red blood cells, white blood cell-free plasma, or platelets could contribute to the prevention of GvHD[31]. We prefer to irradiate erythrocyte suspensions routinely before transfusion in patients who have undergone LT.

In conclusion, although GvHD is a rare complication of LT and the mortality rate remains very high, clinical features represent an important tool for early diagnosis. The prognosis remains poor and further research is needed to clarify the pathogenesis of GvHD and to provide new therapeutic agents for treating this condition effectively.

COMMENTS

Background

Graft-versus-host disease (GvHD) occurs when the donor's immunocompetent cells react against the recipient's cellular antigens. GvHD is a well-known complication in patients who undergo bone-marrow transplantation. However, few reports have described GvHD after liver or other solid organ transplantation. The prognosis for GvHD after liver transplantation (LT) is rather poor, and mortality rates mentioned in the literature range from 75% to 91.6%. Therefore, it is important to determine the factors that affect the prognosis of the disease.

Research frontiers

The authors performed an extensive literature review regarding the development of GvHD after LT that included articles in the PubMed, Medline, EBSCO, EMBASE, and Google Scholar databases published between November 2011 and March 1988.

Innovations and breakthroughs

This study is the most extensive literature review examining factors affecting mortality in patients who develop GvHD after LT.

Applications

Univariate analyses showed that pancytopenia, diarrhea, an age difference between the recipient and donor, and time from first symptom occurrence to diagnosis or treatment were significant risk factors for mortality, and multivariate analysis demonstrated that an age difference between the recipient and donor was an independent risk factor for mortality.

Terminology

GvHD can be divided into acute and chronic forms depending on the timing and character of alloimmune activity. The acute form comprises all manifestations that occur during the first 100 d after transplantation, and the chronic form includes all manifestations that occur after 100 d. GvHD can also be divided into humoral and cellular forms. The humoral form is characterized by hemolysis and fever and occurs in patients transplanted with ABO-incompatible or non-identical grafts. The cellular form occurs when immunocompetent donor lymphocytes originating from the transplanted liver undergo activation and clonal expansion, allowing them to mount a destructive cellular immune response against recipient tissues.

Peer review

This is a good descriptive study in which authors determine the factors affecting mortality in patients who developed GvHD after LT. The results are interesting and showed that an age difference between the recipient and donor is an independent risk factor for mortality in patients who develop GvHD after LT.

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BRIEF ARTICLE

Short-term effectiveness of radiochemoembolization for selected hepatic metastases with a combination protocol

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Abstract

AIM: To introduce the combination method of radiochemoembolization for the treatment of selected hepatic metastases.

METHODS: Twenty patients with biopsy proven hepatic metastases were selected from those who underwent transarterial radiochemoembolization, a novel combination protocol, between January 2009 and July 2010. Patients had different sources of liver metastasis. The treatment included transarterial administration of three chemotherapeutic drugs (mitomycin, doxorubicin and cisplatin), followed by embolization with large (50-150 μm) radioisotope particles of chromic 32P. Multiphasic computer tomography or computer tomography studies, with and without contrast medium injections, were performed for all patients for a short-term period before and after the treatment sessions. The short-term effectiveness of this procedure was evaluated by modified response evaluation criteria in solid tumors (mRECIST), which also takes necrosis into account. The subjective percentage of necrosis was also assessed. The response evaluation methods were based on the changes in size, number, and the enhancement patterns of the lesions between the pre- and post-treatment imaging studies.

RESULTS: Patients had liver metastasis from colorectal carcinomas, breast cancer, lung cancer and carcinoid tumors. The response rate based on the mRECIST criteria was 5% for complete response, 60% for partial response, 10% for stable disease, and 25% for progressive disease. Regarding the subjective necrosis percentage, 5% of patients had complete response, 50% had partial response, 25% had stable disease, and 20% had progressive disease. Based on traditional RECIST criteria, 3 patients (15%) had partial response, 13 patients (65%) had stable disease, and 4 patients (20%) had disease progression. In most patients, colorectal carcinoma was the source of metastasis (13 patients). Based on the mRECIST criteria, 8 out of these 13 patients had partial responses, while one remained stable, and 5 showed progressive disease. We also had 5 cases of breast cancer metastasis which mostly remained stable (4 cases), with only one partial response after the procedure. Six patients had bilobar involvement; three of them received two courses of radiochemoembolization. The follow up imaging study of these patients was performed after the second session. In the studied patients there was no evidence of extrahepatic occurrence, including pulmonary radioactive deposition, which was proven by Bremsstrahlung scintigraphy performed after the treatment sessions. For the short-term follow-ups for the 2 mo after the therapy, no treatment related death was reported. The mostly common side effect was post-embolization

syndrome, presented as vomiting, abdominal pain, and fever. Nineteen (95%) patients experienced this syndrome in different severities. Two patient had ascites (with pleural effusion in one patient) not related to hepatic failure. Moreover, no cases of acute liver failure, hepatic infarction, hepatic abscess, biliary necrosis, tumor rupture, surgical cholecystitis, or non-targeted gut embolization were reported. Systemic toxicities such as alopecia, marrow suppression, renal toxicity, or cardiac failure did not occur in our study group.

CONCLUSION: Radiochemoembolization is safe and effective for selected hepatic metastases in a short-term follow-up. Further studies are required to show the long-term effects and possible complications of this approach.

Key words: Hepatic metastasis; Radiochemoembolization; Phosphorus radioisotopes; Treatment; Outcome

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INTRODUCTION

Although complete surgical resection of the hepatic portion affected by metastasis is usually the best treatment option, most patients with hepatic metastasis are not amenable to resection or have some contraindications to the surgery[1]. As alternatives to standard systemic chemotherapy, some recent palliative therapies have been developed for unresectable hepatic metastases, which include transarterial administration of chemotherapeutic drugs or radiopharmaceuticals, selective tumor vessel embolization and percutaneous tumor ablation with ethanol injection, cryotherapy, radiofrequency, or the use of microwaves[1,2].

Transcatheter arterial chemoembolization (TACE) is a dual minimally invasive therapeutic approach combining transarterial administration of chemotherapeutic drugs and hepatic artery embolization[3]. Although there are many advantages of this combination, it does produce marked tumoral ischemia at the time of drug administration which potentiates the effect of cytotoxic agents and augments tumoral cell apoptosis[4,5].

Radioembolization, on the other hand, is a technique that preferentially targets hepatic lesions by infusing the hepatic arteries that supply the tumor with radioactive microspheres[6]. Traversing the hepatic vascular plexus, these microspheres embed within the tumor arterioles, where they deliver high-energy low-penetrating radiation doses to the tumoral cells, while the normal hepatic tissue is relatively preserved[6,7]. As can be determined from the method's name, radioembolization has also microembolic effects and leads to subsequent vessel occlusion[8].

Regarding the effectiveness of radioembolization and chemoembolization for hepatic malignant neoplasms[2,9], we assumed that the local combination of the two methods (i.e., radiochemoembolization) would be more effective. It has been shown that radioembolization in combination with systemic chemotherapy is an effective first-line therapy for liver metastases[10]. The most commonly used agent for radioembolization of hepatic tumors is Yttrium-90 (^{90}Y) in the form of ^{90}Y microspheres[9], which has a half-life of 64 h[11]. In this study, however, a phosphorus-32 containing particle was adopted as the radiopharmaceutical. In the process of phosphorus-32 (^{32}P) decay, the molecule emits relatively high energy beta particles[12]. Although there are reports of safe clinical ^{32}P application for hepatic tumors[12-16], larger particles were used in the current study to reduce systemic toxicities even more by decreasing hepatic-to-systemic shunt. In addition, the higher half-life of ^{32}P (14.3 d[17]) would provide a longer irradiation time in order to achieve chemo-radiation effects.

The primary purpose of this study is to introduce the radiochemoembolization method for the treatment of hepatic metastases. Short-term effectiveness of this treatment based on imaging criteria was also assessed. As World Health Organization (WHO) criteria and response evaluation criteria in solid tumors (RECIST) guidelines are based solely on the degree of tumor shrinkage for assessing tumor response, we used other criteria like modified RECIST (mRECIST), which includes the degree of necrosis to show the effectiveness of the therapy[18]. Finally, *via* a brief review of the literature concerning ^{32}P application and TACE, possible limitations, concerns, and complications that may be encountered with radiochemoembolization were addressed.

MATERIALS AND METHODS

This was a single institution clinical study approved by the ethics committee of our imaging center. A written consent form was obtained from all patients and they were all informed about the novelty of the method. This paper reports the results of 20 patients who underwent radiochemoembolization between January 2009 and July 2010. The inclusion criteria included: biopsy proven hepatic metastatic lesion/lesions from any source; contraindication to ablative therapies and resection; an eastern cooperative oncology group performance status score of 0 to 2[19]; and the patient needed to be at least 18 years of age. Although more than 20 patients met these criteria and received radiochemoembolization, another inclusion criterion was added to only report the results of patients

who had available contrast-enhanced computer tomography (CT) or magnetic resonance imaging (MRI) 1 to 2 mo previous and after the treatment session. Only 20 patients such patients were qualified. Exclusion criteria were: bleeding diathesis that could not be controlled; significant extra-hepatic involvement, generally more than 50% of the whole tumoral bulk outside the liver; imminent threat to the patient's life caused by the disease; greater than 75% involvement of the hepatic parenchyma; severe hepatic dysfunction; and an active uncontrolled infection.

Patients fasted overnight and received a prophylactic antibiotic (ceftriaxone, 1 g) and antiemetics (granisetron, 3 mg; dexamethasone, 8 mg). During the procedure, fentanyl or pethidine were infused to alleviate the pain caused by embolization. All procedures were performed in the angiography room under aseptic conditions. Intravenous hydration was started 1 h before the procedure.

In this study, ^{32}P-containing particles were used (Nuclear Science and Technology Institute, Iran) with $Cr^{32}PO4$ as the active component. These particles had a grain size of 50-150 μm, significantly larger than previously used colloidal ^{32}P particles also based on $Cr^{32}PO4$[13,14,20]. The physical half-life period of ^{32}P is 14.28 d, with an average penetration distance of 3-4 mm in soft tissues (maximum 8 mm)[13]. Ranging from 0.185 to 0.444 GBq, the dose of injected solution was calculated based on liver volume (not tumor burden) which was estimated with CT or MRI. The prepared ^{32}P solution was dissolved in 1-3 mL of radiographic contrast. The chemotherapeutic mixture consisted of 50 to 100 mg of cisplatin, 50 mg of doxorubicin, and 8-10 mg of mitomycin-C dissolved in 10 mL of radiographic contrast and 10 mL of normal Saline.

Using the Seldinger technique, a catheter was introduced through the femoral artery and selective catheterization of the hepatic artery was performed. A 3-F hydrophilic microcatheter (Cook, United States) used with a 0.014 or 0.021 guide wire was suffice to catheterize the desired artery. This standard catheter allows rapid injection of viscous radiochemoembolic emulsions and is unlikely to clog with particles. A digital subtraction angiography was performed to confirm that there was no hepatic arteriovenous fistula or duodenogastric reflux. For patients who had bilobar involvement, the treatment mixture was infused in both lobes simultaneously or separately in two sessions (3-4 wk apart) depending on the patient's liver function test and number of metastases. Only in one case (case 4) was coil embolization of gastroduodenal artery was performed before radiochemoembolization.

After placing the catheter in a suitable location, the chemotherapeutic mixture was infused and continuously monitored *via* fluoroscopy to avoid reflux into the untargeted arterial bed. Following this step, again under fluoroscopic surveillance, chromic ^{32}P solution was infused for vessel occlusion. If reflux happened, the infusion would be paused until the arterial flow resumed and then restarted at a lower speed.

After the procedure, intravenous hydration, antibiotics, and antiemetic therapies were continued for 24 h and analgesics were supplied for control of pain as needed. All the patients were discharged on the day after the procedure. Oral antibiotics were continued for 5 d, as well as oral antiemetics and analgesics if needed. Twenty-four to 72 h after radiochemoembolization of hepatic tumors, bremsstrahlung scintigraphy was performed in all patients to document ^{32}P particles that accumulated in tumoral locations of the liver, and also to ensure that there were no extrahepatic radioactive deposits.

For evaluating the short-term effectiveness of radiochemoembolization by means of imaging studies, two scans, whether CT or magnetic resonance (MR), were performed 1 to 2 mo before and after the treatment session. CT examinations were performed using a multidetector scanner (Sensation 64, Siemens, Germany), with 5-mm sections (120 kV, 250 mAs). Triphasic liver imaging (including unenhanced, arterial and portal venous phase images) was acquired. Contrast-enhanced scans were performed after approximately 30 s in the arterial phase and after 70 s in the venous phase from the injection of the contrast agent iohexol (Omnipaque 350, Amersham Health; 125 mL at a rate of 3-5 mL/s). MR studies were performed using a 1.5 Tesla machine (Magnetom Symphony, Siemens, Germany). The protocol consisted of axial and coronal thin-section T2-weighted HASTE, axial unenhanced spoiled-T1-weighted gradient echo with fat suppression, and dynamic axial fat-suppressed contrast-enhanced spoiled-T1-weighted gradient-echo sequences for the arterial and venous phases (45, 60 and 90 s) and also the delayed phase (2-5 min) after contrast infusion. The contrast agent was gadopentetate dimeglumine (Magnevist, Berlex Pharmaceuticals, 20 mL), followed by 20 mL of saline flush.

Evaluation of tumor response to therapy was based on mRECIST criteria and subjective percentage of necrosis. Firstly, up to two hepatic target lesions were selected in pre-treatment imaging studies. Said lesions must (1) be capable of being accurately measured in at least one dimension as 1 cm or more; (2) be suitable for repeat measurement; and (3) have intratumoral enhancement after contrast injection. If more than two lesions could met these criteria, the one with larger enhancing portions would be selected. All other hepatic lesions were only recorded and not measured at the baseline; their presence or absence would be noted in the follow-up exams. A viable tumor was defined as a portion of the target lesion which had an uptake of the contrast agent in any phase of the contrast enhanced studies. For the mRECIST criteria, the change in the longest diameter of viable tumors was considered for the evaluation of the response to treatment. On the other hand, necrosis was a portion of the target lesions which remained without contrast enhancement. Based on these necrotic portions, a subjective percentage of necrosis was attributed to each target lesion. Every individual patient had a sum of longest viable tumor diameters and necrosis percentages of target lesions. According to the changes in these amounts, responses were categorized into complete response, partial response, stable disease and progressive disease (Table 1).

Table 1 Assessment of target lesion response

mRECIST		RECIST		Subjective percentage of necrosis	
CR	Disappearance of any intratumoral enhancement in all target lesions	CR	Disappearance of all target lesions	CR	Disappearance of any intratumoral enhancement in all target lesions
PR	At least a 30% decrease in the sum of diameters of viable target lesions	PR	At least a 30% decrease in the sum of diameters of target lesions, taking as reference the baseline sum of the diameters of target lesions	PR	At least a 30% increase in the sum of target lesion necrosis percentages
SD	Any cases that do not qualify for either partial response or progressive disease	SD	Any cases that do not qualify for either partial response or progressive disease	SD	Any cases that do not qualify for either partial response or progressive disease
PD	An increase of at least 20% in the sum of the diameters of viable target lesions or new lesion appearance	PD	An increase of at least 20% in the sum of the diameters of target lesions or new lesion appearance	PD	A decrease of at least 20% in the sum of target lesion necrosis percentages or new lesion appearance

mRECIST: Modified response evaluation criteria in solid tumors; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

Table 2 Description of lesions for each patient and local tumor control outcomes with regard to different criteria

No.	Sex	Age (yr)	No. of lesions	Location	Primary source	New lesion	mRECIST	RECIST	Necrosis	Sum of viable diameters (mm)		Sum of necrosis percentages (%)		Sum of diameters (mm)	
										Baseline	Post-procedural	Baseline	Post-procedural	Baseline	Post-procedural
1	F	41	Multi	U	BRE	No	PR	SD	PR	57	18	0	100	57	44
2	F	29	Multi	U	BRE	No	SD	SD	SD	34	34	5	5	34	34
3	F	35	1	U	BRE	No	PR	SD	PR	50	33	30	50	60	45
4	F	39	Multi	B	BRE	No	PR	SD	PR	57	39	20	100	65	54
5	F	38	Multi	B	BRE	Yes	PD	PR	PD	72	-[1]	30	-	72	-
6	F	27	2	U	CAR	No	CR	PR	CR	92	0	0	200	92	42
7	M	81	3	U	CRC	No	PR	SD	PR	73	23	40	95	107	95
8	M	40	1	U	CRC	No	PR	PR	SD	55	33	5	5	55	33
9	F	41	1	U	CRC	No	PR	SD	PR	65	45	40	80	80	68
10	F	47	1	U	CRC	No	PR	SD	PR	144	103	50	80	226	217
11	F	59	1	U	CRC	Yes	PD	PD	PD	70	-	0	-	70	-
12	M	60	Multi	U	CRC	Yes	PD	PD	PD	50	-	0	-	50	-
13	M	57	1	U	CRC	Yes	PD	PD	PD	25	-	70	-	35	-
14	M	55	1	U	CRC	No	PD	PD	SD	56	102	5	5	56	102
15	M	77	Multi	U	CRC	No	SD	SD	SD	107	107	15	15	116	116
16	F	62	Multi	B	CRC	No	PR	SD	SD	40	7	155	195	68	55
17	F	57	Multi	B	CRC	No	PR	SD	PR	50	35	90	180	65	55
18	M	74	Multi	B	CRC	No	PR	SD	PR	213	116	10	130	213	159
19	F	48	Multi	B	CRC	No	PR	SD	PR	245	160	5	120	265	260
20	F	51	Multi	U	LUN	No	PR	SD	PR	142	61	20	175	150	127

[1]For patients with new lesions in the follow-up scan, the measurements were not performed and the patient was marked as progressive disease (PD); CR: Complete response; PR: Partial response; SD: Stable disease; U: Unilobar; B: Bilobar; F: Female; M: Male; BRE: Breast cancer; CRC: Colorectal carcinoma; CAR: Carcinoid tumor; LUN: Lung cancer; mRECIST: Modified response evaluation criteria in solid tumors.

RESULTS

Patients had different sources of liver metastasis, but most were from colorectal cancer. None of our patients had a history of surgery for hepatic metastases. The demographic, clinical, radiological and response data of the studied patients are shown in Table 2.

The mean and median of the baseline total viable diameters (i.e., the sum of the maximum diameters of the viable portions of target lesions in each patient) were 84.85 mm and 61 mm (range: 25-245 mm), respectively. The response rate, based on mRECIST criteria, was 5% for complete response (Figure 1A and B), 60% for partial response (Figure 1C and D), 10% for stable disease, and 25% for progressive disease (Figure 1E and F).

The baseline sum of the estimated percentage of necrosis in target lesions was calculated for each patient, and had a mean and median of 29.5% and 17.5%, respectively (range: 0% to 155%). Regarding the necrosis percentage, 5% of patients had complete response, 50% had partial response, 25% had stable disease and 20% had progression. Based on traditional RECIST criteria 3 patients (15%) had partial response, 13 patients (65%) had stable disease, and 4 patients (20%) had disease progression.

In most patients, colorectal carcinoma was the source of metastasis (13 patients). Based on mRECIST, 8 out of these 13 patients had partial responses while one remained stable and 5 showed progressive disease. We also had 5 cases of metastasis from breast cancer, which mostly remained stable (4 cases) with only case of one partial response after the procedure.

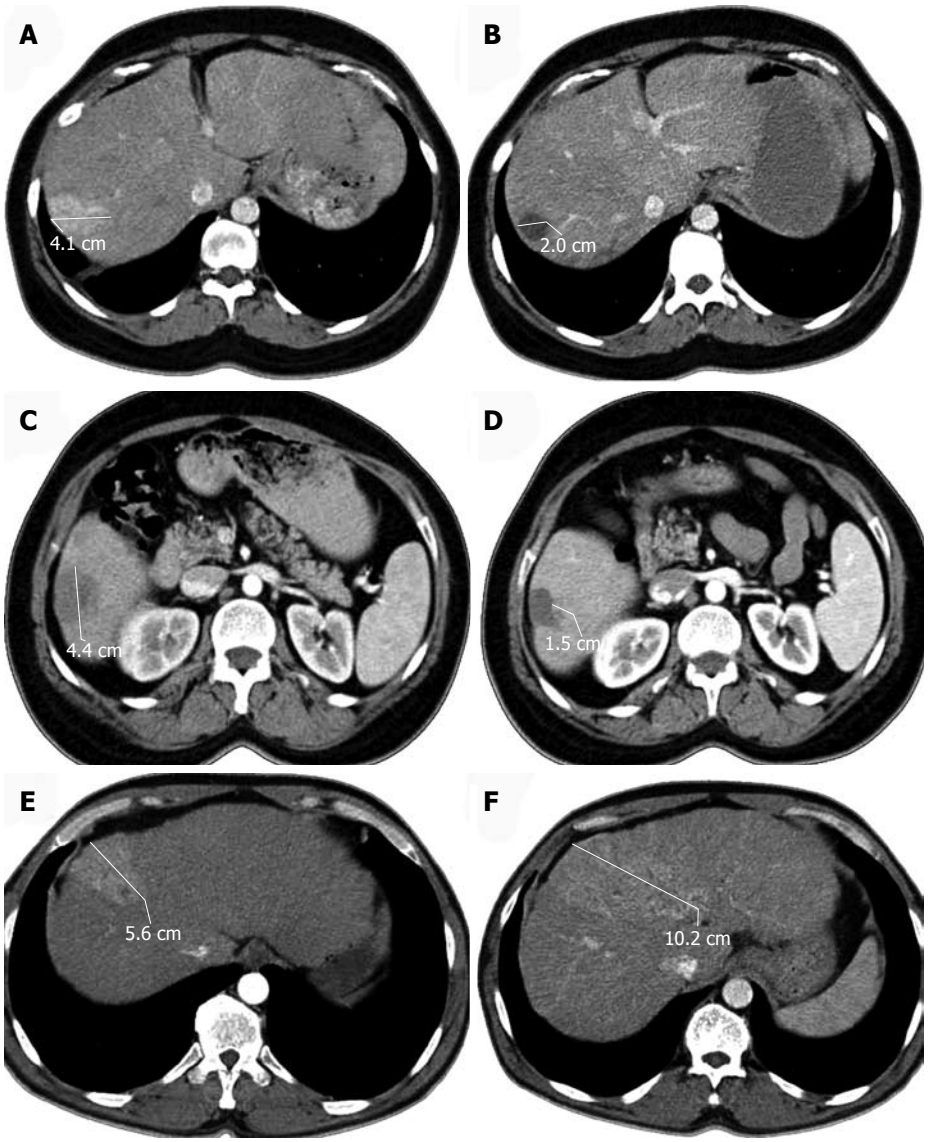

Figure 1 Radiochemoembolization images. A, B: Pre- (A) and post- (B) radiochemoembolization images in a 27 year-old female with a metastatic carcinoid tumor. There is no evidence of enhancing of the viable tumor after treatment. Based on modified response evaluation criteria in solid tumors (mRECIST) criteria, the response is complete, but regarding RECIST criteria we have a partial response; C, D: Pre- (C) and post- (D) radiochemoembolization images in a 41 year-old female with a metastatic carcinoid tumor. Based on modified response evaluation criteria in solid tumors criteria the response is partial; E, F: Pre- (E) and post- (F) radiochemoembolization images in a 55 year-old male with a metastatic colorectal carcinoma. Based on modified response evaluation criteria in solid tumors criteria the response is progressive.

Six patients had bilobar involvement, with three of them receiving two courses of radiochemoembolization. The follow-up imaging study of these patients was performed after the second session. In the studied patients there was no evidence of extrahepatic occurrence (such as pulmonary radioactive deposition), which was proven by Bremsstrahlung scintigraphy performed after the treatment.

For short-term follow-ups for the 2 mo after the therapy, no treatment-related death was reported. The most common side effect was post-embolization syndrome, presented as vomiting, abdominal pain and fever. Nineteen (95%) patients experienced this syndrome in different severities. Two patient had ascites (with pleural effusion for one patient) not related to hepatic failure. Moreover, no cases of acute liver failure, hepatic infarction, hepatic abscess, biliary necrosis, tumor rupture, surgical cholecystitis, or non-targeted gut embolization were reported[2]. Systemic toxicities, such as alopecia, marrow suppression, renal toxicity, or cardiac failure did not occur in our study group.

DISCUSSION

Liver metastases are one of the most difficult therapeutic challenges in oncological management, and are not usually amenable to resection. Many studies have been performed to find more effective palliative options for non-operable metastatic tumors. Although systemic chemotherapy still has a role[10,21], there are attempts at focusing treatment on hepatic tumors[22]. The present study introduced a novel combination of two effective treatment

options, TACE and radioembolization, for metastatic hepatic lesions.

In the current study, large-molecule chromic phosphate containing ^{32}P particles were used. Colloidal ^{32}P, another chromic phosphate-containing agent, has been previously used for radiosynovectomy *via* intrasynovial injection[23], treatment of stage I and II ovarian carcinoma *via* intraperitoneal instillation[24,25], and for regional radiotherapy of some tumors *via* direct intratumoral injection[13,20]. The usual forms of colloidal ^{32}P particles are small, approximately 1 μm[14,26], and might leak into systemic circulation, causing irradiation to undesired parts of body and toxicity. For intrasynovial and intraperitoneal application, as the risk of leakage is low, the colloidal ^{32}P solution is safely used[23]. For direct intratumoral injection on the other hand, the retention of colloidal ^{32}P radioactivity at the site of a solid tumor requires the co-administration of macroaggregated albumin[27]. However, the risk of leakage from the injection site is still present due to intratumoral interstitial pressure[14].

There was only one report of intravascular injection of ^{32}P colloid in our literature review. Kim *et al*[28] administered colloidal ^{32}P *via* the portal vein to prevent growth of occult metastases in the liver. They concluded that the mentioned approach would be expected to prevent liver metastases of completely resected colorectal cancers. Other studies on radioactive phosphorus use phosphorus-32 glass microspheres (^{32}P-GMS) with grain sizes of 46-76 μm to reduce systemic toxicity[13]. Although transarterial administration of this compound has been used safely for hepatic primary or metastatic tumors[13,15,16], the main use for a radioactive pharmaceutical for this purpose is a ^{90}Y microsphere with a particle size of 20-35 μm[9]. There has also been an early report of ^{90}Y systemic leakage[29].

In terms of systemic toxicity, the used compound did not have a higher risk than ^{32}P-GMS or ^{90}Y microspheres. We can, however, raise an advantage for ^{32}P over ^{90}Y regarding our purpose. The half life of this radioactive element (14.28 d) provides a significantly longer period of irradiation than ^{90}Y with its half-life of 64 h[11,13]. Considering the 2 half-lives, there was almost 28 d of radiation for the optimal chemoradiation effect in the presence of chemotherapy drugs.

Another consideration is our chemotherapeutic mixture. Although there is no consensus on the best chemotherapeutic agent for TACE, doxorubicin is the most commonly used drug for the purpose[30]. The most commonly combined drug regimen for TACE, including cisplatin, doxorubicin, and mitomycin C[2,30] was used in this study. In combination with radiotherapy, however, we needed to find some supports and check if there were previous contraindications in the literature. Cisplatin is similar to other platinum-based agents that act as a radiosensitizer[31]. There was one clinical trial combining hepatic radioembolization with ^{90}Y and a systemic chemotherapy regimen containing the platinum-based agent, oxaliplatin[32]. Concerning doxorubicin, which is also a potent radiosensitizer[33], we found no previous usage for hepatic malignancies in combination with radiotherapy, although its co-administration has been used in other body parts[34-36]. Another study on nude mice for medullary thyroid cancer showed the combination of radioimmunotherapy and doxorubicin chemotherapy had synergistic therapeutic efficacy, which may be due to the radiosensitizing effect of doxorubicin[37]. Like doxorubicin, mitomycin C has also had concurrent administration with radiotherapy in several studies[38-40]. Therefore, there is no proven contraindication to applying radiotherapy along with this chemotherapeutic regimen. Moreover, it is expected that in the case of cisplatin and doxorubicin, which are radiosensitizers, the effect of therapy would be more effective than just the addition of TACE and radioembolization effects.

The mRECIST criteria were originally designed for hepatocellular carcinoma (HCC) and are based on the changes in the viable portion of hepatic lesions. Older methods of image-based response evaluation of solid tumors only assess the change in anatomic size of target lesions[18]. Measurements were either by the bilinear product approach (WHO criteria) or by single linear summation (RECIST criteria)[41]. As acknowledged before, relying solely on the changes in tumor size can be misleading[42]. Modified RECIST and a subjective percentage of necrosis criteria take tumor necrosis induced by treatment into account[18].

Studies which used ^{90}Y radioembolization for metastatic hepatic lesions from mixed sources generally relied on WHO and RECIST criteria to assess the treatment response (Table 3). Expanding the response rate to cases with complete or partial response, there were reports of 13% to 42.8% responsiveness with regard to WHO and RECIST criteria. Only Peynircioğlu *et al*[43] reported that all of their patients had at least a partial response in target lesions. Considering necrosis in combination with anatomic size, Miller *et al*[7] showed an increase in response rate from 19% to 50%. The studies on metastatic hepatic lesions using a chemotherapy regimen of doxorubicin, cisplatin, and mitomycin-c for TACE are summarized in Table 4. Only papers which reported an imaging-based response rate were included. WHO and RECIST criteria showed a response rate that differed from 8% to 60% in these studies. A paper by Artinyan *et al*[44] on mixed-source hepatic metastases showed a response rate of 14.8%.

Firusian *et al*[20] reported 5 cases of hepatic metastasis for which direct intratumoral colloidal ^{32}P injection led to three complete and two partial responses. No toxicity was encountered in these 5 patients and there were no alterations in hepatic function. In a study by Gao *et al*[13] on 60 patients with refractory solid tumors, including 25 cases of HCC and 5 cases of hepatic metastatic carcinoma, they administered ^{32}P-GMS *via* the hepatic artery for thirty-two cases. Among all 60 patients, 31 cases achieved complete response (51.7%), 25 cases partial response (41.7%) and 4 cases no effect. Most patients had post-procedural nausea and vomiting. There were also reports of discomfort or pain in the right upper abdominal quad-

Table 3 Studies on Yttrium-90 radioembolization for metastatic hepatic lesions from mixed sources

Study	Procedure	Agent	Absorbed dose or mean activity delivered[1]	Number of patients	Response criteria	Response measured at (months post treatment)	Response rate	Complications
Blanchard *et al*[49], 1989	Radioembolization	^{90}Y plastic microspheres	NA	15	WHO	NA	Partial response in 5 (33.3%), minimal response in 2 (13.3%)	Gastritis or gastric ulceration in 6 (in three this was proven to be due to unintended infusion of microspheres into the gastric circulation)
Andrews *et al*[50], 1994	Radioembolization	^{90}Y glass microspheres	150 Gy	24	WHO	2	Partial response in 5 (20.8%), minimal response in 4 (16.7%), stable disease in 7 (29.2%), progressive disease in 8 (33.3%)	Mild gastrointestinal symptoms in 4 (unrelated to treatment)
Miller *et al*[7], 2007	Radioembolization	^{90}Y glass microspheres	100-120 Gy	42	WHO	2.3[2]	Complete/partial response in 8 (19%), stable disease in 22 (52%), progressive disease in 23	Radiation cholecystitis in 10, liver edema in 18
					RECIST	3.9[2]	Complete/partial response in 10 (24%), stable disease in 21 (50%), progressive disease in 23	
					Necrosis	1[2]	Complete/partial response in 19 (45%)	
					Combined	1.1[2]	Complete/partial response in 21 (50%), stable disease in 11 (26%)	
Sato *et al*[8], 2008	Radioembolization	^{90}Y glass microspheres	112.8 Gy/ 1.83 GBq	137	WHO	1-3	Complete response (2.1%), partial response (40.7%)	Fatigue (56%), vague abdominal pain (26%), nausea (23%)
Lim *et al*[51], 2005	Radioembolization	^{90}Y resin microspheres	NA	46	RECIST	2	Partial response in 12 (27%), stable disease in 12 (27%), progressive disease in 19 (44%)	Between 2 and 8 wk of lethargy, anorexia, nausea and right upper quadrant pain in most patients, severe gastric/duodenal ulceration in 4 (8%), portal hypertension in 1, radiation hepatitis in 1
Yu *et al*[52], 2006	Radioembolization	^{90}Y resin microspheres	42 Gy	49	RECIST	NA	Response rate of 29%	Fatigue in 18 (37%), vague abdominal pain in 10 (20%), nausea/vomiting in 10 (20%), ascites and/or leg edema in 3 (6%)
Szyszko *et al*[53], 2007	Radioembolization	^{90}Y resin microspheres	1.9 GBq	21	RECIST	1-2	Partial response in 2 (13%), stable disease in 9 (60%), progressive disease in 4 (27%)	NA
Stuart *et al*[54], 2008	Radioembolization	^{90}Y resin microspheres	NA	30	RECIST	NA	Partial response or stable disease in 14 (47%)	Gastrointestinal ulceration in 1 (3%)
Kennedy *et al*[55], 2009	Radioembolization	^{90}Y resin microspheres	1.1 ± 0.6 GBq	502[3]	RECIST	3	Complete response in 23 (4.5%), partial response in 48 (9.5%), stable disease in 386 (76.8%), progressive disease in 45 (9%)	Fatigue and upper abdominal pain (29%), gastritis and overt gastric ulceration (2%), severe liver disease (4%)
Peynircioğlu *et al*[43], 2010	Radioembolization	90Y resin microspheres	1.24 GBq	10	RECIST	1-2	All patients had at least partial response of the target lesions	Post-procedural mild to moderate fatigue in all patients for 7 d, with mild to moderate fever and abdominal pain in some patients
Omed *et al*[56], 2010	Radioembolization	^{90}Y resin microspheres	NA	11	RECIST	NA	Partial response (20%), stable disease (50%), progressive disease (30%)	No major complications, 82% of patients experienced side-effects, mainly nausea, vomiting and abdominal pain
Cianni *et al*[57], 2010	Radioembolization	^{90}Y resin microspheres	1.64 Gbq	110	RECIST	2	Complete/partial response in 45, stable disease in 42, progressive disease in 23	Hepatic failure in 1, gastritis in 6, cholecystitis in 2

[1]The absorbed dose in Gy and/or the mean delivered activity in Gbq are provided with respect to their availability; [2]median; [3]the total number of patients in the study was 680, but the response evaluation criteria in solid tumor (RECIST) criteria were only available for 502 patients. ^{92}Y: Yttrium-90; WHO: World Health Organization; NA: Not available.

rant within 1 wk after treatment[13].

There are significant differences between lesion outcomes rated by the mRECIST and RECIST criteria in our series. In agreement with many other reports, in short-term follow-up the degree of necrosis is a major factor for response evaluation and a criteria lacking this factor may

Table 4 Studies on chemoembolization for metastatic hepatic lesions with cisplatin, doxorubicin and mitomycin

Study	Primary diagnosis	Procedure	Chemotherapeutic agents	Embolic material	Number of patients	Response criteria	Response measured at months treatment	Response rate	Complications
Diaco *et al*[58], 1995	Carcinoid tumor	Chemoembolization	Cisplatin, doxorubicin, mitomycin	NA	10	WHO	NA	Partial response (60%), stable disease (30%)	NA
Drougas *et al*[59], 1998	Carcinoid tumor	Chemoembolization	Doxorubicin (60 mg), cisplatin (100 mg), and mitomycin (30 mg)	Polyvinyl alcohol	13[1]	WHO	3	Partial response in 1 (8%), minimal response in 10 (77%), stable disease in 1 (8%), progressive disease in 1 (8%)	Nausea/vomiting in 100%, increased transaminases in 100%, pain in 100%, fever in 29%, myelosuppression in 29%, arterial thrombosis in 8%, dysrhythmia in 8%, mental status changes in 4%
Tellez *et al*[60], 1998	Colorectal carcinoma	Chemoembolization	Cisplatin, doxorubicin, mitomycin	Angiostat (a bovine collagen material)	27	Designed by authors[2]	NA	Radiological response in 17 of 27 patients (63%)	Fever in 83%, RUQ pain in 100%, nausea/vomiting in 83%, gastritis in 17%, lethargy in 60%
Buijs *et al*[45], 2007	Breast cancer	Chemoembolization	Doxorubicin (50 mg), cisplatin (100 mg), and mitomycin (10 mg) in a 1:1 mixture with iodized oil	300- to 500-μm embolic microspheres	14[3]	RECIST	1-2	No complete response, partial response in 7 lesions (26%)[6]	NA
Ruutiainen *et al*[61], 2007	Neuroendocrine tumor	Chemoembolization	Cisplatin, doxorubicin, mitomycin in a mixture with iodized oil	150- to 250-μm granular polyvinyl alcohol particles	44	RECIST	1	88% partial response/stable disease	High incidence of postembolization syndrome, severe pain in 3 sessions, severe nausea in 1 session, severe vomiting in 1 session, severe GGT/ALP elevation in 4 sessions, severe AST elevation in 1 session, severe ALT elevation in 1 session, severe infection in 1 session
Artinyan *et al*[44], 2008	Mixed	Chemoembolization	Doxorubicin (50 mg), mitomycin (10 mg), and cisplatin (150 mg)	Polyvinyl alcohol microspheres (300-700 μm)	61[4]	RECIST	At least 1	Partial response in 9 (14.8%), progressive disease in 3 (4.9%)	Bleeding in 2 patients (2%), renal failure in 6 patients (5%), hepatic failure in 7 patients (6%), infection in 3 patients (3%), mortality in 30 d in 7 patients (6%)
Buijs *et al*[48], 2008	Ocular melanoma	Chemoembolization	Doxorubicin (50 mg), cisplatin (100 mg), and mitomycin (10 mg) in a 1:1 mixture with iodized oil	300- to 500-μm embolic microspheres	6[5]	RECIST	1-2	No complete response, partial response in 8 lesions[6]	NA
Albert *et al*[62], 2011	Colorectal carcinoma	Chemoembolization	Cisplatin, doxorubicin, mitomycin in a mixture with ethiodized oil	Polyvinyl alcohol	95[6]	RECIST	NA	Partial response in 9 (14.8%), stable disease in 49 (80.3%), progressive disease in 3 (4.9%)	NA

[1]There were 15 patients at first, however one died before the follow up imaging and one patient's follow-up was out of state. Three patients only received hepatic artery embolization; [2]at least 75% decrease in the density of lesions consistent with necrosis or 25% decrease in the size of the lesions without the development of concomitant lesions; [3]twenty-seven lesions; [4]the total number of patients was 119, but for 61 patients the response evaluation criteria in solid tumors (RECIST) criteria were available; [5]twenty-one lesions, reporting by patient, all patients were considered non-responders to transcatheter arterial chemoembolization because the total tumor burden did not decrease by 30% in any given patient; [6]ninety-five of 141 treatment cycles were evaluable for response. The response rate is calculated per treatment cycle. NA: Not available; RUQ: Right upper quadrant; WHO: World Health Organization; GGT/ALP: Gamma-glutamyl transpeptidase/alkaline phosphatase; AST/ALT: Aspartate aminotransferase/alanine aminotransferase.

underestimate the effectiveness of the therapy[7,42,45-48]. There were differences in the results of mRECIST and necrosis percentage criteria for 3 patients. In these cases the change was in the anatomical size of lesions without a significant change in necrosis percentage. This finding shows that, in addition to its inherent flaw of being subjective, necrosis percentage cannot always reveal the real response. It seems that criteria that gather both anatomic sizes and the degree of necrosis (e.g., mRECIST) are more accurate. In 2 patients with newly appeared lesions (cases 12 and 13) after the treatment, these new lesions were in the lobe other than the one that underwent radiochemoembolization. These lesions could be new metastatic lesions or previously non-visible metastatic

foci in the non-treated lobe. For that reason, assessing the response by patient and not by lobe might have shown lower response rates in this study.

Our patients, like in most other studies on radioembolization and TACE, generally had constitutional and mild gastrointestinal symptoms after treatment sessions. There was no severe toxicity in the short-term follow-up of our series. Various degrees and severities of complications have been previously encountered after TACE and radioembolization procedures (Tables 3 and 4).

One limitation of the current study is the lack of control groups which only receive either TACE or radioembolization. Therefore, statistical comparison between the methods is impossible. However, many studies in this field perform new treatment strategies without a sample group and compare their result with the literature (Tables 3 and 4). Other weaknesses of this study were the short-term follow-up period and the mixed sources of hepatic metastases. The metastatic foci from different sources may have different responses to the administrated therapy. Longer than 2 mo period imaging results, long-term survival rates, time to progression (i.e., the post procedural elapsed time after which imaging studies show progressive disease), and also response rates in larger series of patients with single source of metastasis remain to be reported on the forthcoming steps after completion of regular long-term imaging evaluations and follow-ups.

In conclusion, this study introduces a new treatment approach for hepatic metastatic lesions on a rational basis. This was a combination of TACE and radioembolization which have been used individually for such lesions. It also shows that in short-term follow-ups this method is safe and effective, with a response rate of 65% with regards to the mRECIST criteria. Further studies are required to show the long-term effects and possible complications of this approach.

COMMENTS

Background

Liver metastases are a therapeutic challenge in oncological management. Surgery is frequently impossible due to disease extent and systemic chemotherapy usually fails. Transcatheter arterial chemoembolization and radioembolization are two separately used locoregional palliative therapies for metastatic hepatic lesions.

Research frontiers

Designing new treatments for patients with multiple or unresectable liver metastases is an interesting field of oncology. Transcatheter arterial administration of therapies is rapidly developing with the aid of diverse chemotherapeutic drugs and radiopharmaceuticals. Application of microsphere and particle technology is an evolving area of interventional oncology.

Innovations and breakthroughs

This study presents a novel image-guided combination of transcatheter arterial chemoembolization and radioembolization for advanced hepatic metastases, referred to as radiochemoembolization, which substantially intensifies local treatment effect. The authors used chromic phosphorus-32 molecules embedded in large particles with greater local effects and less systemic toxicities.

Applications

By showing the short-term effectiveness of the new combination method, the study opens the way for further research studies to assess the effects and complications of radiochemoembolization more thoroughly. After the release of results from the ongoing and upcoming studies by the authors and other scientists, an adjusted form of radiochemoembolization might play an important role in the treatment of hepatic metastases.

Terminology

Radiochemoembolization is a newly designed combination of radioembolization and transcatheter arterial chemoembolization, which are minimally invasive therapeutic approaches for administrating radioactive microspheres and chemotherapeutic drugs transarterially.

Peer review

This paper presents the short-term effectiveness of radiochemoembolization for selected hepatic metastases. This is the first report in which the novel combination of two commonly used effective options of treatment is described.

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BRIEF ARTICLE

Adenosine deaminase activity in tuberculous peritonitis among patients with underlying liver cirrhosis

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Abstract

AIM: To investigate the value of adenosine deaminase (ADA) for early detection of tuberculous peritonitis (TBP) among cirrhotic patients.

METHODS: We retrospectively analyzed 22 patients with TBP from July 1990 to June 2010. Twenty-five cirrhotic patients with uninfected ascites were prospectively enrolled as the cirrhosis control group from July 2010 to June 2011. An additional group of 217 patients whose ascites ADA levels were checked in various clinical conditions were reviewed from July 2008 to June 2010 as the validation group.

RESULTS: The mean ascites ADA value of cirrhotic patients with TBP (cirrhotic TBP group, n = 8) was not significantly different from that of non-cirrhotic patients (non-cirrhotic TBP group, n = 14; 58.1 ± 18.8 U/L *vs* 70.6 ± 29.8 U/L, P = 0.29), but the mean ascites ADA value of the cirrhotic TBP group was significantly higher than that of the cirrhosis control group (58.1 ± 18.8 U/L *vs* 7.0 ± 3.7 U/L, P < 0.001). ADA values were correlated with total protein values (r = 0.909, P < 0.001). Using 27 U/L as the cut-off value of ADA, the sensitivity and specificity were 100% and 93.3%, respectively, for detecting TBP in the validation group.

CONCLUSION: Even with lower ADA activity in ascites among cirrhotic patients, ADA values were significantly elevated during TBP, indicating that ADA can still be a valuable diagnostic tool.

Key words: Extrapulmonary; Tuberculosis; Ascites; Cirrhosis; Peritonitis

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INTRODUCTION

Tuberculous peritonitis (TBP) is one of the most frequent extra-pulmonary locations of tuberculosis, and the mortality rate may exceed 50% without prompt treatment[1,2]. The gold standard for diagnosing TBP is culture

of *Mycobacterium* in ascites fluid or peritoneal biopsy, but the cultures are time-consuming and have low positivity rates, with a mean sensitivity of 43%-83% depending on the quality of samples cultured and methods utilized[3,4]. In addition, caseous granulomas of peritoneal biopsies obtained by either laparoscopy or laparotomy are alternative methods for rapid primitive diagnosis, but the procedures are invasive and may increase rates of morbidity and mortality[4-6]. The high mortality rate in untreated patients warrants a quick and noninvasive test for diagnosing TBP[1,3,7], and adenosine deaminase (ADA) in ascites is an available test that has been proposed to be useful for rapid diagnosis[1,4,6,8,9]. By analyzing ADA level in ascites, the sensitivity and specificity for diagnosing TBP have been reported to be as high as 100% and 97%, respectively[2,8,10-12].

The risk of TBP is increased in patients with underlying liver cirrhosis[4,13], and the percentage of underlying cirrhosis among patients with TBP could be as high as 50% in the United States[14]. However, concerns regarding the sensitivity of ascites ADA in detecting TBP have been raised among patients with underlying liver cirrhosis[3,15], and the low sensitivity is thought to be caused by the concomitant immunocompromised status and dilutional phenomenon in advanced liver disease[3]. In addition, cirrhotic patients always have a low protein level in ascites, and the correlation between total protein and ADA activity has been discussed in previous studies[3,10,15]. However, the laboratory analysis of ascites might be confounded by heterogenous clinical conditions in the control groups, and previous studies show conflicting results for cirrhotic patients with TBP[4]. Therefore, in the present study, we employed a prospectively-enrolled cirrhosis control group in order to investigate the laboratory changes in ascites and the clinical utility of measuring ADA activity in cirrhotic patients with TBP.

MATERIALS AND METHODS

Retrospective tuberculous peritonitis cohort

All patients diagnosed with TBP at Taichung Veterans General Hospital, a tertiary referral center in central Taiwan, from July 1990 to June 2010 were retrospectively analyzed and formed the TBP group. The patients' medical records were reviewed and demographic, laboratory, microbiological, histological and laparoscopic features were collected. A definite diagnosis of TBP was based on one or more of the following: (1) positive TB cultures of ascites or peritoneal biopsy; (2) characteristic finding of caseous granulomas on histology of peritoneal biopsy; and (3) clinical judgment of TBP by a physician based on ascites data followed by a good response to anti-tuberculous treatment. A good response to therapy was defined as complete resolution or clinical improvement during the follow-up period. Exclusion criteria included (1) ascites ADA data unavailable; (2) ascites ADA analyzed after anti-tuberculous treatment; and (3) patients with concomitant end-stage renal disease under continuous ambulatory peritoneal dialysis, whose ascites might be diluted by dialysate. In addition, for further analysis of the role of liver cirrhosis, patients were evaluated to determine whether or not they had concurrent liver cirrhosis. Liver cirrhosis was defined as typical morphologic change such as blunted edge of liver, irregular liver surface and highly coarse liver parenchyma[16], with or without evidence of portal hypertension, such as previous history of ascites before TBP, splenomegaly or esophageal/gastric varices recognized by imaging techniques or endoscopy.

Prospective control cohort of cirrhosis

Patients with liver cirrhosis and uninfected ascites were prospectively enrolled as the cirrhosis control group from July 2010 to June 2011, and medical history, etiology of cirrhosis, and Child-Pugh's classification, as well as imaging features were recorded. Patients with primary or metastatic liver malignancy, peritoneal carcinomatosis, congestive heart failure, spontaneous bacterial peritonitis, nephritic syndrome, renal failure under dialysis, or evidence of peritonitis were excluded. Ascites was obtained and analyzed with regard to parameters including ADA, albumin, total protein, lactate dehydrogenase, glucose, cell counts, cytology, ordinary and anaerobic culture, acid-fast stain, bacteria and tuberculosis cultures.

Validation cohort

Patients whose ascites ADA levels were detected by a clinical need for differential diagnosis from July 2008 to June 2010 comprised the validation group and their medical records were retrospectively reviewed. The patients in the cirrhosis control group were not included in the validation cohort. Further analysis of subgroups according to etiology of ascites was performed.

Method of adenosine deaminase determination

ADA activity was determined using a method similar to that described by Slaats *et al*[17] with an autoanalyzer (Hitachi 7170, Japan) and ADA-N kit (Denka Seiken Co. Ltd, Japan). The kinetic method estimated ADA activity by coupling the liberated NH_3 to oxoglutarate with glutamate dehydrogenase, which leads to a decrease in the reduced form of nicotinamide adenine dinucleotide phosphate absorbance at 340 nm[3,17]. The ratio of decreased absorbance reflects the activity of ADA.

Ethics

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. This study was approved ethically by the Institutional Review Board of Taichung Veterans General Hospital (C10124).

Statistical analysis

The discrete variables are presented with number and percentage; continuous variables are presented with mean ± SD. The continuous variables were compared by Mann-Whitney *U* test. The discrete variables were compared by

Table 1 Comparisons between the tuberculous peritonitis without and with cirrhosis group

Demographic data	TBP without cirrhosis (n = 14)	TBP with cirrhosis (n = 8)	P value
Age (yr)	63.3 ± 22.8	66.5 ± 9.7	0.65
Sex			0.64
Male	10 (71.4)	5 (62.5)	
Female	4 (28.6)	3 (37.5	
Ascites data			
ADA (U/L)	70.6 ± 29.8	58.1 ± 18.8	0.29
SAAG (g/dL)	0.9 ± 0.3	1.3 ± 0.6	0.10
Total protein (mg/dL)	4781.8 ± 1645.5	3400.0 ± 1000.0	0.04
WBC (/mm^3)	1411.1 ± 1291.2	1489.9 ± 759.3	0.42
Lymphocytes (/mm^3)	1078.3 ± 935.6	1135.0 ± 696.1	0.71

Data are presented as mean ± SD or n (%). TBP: Tuberculous peritonitis; ADA: Adenosine deaminase; SAAG: Serum ascites albumin gradient; WBC: White blood cells.

Table 2 Comparisons between the tuberculous peritonitis with cirrhosis group and the cirrhosis control group

Demographic data	TBP with cirrhosis (n = 8)	Cirrhosis control (n = 25)	P value
Age (yr)	66.5 ± 9.7	59.8 ± 13.5	0.65
Sex			1.00
Male	5 (62.5)	15 (60.0)	
Female	3 (37.5)	10 (40.0)	
Child's classification			0.99
A	1 (12.5)	3 (12.0)	
B	4 (50.0)	12 (48.0)	
C	3 (37.5)	10 (40.0)	
Ascites data			
ADA (U/L)	58.1 ± 18.8	7.0 ± 3.7	< 0.001
SAAG (g/dL)	1.3 ± 0.6	2.1 ± 0.5	0.01
Total protein (mg/dL)	3400.0 ± 1000.0	1176.0 ± 636.6	< 0.001
WBC (/mm^3)	1489.9 ± 759.3	174.7 ± 159.6	< 0.001
Lymphocytes (/mm^3)	1135.0 ± 696.1	113.3 ± 127.1	< 0.001

Data are presented as mean ± SD or n (%). TBP: Tuberculous peritonitis; ADA: Adenosine deaminase; SAAG: Serum ascites albumin gradient; WBC: White blood cells.

χ^2 test and Fisher's exact test. Diagnostic utility of ADA for TBP was evaluated at various cutoff values by the sensitivity and specificity. These were assessed based on comparisons of relative operating characteristic curves. Spearman's linear regression was used to evaluate the correlation between total protein of ascites and serum. $P < 0.05$ was considered to be statistically significant.

RESULTS

A computerized database search identified 29 consecutive patients who were diagnosed with TBP from July 1988 to June 2010 and who met the inclusion criteria. Seven patients (6 patients without ADA data and 1 patient under continuous ambulatory peritoneal dialysis) were excluded according to the exclusion criteria of this study, and none had concurrent liver cirrhosis. A total of 22 patients with TBP were included in the final analysis and formed the TBP group, which was further divided into two subgroups: (1) cirrhotic TBP group: concomitant cirrhosis was diagnosed in 8 of 22 patients; and (2) non-cirrhotic TBP group: no evidence of cirrhosis among the other 14 of 22 patients. The demographic and ascites data of the two TBP subgroups were compared (Table 1), and there were no significant differences in ascites ADA, white blood cell (WBC) count and lymphocyte count between the two subgroups. However, the mean total protein concentration of ascites in the non-cirrhotic TBP group was significantly greater than that in the cirrhotic TBP group ($P < 0.05$).

The demographic and ascites data of the cirrhotic TBP group and the cirrhosis control group were compared (Table 2), and there were no significant differences in demographic data between the two groups. However, the mean values of ADA, total protein, WBC counts and lymphocyte counts in ascites of the cirrhotic TBP group were significantly higher than those of the cirrhosis control group ($P < 0.05$).

The distribution of ascites ADA values among the patient groups were compared (Figure 1), and the values of ascites ADA in the cirrhosis control group were markedly different from those of the other two groups with no overlapping area. Using 27 U/L as a cut-off value of ADA in ascites (the lowest value in the TBP group), no patient in the cirrhosis control group was found to have an ascites ADA concentration higher than this value.

The validation group included 217 patients, and subgroup analysis was performed according to the different etiologies of ascites (Figure 2). Using 27 U/L as a cut-off value of ascites ADA in the validation group, the sensitivity in detecting TBP was 100% and specificity was 93.3%. There were fifteen (6.9%) patients (one hepatocellular carcinoma, four malignancy other than hepatoma, five intra-abdominal infection, three nephrogenic ascites, one cardiogenic ascites and one hepatocellular carcinoma mixed with spontaneous bacterial peritonitis) who had ascites ADA levels higher than 27 U/L but did not have TBP.

Spearman's correlation analysis revealed that ascites ADA levels of all patients were strongly correlated with ascites total protein amounts ($r = 0.909$, $P < 0.001$) (Figure 3).

DISCUSSION

The utility of ascites ADA in the differential diagnosis of TBP for patients with underlying liver cirrhosis remains controversial due to conflicting results in previous studies. This is the first study to investigate cirrhotic patients with TBP involving a prospectively-enrolled cirrhosis control group, which was included to avoid the potential confounding effects of heterogeneous clinical conditions. In this study, even though lower levels of ascites ADA were found in the cirrhotic TBP group, their mean ascites ADA value was not significantly different from that of the non-cirrhotic TBP group. Moreover, ascites ADA values of cirrhotic patients with TBP were notably higher than those of the cirrhosis control group, and every pa-

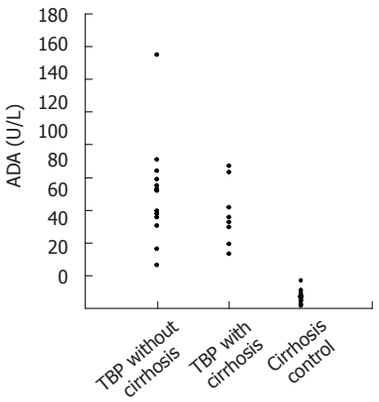

Figure 1 Adenosine deaminase distribution in the study groups. ADA: Adenosine deaminase; TBP: Tuberculous peritonitis.

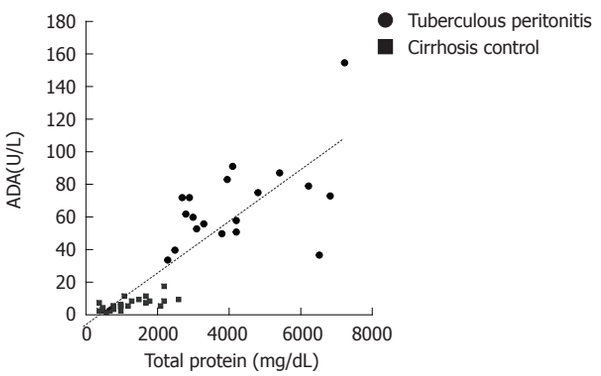

Figure 3 Correlation between adenosine deaminase and total protein in ascites among patients with tuberculous peritonitis. ADA: Adenosine deaminase.

tient in the cirrhosis control group had an ascites ADA level lower than the lowest value in the TBP group (27 U/L). Therefore, these data provide convincing evidence that ascites ADA may be significantly raised in TBP patients with underlying cirrhosis, and thus TBP should be considered in the differential diagnosis.

Previous studies showed high sensitivity and specificity in detecting TBP by checking ADA activity in ascites, based on ADA cut-off values of 36-40 IU/L[2,10,11,18]. However, Hillebrand *et al*[3] used only 7 IU/L as the cut-off value of ADA, and they found the sensitivity of ADA was only 30% in the setting of cirrhosis. There was considerable overlap in the ADA activity between TBP and sterile ascites among cirrhotic patients. Furthermore, Hillebrand *et al*[3] postulated that lower sensitivity and cut-off value might be caused by the higher proportion of cirrhosis (59%) in their patients with TBP. In contrast, Burgess *et al*[4] reported a sensitivity of 94% and a specificity of 92% for cirrhotic patients with TBP using a cut-off value for ADA of 30U/L. In the current study, there was no overlapping phenomenon in the ADA activity between TBP and sterile ascites among cirrhotic patients, and the sensitivity and specificity in detecting TBP were also high (100% and 93.3%, respectively) in the validation group using a cut-off value for ADA of 27 U/L. In

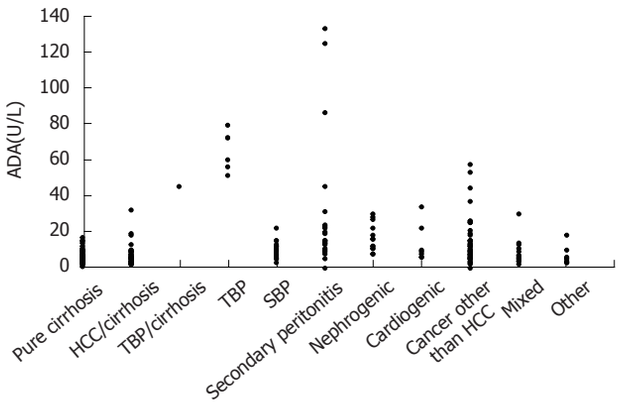

Figure 2 Adenosine deaminase distribution for various ascites etiologies in the validation group. ADA: Adenosine deaminase; TBP: Tuberculous peritonitis; SBP: Spontaneous bacterial peritonitis; HCC: Hepatocellular carcinoma.

addition, Hillebrand *et al*[3] determined ADA activity by detecting the decrease in adenosine concentration under the action of ADA, but this method was different from the measurement techniques applied in most previous studies. The methods employed by Slaats *et al*[17] or Giusti *et al*[19] have been extensively described in the literature[2,4,9,18], and involve determination of ADA activity by changes of NH_3 or NADH, respectively, after interacting ADA with adenosine. The different techniques used might affect the sensitivity and specificity of the ADA test, so the correlation of ADA values obtained from different methods need further investigation.

As shown in our validation group and in previous studies[2-4], false positive findings of ascites ADA are still possible. Peritoneal carcinomatosis and secondary bacterial peritonitis are the two most likely etiologies after TBP. ADA plays an important role in regulating the level of adenosine, and its primary function in humans is development and maintenance of the immune system[20,21]. ADA is involved in proliferation and differentiation of T lymphocytes, and diseases such as malignant conditions, collagen vascular diseases and some microorganism infections that are associated with lymphocytosis may increase ADA levels[22]. Therefore, for patients with elevated ascites ADA, further differential diagnosis by ascites cytological examination or radiological imaging studies should be performed, and laparoscopic visualization and biopsy may be considered for equivocal cases. Furthermore, due to the high negative predictive value (100%) found in this study, invasive procedures such as laparoscopic peritoneum biopsy or laparotomy, which are relatively high risk in cirrhosis patients, may be unnecessary and should be avoided when ascites ADA activity is low. In addition, the mean time to develop positive tuberculosis culture from ascites was 36.4 ± 18.2 d in our study, but the mean time in previous studies on ascites ADA was only 3.0 ± 0.5 d. Testing for ADA in ascites has high sensitivity and specificity, and may therefore be useful as a rapid test for diagnosis. Although TB polymerase chain reaction (PCR) has been used as a rapid diagnostic tool for pulmonary TB, PCR cannot be suggested for diagnosing TBP due to its

low sensitivity rate[9,23]. In endemic area of tuberculosis, DNA from dead TB bacilli may also give a false-positive result[9]. In addition, testing for ADA may also be currently more widely available than other valuable clinical tests such as interferon-gamma[24].

In this study, ADA activity was found to be strongly correlated with total protein in ascites in the TBP and control groups, and this finding was compatible with the simultaneously elevated total protein and ADA found in cirrhotic patients suffering from TBP. It is interesting to note that Fernandez-Rodriguez *et al*[15] found ADA was correlated with total protein in ascites among patients with TBP ($r = 0.842$), but the correlation was non-significant when the control group was included in the analysis. This inconsistent finding might be explained by heterogeneous etiologies and complex clinical conditions in the control group. Elevated total protein in the body fluid has been one of the diagnostic markers for inflammatory exudate[25], and it is also an important finding in ascites of TBP[5,15]. ADA is related to activation and differentiation of mononuclear leukocytes, and it is secreted during immune responses[3,21,26]. Lymphocytes are the predominant cells in ascites of TBP, and simultaneously elevated total protein and ADA can be explained by lymphocytic inflammation. Moreover, although mean total protein in the cirrhotic TBP group was significantly lower than that of the non-cirrhotic TBP group in this study, mean ADA levels were not significantly different. Compared with the cirrhosis control group, the WBC and lymphocyte counts were significantly higher in the cirrhotic TBP group, and the immune responses were predominant. Even in cirrhotic patients with a relatively immunocompromised status, TBP still activated strong immune reactions, which resulted in a sharp elevation of ADA in ascites, and this phenomenon indicates that ascites ADA may still be a valuable tool in the differential diagnosis of TBP.

We acknowledge several limitations in this study. Firstly, this study reflects the experience of a single medical center with a relatively small sample size, but the statistical power of the analysis was adequate. Secondly, cases with TBP were obtained from a computerized database search and retrospectively analyzed, so some TBP cases may have been missed due to incorrect coding in this long-term cohort study. Thirdly, we compared a historical TBP cohort with a prospectively-enrolled cirrhosis control group, and different time periods of study might produce some bias. However, patients were consecutively recruited, and the modalities for diagnosing TBP were not changed during the study period. The possible bias should be minimized.

In conclusion, even with lower ascites ADA activity in liver cirrhosis, ascites ADA levels could be significantly elevated due to strong immune responses when cirrhotic patients suffer from TBP. Owing to its high sensitivity and specificity, ascites ADA may be a valuable tool in the differential diagnosis of TBP in patients with underlying cirrhosis, and concomitant cirrhosis should not limit its clinical utility.

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COMMENTS

Background

The risk of tuberculous peritonitis (TBP) is increased in patients with underlying liver cirrhosis. Adenosine deaminase (ADA) in ascites has been proposed to be a useful test for early detection of TBP, but its value among patients with underlying cirrhosis is uncertain.

Research frontiers

The utility of ascites ADA in the differential diagnosis of TBP in patients with underlying liver cirrhosis remains controversial due to conflicting results in previous studies. In addition, the relationship between ascites ADA and other parameters such as total protein has not been well discussed, and the mechanisms of ADA elevation among cirrhotic patients with TBP need further investigation.

Innovations and breakthroughs

This is the first study to investigate cirrhotic patients with TBP which includes a prospectively-enrolled cirrhosis control group, and ADA activity was strongly correlated with total protein in ascites. Even with lower ascites ADA activity in liver cirrhosis, ascites ADA levels could be significantly elevated due to strong immune responses when cirrhotic patients suffer from TBP.

Applications

Owing to its high sensitivity and specificity, ascites ADA may be a valuable tool in the differential diagnosis of TBP in patients with underlying cirrhosis, and concomitant cirrhosis should not limit its clinical utility. Furthermore, due to the high negative predictive value, invasive procedures such as laparoscopic peritoneum biopsy or laparotomy may be unnecessary when ascites ADA activity is low.

Terminology

ADA is an enzyme involved in purine metabolism, and it can be a product of immune responses relating to T lymphocyte activity in humans.

Peer review

This is a good case-control study and the message is clear. The authors examined ADA activity in TBP among patients with underlying liver cirrhosis, and a prospectively-enrolled cirrhosis control group was conducted to avoid the potential confounding effects of heterogeneous clinical conditions. Due to convincing data in this study, ascites ADA can still be a valuable tool in the differential diagnosis of TBP.

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BRIEF ARTICLE

Volumetric-modulated arc therapy *vs* c-IMRT in esophageal cancer: A treatment planning comparison

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Abstract

AIM: To compare the volumetric-modulated arc therapy (VMAT) plans with conventional sliding window intensity-modulated radiotherapy (c-IMRT) plans in esophageal cancer (EC).

METHODS: Twenty patients with EC were selected, including 5 cases located in the cervical, the upper, the middle and the lower thorax, respectively. Five plans were generated with the eclipse planning system: three using c-IMRT with 5 fields (5F), 7 fields (7F) and 9 fields (9F), and two using VMAT with a single arc (1A) and double arcs (2A). The treatment plans were designed to deliver a dose of 60 Gy to the planning target volume (PTV) with the same constrains in a 2.0 Gy daily fraction, 5 d a week. Plans were normalized to 95% of the PTV that received 100% of the prescribed dose. We examined the dose-volume histogram parameters of PTV and the organs at risk (OAR) such as lungs, spinal cord and heart. Monitor units (MU) and normal tissue complication probability (NTCP) of OAR were also reported.

RESULTS: Both c-IMRT and VMAT plans resulted in abundant dose coverage of PTV for EC of different locations. The dose conformity to PTV was improved as the number of field in c-IMRT or rotating arc in VMAT was increased. The doses to PTV and OAR in VMAT plans were not statistically different in comparison with c-IMRT plans, with the following exceptions: in cervical and upper thoracic EC, the conformity index (CI) was higher in VMAT (1A 0.78 and 2A 0.8) than in c-IMRT (5F 0.62, 7F 0.66 and 9F 0.73) and homogeneity was slightly better in c-IMRT (7F 1.09 and 9F 1.07) than in VMAT (1A 1.1 and 2A 1.09). Lung V30 was lower in VMAT (1A 12.52 and 2A 12.29) than in c-IMRT (7F 14.35 and 9F 14.81). The humeral head doses were significantly increased in VMAT as against c-IMRT. In the middle and lower thoracic EC, CI in VMAT (1A 0.76 and 2A 0.74) was higher than in c-IMRT (5F 0.63 Gy and 7F 0.67 Gy), and homogeneity was almost similar between VMAT and c-IMRT. V20 (2A 21.49 Gy *vs* 7F 24.59 Gy and 9F 24.16 Gy) and V30 (2A 9.73 Gy *vs* 5F 12.61 Gy, 7F 11.5 Gy and 9F 11.37 Gy) of lungs in VMAT were lower than in c-IMRT, but low doses to lungs (V5 and V10) were increased. V30 (1A 48.12 Gy *vs* 5F 59.2 Gy, 7F 58.59 Gy and 9F 57.2 Gy), V40 and V50 of heart in VMAT was lower than in c-IMRT. MUs in VMAT plans were significantly reduced in comparison with c-IMRT, maximum doses to the spinal cord and mean doses of lungs were similar between the two techniques. NTCP of spinal cord was 0 for all cases. NTCP of lungs and heart in VMAT were lower than in

c-IMRT. The advantage of VMAT plan was enhanced by doubling the arc.

CONCLUSION: Compared with c-IMRT, VMAT, especially the 2A, slightly improves the OAR dose sparing, such as lungs and heart, and reduces NTCP and MU with a better PTV coverage.

Key words: Esophageal cancer; Treatment planning; Intensity modulated radiotherapy; Volumetric modulated arc radiotherapy; Normal tissue complication probability

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INTRODUCTION

Esophageal cancer (EC) is one of the most common malignancies in the world. It was estimated that there were 16 470 newly diagnosed cases of EC, and 14 280 cases of death in America in 2008[1]. Squamous cell carcinoma is commonly seen in China, whereas adenocarcinoma is common in Europe and America. Radiotherapy is a major treatment method for EC because more than 60% of the patients are often diagnosed at locally advanced stages which could not be totally resected. Innovative technologies in radiation delivery such as intensity-modulated radiotherapy (IMRT) offer the potential for improved tumor coverage, while reducing the doses delivered to the surrounding normal tissues. Clinical studies have yielded good dosimetry and patient outcome by IMRT[2-6]. There are different IMRT delivery approaches, including "step and shoot", "sliding window" modes and the rotational technique. Volumetric-modulated arc therapy (VMAT), the novel form of IMRT that was first proposed by Yu in 1995[7], allowed for intensity-modulated radiation delivery during gantry rotation with dynamic multi-leaf collimator (MLC) motion, variable dose rates (DR) and gantry speed modulation. VMAT had already been investigated for prostate cancer, small brain tumors and cervix uteri cancer[8-10]. These studies have generally shown that VMAT is able to produce similar or better dose distributions, while achieving a reduction in treatment time and a reduction in monitor units (MU).

We performed a planning study to compare VMAT with conventional sliding window intensity-IMRT (c-IMRT) in EC of all locations and in dose distributions to planning target volume (PTV) and organs at risk (OAR). We also investigated the difference of normal tissue complication probability (NTCP) between the two techniques.

MATERIALS AND METHODS

Patients

Twenty EC patients treated with c-IMRT previously in our department were selected for this study, involving 5 cases of EC located in the cervical, the upper, the middle and the lower thorax, respectively. Five patients were staged Ⅱ, 10 were Ⅲ and 5 were Ⅳ according to the American Joint Committee (AJCC) on Cancer 2006 Guidelines. Details are shown in Table 1.

Target volume and organ at risk delineation

All patients were immobilized in a supine position and computed tomography scanned using a helical scanner (Siemens Somatom, Sensation Open Computed Tomography) with 1.25 mm thick slices over the neck and the entire thorax. The clinical target volume, including the esophageal tumor, with a margin for microscopic tumor extension, and the adjacent regional lymph nodes[11,12], was expanded with a 5-mm margin to create PTV. OAR, such as spinal cord, heart and lung, was outlined on each image. Details of the delineation of these volumes were recently described[13].

Planning techniques and objectives

All the treatment plans were designed to deliver 60 Gy to the PTV in 30 fractions using the Eclipse treatment planning system (Version 8.9, Varian Medical Systems, Palo Alto, CA), with 6 MV photon beam from a Varian Ⅸ (RapidArc) equipped with a Millennium MLC with 120 leaves. The Anisotropic Analytical Algorithm (Version 8.9) photon dose calculation algorithm and dose calculation grid of 2.5 mm were used for both c-IMRT and VMAT. When necessary, field size was minimized to 15.3 cm in the X direction. This dimension corresponded of the maximal displacement of a leaf in a MLC bank. By doing so, all the leave positions were possible during the optimization process increasing the degree of modulation even if in a beam eye view, a part of the volume was excluded of the beam at each gantry position. Globally rotational delivery permitted to irradiate all the volume of the PTV during rotation. All plans aimed to achieve a minimum dose larger than 95% and a maximum dose lower than 107% of the prescribed dose, and no 2-cc region (either in or outside of PTV) may receive > 110% of the dose. With regard to the OAR, the primary objectives were defined as follows: spinal cord: D_{max} < 45 Gy and lungs: V20 < 30%. The secondary objectives were: mean doses of lungs (MLD) < 15 Gy and heart: V40 ⩽ 50%, V50 ⩽ 40%. As a result of tumor coverage requirements, a waiver can be applied on these dose constraints.

VMAT plans

The VMAT plans using full arcs sharing the same isocenter, in which 1A consisting of a single 360° rotation

Table 1 Characteristics of patients (n = 20)

Variables	n
Gender	
Male	16
Female	4
Age range (yr)	45-82
Stage[1]	
Ⅱ	5
Ⅲ	10
Ⅳ	5
Histology	
Squamous carcinoma	18
Adenocarcinoma	2

[1]According to the American Joint Committee on Cancer 2006 Guidelines.

and 2A consisting of two coplanar arcs of 360° with opposite rotation (clock-wise or counter clock-wise), were optimized selecting a maximum DR of 600 MU/min. For 1A, starting at a gantry angle of 179° and rotating counter clockwise at 360° to stop at a gantry angle of 181°, field size and collimator rotation were determined by the automatic tool from Eclipse to encompass the PTV. And 2A, consisting of two coplanar arcs of 360°, was optimized simultaneously with opposite rotation. Since each individual arc is limited to a sequence of 177 control points, the application of two coplanar arcs that increase the modulation factor during optimization, could allow the optimizer to achieve a higher target homogeneity and lower OARs involvement at the same time. For the second arc, the collimator was rotated 5° extra to reduce overlapping tongue and groove effects with the first arc. Details about VMAT optimization process have been published elsewhere[14].

c-IMRT plans

The c-IMRT plans were optimized with a fixed DR of 400 MU/min. The MLC leaf sequences were generated using the dynamic sliding window IMRT delivery technique. Plans were individually optimized using five (5F), seven (7F) and nine (9F) coplanar fields. Beam geometry consisted of each treatment field with the following gantry angles: 0°/50°/153°/204°/310° (5F), 20°/60°/150°/180°/210°/300°/340° (7F), and 0°/35°/70°/130°/160°/200°/230°/290°/325° (9F).

Once the treatment planning was completed, the plan was normalized to cover 95% of the PTV with 100% of the prescribed dose. In the present study, we tried to modify constraints and priority factors in the c-IMRT and VMAT plans to improve the results. These parameters were modified in function of dose-volume histogram (DVH) results for each patient.

Evaluation tools

Analysis was performed on DVH computing several standard parameters[15], Dx was the specific dose computed for a fraction of a target or an organ volume, and Vx was the volume irradiated above a designated dose. For PTV, the mean dose (D_{mean}) was analyzed, and the conformity of dose distribution was assessed by means of conformity index (CI) which was defined as the ratio between the volume receiving at least 95% of 60 Gy and the volume of the PTV. Higher values of CI represented a better PTV conformity. CI = (VT95%/VT) × (VT95%/V95%)[16].

The homogeneity index (HI) of the PTV was computed as D5%-D95% (difference between the dose covering 5% and 95% of the PTV). Lower values of HI represented a more homogenous PTV dose distribution[17].

DVH parameters for OARs (spinal cord, lungs and heart) were calculated and compared. A set of Vx values, D_{mean}, D_{max} and MU was therefore reported.

Radiobiological comparison was analyzed by the NTCP. The risk of developing acute complications and other late complications was assessed using the Lyman-Kutcher-Burman model[18]. The parameters for NTCP calculations (volume effect, slope, and tolerance doses) were taken from Burman *et al*[19] and are shown in Table 2.

Statistical analysis

The Wilcoxon matched-pair signed-rank test was used to compare the results between the VMAT and IMRT plans. Difference was considered statistically significant at $P < 0.05$. All statistical tests were two-sided, and all statistical analyses were done using the SPSS software, Version 11.0 (Chicago, IL).

RESULTS

Target coverage, conformity and dose homogeneity

Clinically acceptable plans of VMAT and c-IMRT were completed by all the 20 patients. The dosimetric results of each position for PTV are listed in Table 3. The results were analogous in the cervical and upper EC, for which PTV was T-shaped from a posteroanterior view, while PTV was I-shaped in middle and lower EC. As the numbers of field in c-IMRT or arc in VMAT were increased, the conformity and homogeneity were improved.

For D_{mean} of PTV, VMAT (1A and 2A) yielded higher values than IMRT (5F, 7F and 9F). There was significant difference between VMAT and c-IMRT (7F and 9F) in cervical and upper thoracic EC and c-IMRT (5F and 7F) in the middle and lower thoracic EC, and only 1A achieved a higher D_{mean} as compared with IMRT ($P < 0.05$).

VMAT had a better CI than c-IMRT. Statistically significant difference was seen between VMAT and c-IMRT (5F, 7F and 9F) in cervical and upper thoracic EC, but between VMAT and c-IMRT (5F and 7F) in middle and lower thoracic EC ($P < 0.05$). Especially in cervical cases, 2A showed the best CI ($P < 0.05$), but there was no significant difference between 1A and 2A in thoracic cases.

Homogeneity was slightly better in c-IMRT than in VMAT. In cervical and upper thoracic EC, HI of 2A and 5F was equivalent, and 7F or 9F showed a significant trend for better results compared with VMAT ($P < 0.05$). In the middle and lower thoracic EC, the trend was not conspicuous, 9F also had a higher HI compared with

Table 2 Parameters used in normal tissue complication probability

Organ	Size factor (*n*)	Slope (m)	TD5/5 (Gy)	TD50/5 (Gy)	End point
Lung	0.87	0.18	17.5	24.5	Pneumonitis
Heart	0.35	0.10	40	48	Pericarditis
Spinal cord	0.05	0.175	47	66.5	Myelitis/necrosis

TD5/5: Tolerance dose leading to 5% complication rates at 5 years; TD50/5: Tolerance dose leading to 50% complication rates at 5 years.

Table 3 Dosimetric results for planning target volume and monitor units

Variable	IMRT-5F	IMRT-7F	IMRT-9F	VMAT-1A	VMAT-2A	$P < 0.05$
D_{mean}						
Cervical	63.68 ± 0.37	63.07 ± 0.36	62.55 ± 0.39	63.97 ± 0.08	63.63 ± 0.49	2A *vs* 7F and 9F
Upper	63.30 ± 0.66	62.74 ± 0.49	62.38 ± 0.34	63.90 ± 0.45	63.43 ± 0.63	1A, 2A *vs* 7F and 9F
Middle	64.12 ± 1.03	64.05 ± 1.27	63.88 ± 1.27	64.83 ± 1.06	64.21 ± 0.59	1A *vs* 5F and 9F
Lower	63.14 ± 0.90	63.20 ± 1.09	62.98 ± 0.87	64.17 ± 1.26	63.98 ± 1.36	1A *vs* 5F, 7F and 9F
HI						
Cervical	1.10 ± 0.01	1.09 ± 0.01	1.07 ± 0.01	1.11 ± 0.00	1.10 ± 0.01	1A, 2A *vs* 7F and 9F
Upper	1.09 ± 0.02	1.08 ± 0.01	1.07 ± 0.01	1.10 ± 0.01	1.09 ± 0.02	1A *vs* 7F and 9F; 2A *vs* 9F
Middle	1.11 ± 0.02	1.11 ± 0.03	1.11 ± 0.03	1.12 ± 0.02	1.11 ± 0.01	1A *vs* 9F
Lower	1.09 ± 0.02	1.09 ± 0.03	1.09 ± 0.02	1.11 ± 0.04	1.11 ± 0.03	1A *vs* 9F
CI						
Cervical	0.63 ± 0.03	0.66 ± 0.02	0.74 ± 0.04	0.78 ± 0.03	0.80 ± 0.03	2A *vs* 5F, 7F, 9F and 1A
Upper	0.62 ± 0.04	0.66 ± 0.03	0.73 ± 0.02	0.79 ± 0.03	0.80 ± 0.02	1A, 2A *vs* 5F, 7F and 9F
Middle	0.62 ± 0.09	0.67 ± 0.08	0.71 ± 0.10	0.76 ± 0.05	0.74 ± 0.08	1A, 2A *vs* 5F and 7F
Lower	0.64 ± 0.05	0.67 ± 0.05	0.71 ± 0.05	0.76 ± 0.05	0.77 ± 0.04	1A, 2A *vs* 5F and 7F
MU						
Cervical	1088 (921-1157)	1261 (1094-1393)	1236 (1004-1413)	610 (546 -665)	525 (452-590)	2A *vs* 5F, 7F, 9F and 1A
Upper	1110 (841-1244)	1251 (950-1377)	1334 (1040-1592)	679 (538-825)	682 (475-1004)	1A, 2A *vs* 5F, 7F and 9F
Middle	831 (707-980)	903 (808-1086)	999 (858-1219)	418 (347-459)	431 (376-503)	1A, 2A *vs* 5F, 7F and 9F
Lower	826 (721-966)	923 (720-1234)	1086 (958-1375)	440 (387-540)	419 (347-531)	1A , 2A *vs* 5F, 7F and 9F

IMRT: Intensity modulated radiotherapy; VMAT: Volumetric modulated arc therapy; F: Coplanar field; A: Arc; HI: Homogeneity index; CI: Conformity index; D_{mean}: Mean dose; MU: Monitor units.

other plans, and significant difference was found only in 9F and 1A ($P < 0.05$). Figure 1 depicts the dose distribution of c-IMRT and VMAT in a cervical EC patient.

OAR

The absolute plan parameters for lungs, heart and spinal cord are summarized in Table 4. DVH of OAR in one patient were shown in Figure 2.

The reduction trend of lung parameters (V5, V10, V20 and V30) was similar between the two techniques, except for MLD. In cervical and upper thoracic EC, MLD, V20 and V30 by VMAT were reduced by 0.6%-2.9%, 2.1%-10.7% and 13.2%-17.3%, respectively. V5 and V10 of lung by VMAT in cervical and upper thoracic EC were increased by 5.5%-7.7% and 10.5%-12.6%, respectively. In middle and lower thoracic EC, VMAT resulted in increased V5 (10.6%-13.3%), V10 (18.4%-21.8%) and MLD (2%-2.3%), but decreased V20 (5.5%-15.5%) and V30 (13.2%-18.2%). Statistically significant difference was found between VMAT (1A or 2A) and c-IMRT (5F, 7F and 9F) for V5, V10, V20 and V30, but for MLD, there was significant difference between VMAT (1A or 2A) and c-IMRT (5F, 7F) ($P < 0.05$).

For the heart, VMAT reduced V30, V40 and V50 as compared with c-IMRT, especially in thoracic cases. V30 by VMAT was reduced by 33.5%, 10.7% and 21.6%, V40 by 36%, 15.1% and 21.7%, and V50 by 39.3%, 29.7% and 38% in the upper, middle and lower thoracic EC, respectively. VMAT (1A or 2A) reduced V30 and V40 (5F, 7F and 9F) in thoracic EC, and V50 in middle and lower thoracic EC ($P < 0.05$). However, no difference was found in the D_{max} of spinal cord between VMAT and c-IMRT.

NTCP results are shown in Table 5. VMAT (1A or 2A) significantly lowered the NTCP in comparison with c-IMRT (5F, 7F and 9F) in cervical and upper thoracic cases, while there was significant difference between 2A and 5F in middle and lower thoracic cases ($P < 0.05$). The trend of cardiac NTCP in VMAT was similar with lungs in thoracic EC, especially in middle and lower thoracic EC ($P < 0.05$).

It was worth noting that D_{mean} and maximal doses to the humeral head (HH_{mean} and HH_{max}) in VMAT were dramatically increased in comparison with c-IMRT in cervical and upper thoracic EC (Table 4). Compared with c-IMRT, HH_{mean} in VMAT was increased by almost three times in cervical EC and four times in upper thoracic EC ($P < 0.05$). HH_{max} in VMAT was twice higher in cervical EC and three times higher in upper thoracic EC than that in c-IMRT (5F and 7F) ($P < 0.05$).

Table 4 Dosimetric comparison for organs at risk of conventional sliding window intensity-modulated radiotherapy and volumetric-modulated arc therapy in cervical and upper thoracic esophageal cancer, and in middle and lower thoracic esophageal cancer, mean value (range)

Organ	Variable	c-IMRT (5F, 7F, 9F)	VMAT (1A, 2A)	Relative reduction (%)	$P < 0.05$
In cervical and upper thoracic					
Lung (Gy)	MLD[1]	12.65 (12.38-13.04)	12.57 (12.35-12.79)	0.6	2A *vs* 7F, 9F; 1A *vs* 7F
	MLD[2]	14.35 (13.91-14.76)	13.94 (13.74-14.14)	2.9	2A *vs* 7F, 9F
	V5[1]	48.52 (46.28-50.52)	51.20 (51.03-51.37)	-5.5	1A, 2A *vs* 5F, 7F
	V5[2]	61.53 (57.51-65.06)	66.25 (66.07-66.43)	-7.7	1A , 2A *vs* 5F, 7F
	V10[1]	39.30 (37.41-41.58)	43.44 (43.23-43.65)	-10.5	1A , 2A *vs* 5F, 7F; 2A *vs* 9F
	V10[2]	48.17 (44.18-52.07)	54.25 (53.70-54.79)	-12.6	1A , 2A *vs* 5F, 7F, 9F
	V20[1]	24.70 (23.93-25.16)	24.17 (23.08-25.26)	2.1	1A *vs* 5F
	V20[2]	25.58 (24.29-26.23)	22.85 (21.94-23.76)	10.7	NS
	V30[1]	14.96 (14.42-15.33)	12.99 (12.99-12.99)	13.2	1A, 2A *vs* 5F, 7F, 9F
	V30[2]	14.52 (14.27-15.00)	12.01 (11.58-12.04)	17.3	1A, 2A *vs* 5F, 7F; 2A *vs* 9F
Heart (%)	V30[1]	6.83 (5.7-7.41)	5.30 (4.57-5.62)	22.4	NS
	V30[2]	15.20 (13.62-16.96)	10.11 (9.98-10.24)	33.5	1A *vs* 7F, 9F, 2A
	V40[1]	4.33 (3.55-5.08)	2.68 (2.45-2.91)	38.1	NS
	V40[2]	8.32 (7.00-10.33)	5.30 (5.08-5.22)	36	1A *vs* 5F, 7F, 9F; 2A *vs* 5F
	V50[1]	2.62 (2.07-3.09)	1.44 (1.43-1.45)	45	NS
	V50[2]	4.33 (3.44-5.53)	2.63 (2.59-2.67)	39.3	NS
Spinal cord (Gy)	D_{max}[1]	37.92 (37.53-38.32)	37.74 (37.31-38.17)	0.5	NS
	D_{max}[2]	37.85 (37.41-38.43)	38.41 (38.05-38.76)	-1.5	NS
Head of humerus	D_{max}[1]	10.00 (5.97-17.59)	21.88 (20.80-22.95)	-118.8	1A , 2A *vs* 5F, 7F, 9F
(Gy)	D_{max}[2]	7.57 (6.47-9.61)	26.44 (26.35-26.52)	-249.3	1A , 2A *vs* 5F, 7F
	D_{mean}[1]	3.24 (2.07-4.71)	12.27 (11.85-12.69)	-278.7	2A *vs* 5F, 7F, 9F, 1A
	D_{mean}[2]	2.89 (1.47-4.86)	15.26 (14.61-15.90)	-428	2A *vs* 5F, 7F, 9F, 1A
In middle and lower thoracic					
Lung (Gy)	MLD[3]	15.03 (14.86-15.27)	15.38 (15.24-15.51)	-2.3	1A, 2A *vs* 5F, 7F
	MLD[4]	15.37 (15.01-15.81)	15.67 (15.51-15.82)	-2	1A *vs* 5F
	V5[3]	74.86 (71.00-79.05)	82.83 (82.72-82.93)	-10.6	1A, 2A *vs* 5F, 7F, 9F
	V5[4]	79.36 (72.43-85.85)	89.91 (89.77-90.04)	-13.3	1A, 2A *vs* 5F, 7F, 9F
	V10[3]	55.14 (52.64-58.66)	65.26 (64.75-65.76)	-18.4	1A, 2A *vs* 5F, 7F, 9F
	V10[4]	59.56 (54.27-64.76)	72.54 (72.04-73.03)	-21.8	1A, 2A *vs* 5F, 7F; 2A *vs* 9F
	V20[3]	24.39 (23.84-24.96)	23.05 (22.28-23.81)	5.5	2A *vs* 7F, 9F, 1A
	V20[4]	25.32 (24.48-26.66)	21.40 (20.69-22.11)	15.5	1A, 2A *vs* 5F
	V30[3]	12.64 (12.08-13.01)	10.97 (10.88-11.06)	13.2	1A,2A *vs* 5F, 7F; 2A *vs* 9F
	V30[4]	11.13 (10.55-12.20)	9.10 (8.58-9.62)	18.2	1A, 2A *vs* 5F, 7F; 2A *vs* 9F
Heart (%)	V30[3]	47.30 (46.61-48.20)	42.26 (40.72-43.79)	10.7	1A *vs* 5F, 7F, 9F
	V30[4]	72.16 (67.81-78.60)	56.55 (55.52-57.57)	21.6	1A *vs* 5F, 7F, 9F; 2A *vs* 5F
	V40[3]	26.89 (24.86-28.14)	22.84 (20.98-24.69)	15.1	1A *vs* 7F
	V40[4]	33.53 (30.63-38.19)	26.27 (26.21-26.32)	21.7	2A *vs* 5F
	V50[3]	14.06 (12.50-16.06)	9.89 (8.93-10.85)	29.7	1A *vs* 5F, 7F, 9F
	V50[4]	17.99 (14.48-22.87)	11.16 (10.93-11.39)	38	1A, 2A *vs* 5F; 2A *vs* 7F, 9F
Spinal cord (Gy)	D_{max}[3]	39.03 (38.91-39.15)	38.70 (38.54-38.86)	0.8	NS
	D_{max}[4]	37.61 (37.45-37.91)	37.99 (37.78-38.20)	-1	NS

Wilcoxon's signed ranks test. [1]Cervical thoracic esophageal cancer; [2]upper thoracic esophageal cancer; [3]middle thoracic esophageal cancer; [4]lower thoracic esophageal cancer. c-IMRT: Conventional sliding window intensity-modulated radiotherapy; VMAT: Volumetric-modulated arc therapy; MLD: Mean dose of lungs; D_{max}: Maximal dose; D_{mean}: Mean dose; F: Coplanar field; A: Arc; Vx: The percentage of organ receiving a dose > X Gy; NS: Not significant.

Monitor units

The c-IMRT plans required an increased MU per fraction when the field was increased whereas the VMAT plans usually resulted in lower MU when rotating arcs were increased. 1A plans required at least 50% or 60% less than 9F in cervical and upper EC or middle and lower EC ($P < 0.05$). The difference between VMAT (1A and 2A) and c-IMRT (5F, 7F and 9F) remained significant ($P < 0.05$) in all the cases. Detailed information about MU is shown in Table 3.

DISCUSSION

In the present study, VMAT proved to be slightly better than c-IMRT for targeting dose distribution in EC of all locations, and to have equivalent or better OAR dose sparing and lower NTCP. We initiated a dosimetric and radiobiological comparison in the EC of all locations in this study. The results indicated that VMAT could generate better radiotherapeutic plans than sliding window IMRT.

VMAT is a complex form of IMRT that allows dose delivery in single or multiple arcs. Two arcs allowed superior modulation factor during optimization due to the independent optimization, and unrelated sequence of MLC shape, gantry speed and dose rate combinations. This approach provided adequate coverage of PTV and spare of OARs at least equivalent to c-IMRT,

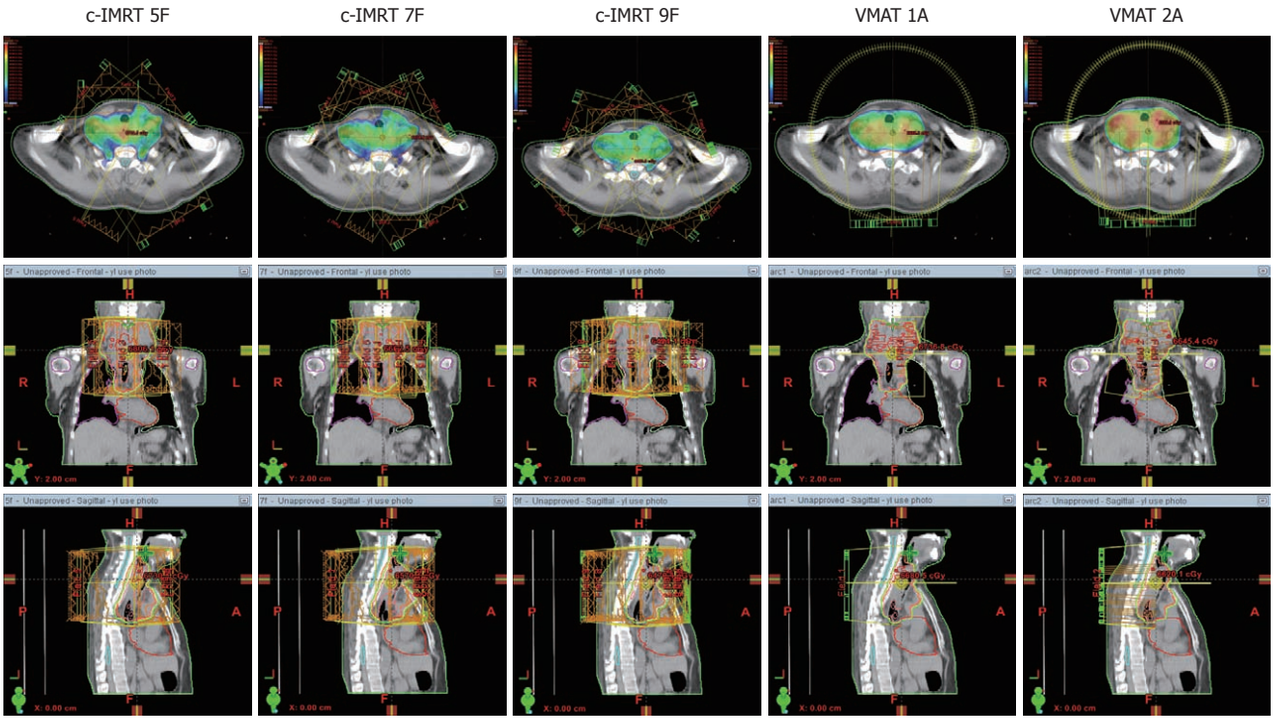

Figure 1 Dose distributions in a cervical esophageal cancer patient planed by conventional sliding window intensity-modulated radiotherapy (5 fields, 7 fields and 9 fields) and volumetric-modulated arc therapy (1 arc and 2 arcs). IMRT: Intensity-modulated radiotherapy; VMAT: Volumetric modulated arc therapy; F: Coplanar field; A: Arc; Orange line: Planning target volume; Blue line: Spinal cord; Color wash areas: Receiving ⩾ 100% of the dose (60 Gy).

Table 5 Normal tissue complication probability results for organs at risk

Organ	IMRT-5F	IMRT-7F	IMRT-9F	VMAT-1A	VMAT-2A	$P < 0.05$
Lung						
Cervical	0.24 ± 0.14	0.24 ± 0.15	0.32 ± 0.22	0.22 ± 0.18	0.13 ± 0.09	1A *vs* 9F; 2A *vs* 5F, 7F, 9F
Upper	0.73 ± 0.62	0.77 ± 0.66	0.84 ± 0.71	0.41 ± 0.37	0.30 ± 0.23	1A *vs* 7F, 9F; 2A *vs* 5F, 7F, 9F
Middle	0.62 ± 0.48	0.59 ± 0.48	0.60 ± 0.47	0.57 ± 0.45	0.50 ± 0.41	2A *vs* 5F
Lower	0.59 ± 0.45	0.59 ± 0.50	0.67 ± 0.46	0.57 ± 0.54	0.46 ± 0.37	2A *vs* 5F
Heart						
Cervical	0	0	0	0	0	NS
Upper	0.02 (0-0.12)	0.02 (0-0.09)	0 (0-0.02)	0	0	NS
Middle	0.61 (0.05-1.07)	0.94 (0.01-4.24)	0.31 (0.01-1.17)	0.13 (0-0.58)	0.21 (0-0.80)	1A *vs* 5F, 7F, 9F
Lower	6.66 (1.14-16.04)	1.97 (0.11-5.09)	1.76 (0.07-5.95)	1.32 (0-5.81)	0.66 (0-1.59)	1A *vs* 5F, 9F; 2A *vs* 5F, 7F, 9F
Spinal cord						
Cervical	0	0	0	0	0	NS
Upper	0	0	0	0	0	NS
Middle	0.002 (0-0.01)	0.006 (0-0.03)	0.008 (0-0.04)	0	0	NS
Lower	0	0	0	0	0	NS

IMRT: Intensity-modulated radiotherapy; VMAT: Volumetric modulated arc therapy; F: Coplanar field; A: Arc; NS: Not significant.

while it could reduce significantly the treatment time and the number of MU required in the morbidities, such as head and neck cancer, intracranial tumor, breast cancer, glioma, and carcinoma of the anal canal[14,17,20,21]. In the present study, VMAT using 2A achieved better results than using 1A.

First, the PTV volumes were larger in cervical and upper EC series due to pathological characteristics and biological behavior of carcinoma in these regions, and presented T-shaped from a posteroanterior view. In head and neck carcinoma, due to more complex target volume, 7F or 9F are constantly used in c-IMRT to meet the requirements of dose distribution, HI and CI of PTV[22,23]. Our results were consistent with this. PTV coverage in c-IMRT with 5F was less qualified in comparison with 7F or 9F. Both VMAT and c-IMRT resulted in abundant D_{mean} in PTV (63.94 Gy and 63.53 Gy in 1A and 2A *vs* 63.49 Gy, 62.90 Gy, 62.46 Gy in 5F, 7F and 9F). VMAT proved to be superior to c-IMRT in terms of MU and CI, but slightly inferior to c-IMRT in terms of HI. We also confirmed that VMAT with 2A achieved better results than 1A in terms of conformity and homogeneity. For the heart, VMAT showed a lower percentage of V30, V40 or V50. For lungs, VMAT provided

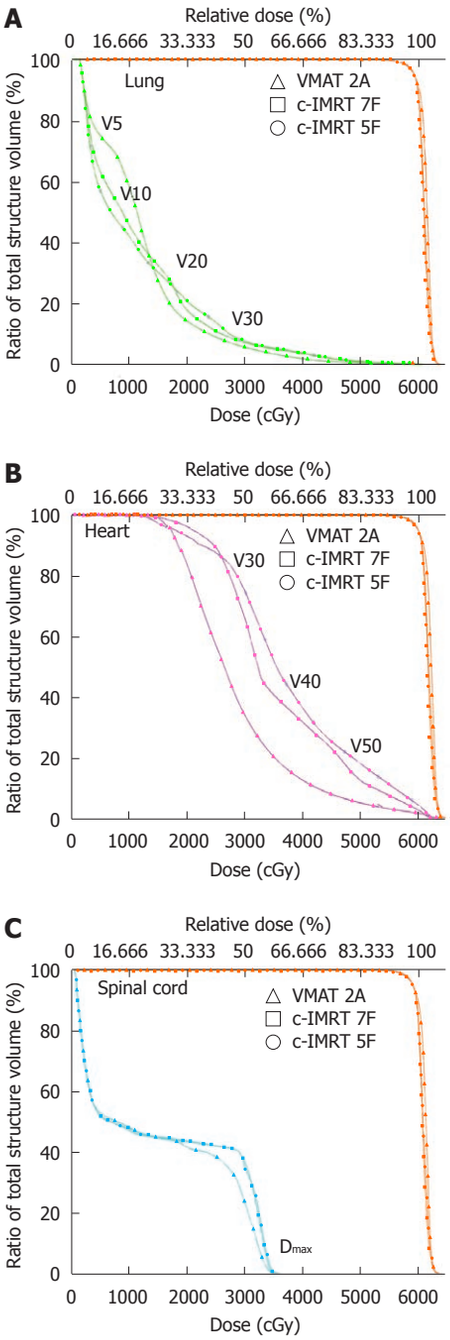

Figure 2 Dose-volume histogram of organs at risk and planning target volume for volumetric-modulated arc therapy and conventional sliding window intensity-modulated radiotherapy in a lower thoracic esophageal cancer patient. Volumetric-modulated arc therapy (VMAT) with double arcs (triangle) and conventional sliding window intensity-modulated radiotherapy (c-IMRT) with 7 fields (squares) and 5 fields (round). The planning target volume is shown in orange, the lungs in green (A), heart in red (B) and spinal cord in blue (C). F: Coplanar field; A: Arc; Vx: The percentage of organ receiving a dose > X Gy.

better sparing in terms of V20 and V30. The results of radiobiological NTCP comparison demonstrated that VMAT was superior to c-IMRT either in lungs or heart ($P < 0.05$). Yin *et al*[24] compared 7F in IMRT with VMAT plans for cervical EC, and found that there were differences between VMAT and IMRT in HI and MU, but not in CI, which are consistent with our results, but lung V5 in VMAT (1A 51.4, Ax 49.3 and 7F 50.9) was reduced while lung V30, V40, V50 and MLD were increased. In our study, lung V5 in VMAT was slightly increased (1A 51.37, 2A 51.03, and 7F 48.77), but V30 (1A 12.99, 2A 12.99, 7F 14.42) and MLD (1A 12.79, 2A 12.35 7F 12.52) were lower than in c-IMRT. The difference in V5, V30 and MLD may be due to that they avoided a certain angle in the VMAT plan with 2A, and this caused the reduction of the volume of irradiated lungs. One of the particular interesting phenomena in VMAT is the increase trends of mean or maximal radiation doses to the humeral head. This may be because that humeral head is adjacent to the target volume in the cervical and upper EC and the rotating mode of VMAT thus increased the irradiation volumes of the humeral head. However, clinical evidence on the acceptable humeral head constraints for IMRT remains scarce in literature. Nevertheless, according to the tolerated doses and clinical data of bone joints, such as femoral head and neck or temporomandibular joint[25,26], the maximum doses to humeral heads in the cervical EC (1A 34.00 Gy and 2A 33.82 Gy) or upper thoracic EC (1A 44.33 Gy and 2A 41.03 Gy) were considered acceptable, but we should pay attention to this performance and its potential risk.

Subsequently, the results obtained in cervical and upper thoracic EC were almost seen in middle and lower thoracic EC, with a PTV of smaller volume but surrounded by more lungs and heart. In thoracic and epigastric cases, except for a few complex cases, 5-7F of c-IMRT could meet most of the clinical dosimetric requirements. In our study, an ideal homogeneity was achieved in 5F or 7F of IMRT. With increase of the fields in c-IMRT or doubling arc in VMAT, dose distribution in PTV became more optimal in terms of better conformity and similar homogeneity. The trends were significantly different ($P < 0.05$) between VMAT(1A and 2A) and c-IMRT(5F and 7F). Recently, Van Benthuysen *et al*[27] demonstrated that VMAT had the advantage to decrease treatment times over c-IMRT, while providing similar OAR sparing and PTV coverage, but lower homogenous dose distribution in lower EC. In our study, we found that in the middle and lower thoracic EC, HI was similar in VMAT and c-IMRT. We did not find a significant difference between 5F or 7F IMRT and VMAT for these trends. For lesions in this region, more volumes of heart and lungs were involved in the irradiation area, mean doses to lungs and heart were elevated markedly and HI was also inferior to cervical and upper EC. Because lung tissue filled with air was significantly less dense than other body tissues, as a result of heterogeneity corrections in radiation treatment planning systems, optimization procedures produced substantial dose non-uniformity in PTV caused by the effect of surrounding lung tissues. To further optimize the dose in the target volume, dose heterogeneity was achieved by loosening the constraints on the maximum doses in PTV. It may result in insufficient dose in PTV or the creation of clinically significant hotspots in the PTV

and surrounding normal tissue structures. The National Comprehensive Cancer Network Guidelines recommend dose limits for selecting critical normal structures, i.e., the spinal cord doses should not exceed 45 Gy, and one-third of the heart should receive less than 50 Gy. The dosimetric parameters of lung injury risk were mainly studied on lung cancer irradiation, the increased risk of radiation pneumonitis correlated with heterogeneous parameters, such as MLD, the percentage of lung volume receiving at least 20 Gy (V20), 13 Gy (V13), 10 Gy (V10) or 5 Gy (V5), in which V20 was a recognized indicator confirmed by several studies. Based on pooled data from 540 patients irradiated for thoracic malignancy, the calculated risk of grade $\geqslant$ 2 pneumonitis was 43%, 18%, and 11% for the MLD of 24-36, 16-24 and 8-16 Gy, respectively. In our study, MLD was controlled below 16 Gy and it was acceptable. For conventionally fractionated regimens (2 Gy/fraction), V20 and MLD were the traditional parameters used to predict for lung toxicity, however, emerging data suggested that percentage of lung volume receiving lower doses may be predictive of pulmonary toxicity. VMAT plans offered the potential to significantly escalate the coverage of the low-dose area (V5 and V10) because all doses were deposited within the plane of the arc, instead of being spread out in non-coplanar directions. Mean V5 in VMAT was beyond 80% and it might increase the potential pulmonary toxicity. Wei *et al*[28] found that V30 > 46% and < 46% was associated with rates of pericardial effusion of 73% and 13%, respectively. The ischemic segments usually occur in volumes irradiated to a dose of 45 Gy or more. In our study, V40 and V50 were achieved in both VMAT and c-IMRT, but V30 was higher due to lower constrained priority. In VMAT, 1A achieved better results than 2A for less irradiation volumes of heart and lungs. In comparison with 5F or 7F, VMAT reduced V20 and V30 of lungs, and V30, V40 and V50 of heart. Besides, Hawkins *et al*[29] evaluated the capability of VMAT to reduce heart and cord dose while maintaining lung V20 < 20% in lower gastroesophageal tumors. IMRT (4F) and VMAT plans showed that VMAT provided a significant reduction in heart V30 (31% *vs* 55%) with a better CI in a shorter time. But V30 (2A 57.57 *vs* 5F 78.6) in our study was higher because our prescribed dose (60 Gy) was higher than theirs (54 Gy). NTCP of heart and lungs were common indicators in radiobiological assessment to indicate the tendency in plan comparison. VMAT had a trend with a lower NTCP of lungs and heart, but statistical significance only existed for lungs between 5F and 2A in lower EC, as for heart, between c-IMRT and 1A in middle thoracic EC, and between c-IMRT and 2A in lower EC ($P < 0.05$). Therefore, regarding the correlation between dosimetric parameters and OAR toxicity, we did find a superior trend in VMAT to c-IMRT. Wang *et al*[30] also conducted a planning comparison for EC between VMAT (1A and 2A) and 7F IMRT, and found that VMAT plan, especially using double arcs, could improve OAR sparing (lung V20 and V30, heart V30 and V40) and lower MUs without compromised target qualities as compared with IMRT. This was consistent with our findings.

VMAT reduced the number of required MU[31], because it was performed simultaneously with rotation by a dynamic MLC adaptation to the target volume during the rotation. Using double arcs, the rotation in clockwise and counter clock-wise directions allows diminished 25 s off-time between the two arcs[32]. The number of MU required is higher due to the sliding window technique. c-IMRT plans in this study offered wider than 15 cm in the direction of the MLC motion necessitating splitting into two sequences and doubling the number of fields. By contrast, one of the drawbacks of c-IMRT was the potential risk of second cancer. Theoretically, the significant reduction of MU by VMAT decreases scattered dose and may reduce the risk of secondary malignancies. The impact of irradiation of healthy tissues at low doses remains unresolved with the use of VMAT.

In conclusion, VMAT treatment plan was slightly better than c-IMRT in terms of PTV coverage. It provided an equivalent or better lungs and heart dose sparing, significant reduction of NTCP and MU per fraction. For cervical and upper EC, PTV was T-shaped across neck and chest, VMAT achieved fairly uniform dose distribution, but the 2A provided the best CI in all plans, and VMAT significantly increased the doses of humeral head. For middle and lower EC, in which PTV involved more lungs, VMAT plans offered the most conformal dose distribution and the potential to significantly escalate the coverage of lungs at low doses.

COMMENTS

Background

Esophageal cancer (EC) is very common in China and other developing countries. Radiotherapy is a major non-invasive treatment method with a high efficacy rate for EC. Innovative technologies have been developed in radiation delivery such as intensity-modulated radiotherapy (IMRT), and volumetric modulated arc therapy (VMAT) is a relatively new form of IMRT. There are several new interesting techniques in IMRT, but few evaluations have been available in term of their efficiency and safety.

Research frontiers

There have been few studies to compare the two radiotherapy techniques, particularly in EC. VMAT had already been investigated for some cancers. In this study, the authors further compared the VMAT plans and the conventional sliding window IMRT plans in EC of different anatomic regions.

Innovations and breakthroughs

Previous dose comparison studies showed that VMAT was able to produce similar or improved dose distributions, while achieving a reduction in treatment time and monitor units (MU). However, results are different in EC about dose to organs at risk (OAR) due to variable limitation conditions. Normal tissue complication probability (NTCP) of OAR in the VMAT plan, a common indicator in radiobiological assessment, is still not clear in EC. In the present study, the authors showed that VMAT, especially 2 arcs, slightly improved the OAR dose sparing for some organs, such as lungs and heart, and reduced the NTCP and MU with a better planning target volume coverage.

Applications

This study provides a new insight into better understanding of the VMAT plan characteristics in EC of different anatomical parts, and lays the foundation for further clinical studies in VMAT.

Terminology

IMRT is a three-dimensional conformal radiotherapy developed based on inverse planning optimization to modulate intensity beams using multi-leaf

collimator (MLC), this technique offers improvement in target dosimetric coverage. There are different IMRT delivery techniques, including "step and shoot", sliding window modes and a rotational technique (VMAT). Conventional sliding window IMRT, in which the leaves are adjusted with fixed gantry, is a common form which is being used in clinical practice. VMAT, in which dose rates, gantry speed and dynamic MLC motion are all variable during gantry arc rotation, is a novel form of IMRT in recent years.

Peer review

IMRT is developing a lot in Radiation Oncology Departments for a few years. There are several very interesting technics but still few real evaluations in terms of efficiency and safety. Comparing two technics is very interesting even if it's "only" a dosimetric comparison. This kind of comparisons of recent technics are not very frequent, particularly in EC but are developing a lot this last few years. The text of this manuscript is very clear and well presented.

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BRIEF ARTICLE

Nur-related receptor 1 gene polymorphisms and alcohol dependence in Mexican Americans

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Abstract

AIM: To investigate the association of polymorphisms of nur-related receptor 1 (*Nurr1*) and development of alcohol dependence in Mexican Americans.

METHODS: Peripheral blood samples were collected from 374 alcoholic and 346 nonalcoholic Mexican Americans; these two groups were sex- and age-matched. Sample DNA was extracted and genomic DNA was amplified by polymerase chain reaction. The *-2922(C) 2-3* polymerase chain reaction products were digested with *Sau96I*, alleles of *1345(G/C)*, and *-1198(C/G)* in the regulatory region as well as *Ex+132 (G/T/A/C)* and *Ex+715(T/-)* in exon 3 were studied by sequencing.

RESULTS: The *C2/C2, C2/C3, C3/C3* genotype distribution of *-2922(C) 2-3* was 34.4%, 38.2% and 27.5% in the nonalcoholic group compared to 23.3%, 51.2% and 25.4% in the alcoholic group (P = 0.001). The *C/C, C/G, G/G* genotype distribution of *-1198(C/G)* was 23.5%, 46.1% and 30.3% in the nonalcoholic group compared to 13.9%, 50.9% and 35.3% in the alcoholic group (P = 0.007). However, the *-1345 (G/C)*, *Ex3+132(G/T/A/C)* and *Ex3+715(T/-)* alleles were not polymorphic in Mexican Americans, and all those studied had *G/G, G/G* and *T/T* genotype for these three alleles, respectively. The *-2922(C) 2-3* did not show allele level difference between alcoholic and nonalcoholic individuals, but *-1198 (C/G)* showed a significant allele frequency difference between alcoholic (39.3%) and nonalcoholic (46.6%) populations (P = 0.005). Excluding obese individuals, significant differences were found at both genotypic and allelic levels for the *-2922(C) 2-3* polymorphism (P = 0.000 and P = 0.049) and the *-1198 (C/G)* polymorphism (P = 0.008 and P = 0.032) between nonobese alcoholics and nonobese controls. Excluding smokers, a significant difference was found only at the genotypic level for the *-2922(C) 2-3* polymorphism (P = 0.037) between nonsmoking alcoholics and nonsmoking controls, but only at the allelic level for the *-1198(C/G)* polymorphism (P = 0.034).

CONCLUSION: Polymorphisms in the regulatory region of *Nurr1* are implicated in pathogenesis of alcohol dependence and the *Nurr1*/dopamine signaling pathway might be important for this dependence development in Mexican Americans.

Key words: Nur-related receptor 1; Polymorphism; Alcohol dependence; Obesity; Smoking; Nuclear receptor

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INTRODUCTION

It is widely accepted that genetic factors play an important role in the development of alcohol dependence. Family clustering surveys have shown that alcohol dependence rates are higher among persons who are biologically related to an alcoholic[1], and that a family alcohol history is a strong predictor of alcohol dependence[2]. Twin pair studies have revealed a significantly greater concordance rate for alcohol dependence in monozygotic compared with dizygotic twins[3]. Half sibling and adoption studies have demonstrated that half brothers with different fathers and adopted sons of alcoholic men show a rate of alcohol dependence more like that of the biological father than that of the foster father[4]. On the other hand, there is no classic Mendelian pattern of inheritance for alcohol dependence. Environmental factors are also linked to alcohol dependence. Thus, alcohol dependence is a polygenic complex disorder resulting from an intricate interaction of multiple genes and various environmental factors.

Genes encoding alcohol metabolizing enzymes alcohol dehydrogenase (*ADH1B)* and aldehyde dehydrogenase (*ALDH2*) are the only genes that have been firmly linked to alcoholism[5,6]. However, data suggest that genes involved in the brain reward pathways are also strong candidates for a predisposition to alcohol dependence. These pathways, in particular, those utilizing dopamine and opioids as neurotransmitters, mediate positive reinforcement of activities, such as eating, love and reproduction. When engaging in such activities, the "natural reward" center releases dopamine from brain pathways in the nucleus accumbency and frontal cortex[7,8]. However, these same pathways can also be activated by "unnatural rewards" such as alcohol abuse. Only a minority of individuals become addicted to these various substances and certain types of behavior, therefore, it should be possible to identify factors, such as genes, that distinguish them from others not affected. Dopamine is the primary neurotransmitter in the reward pathway, genes that control dopamine signaling, including dopamine receptors and transporters, are particularly tempting candidates. Alcohol dependence is likely to be a polygenic and multifactorial disease, therefore, certain genes might have a small effect, whereas others have a greater impact in terms of increasing the risk for this disorder.

Our previous studies have shown that certain alleles of the brain dopamine receptors 2 and 4 (*DRD2* and *DRD4*) and of the serotonin transporter (*5-HTT*) as well as of opioid receptor are associated with alcohol dependence[9-13]. These findings indicate the importance of polymorphism of the gene in the reward pathway in contributing to the development of alcohol dependence in Mexican Americans.

Alcohol consumption, compulsive overeating and smoking are all associated with dysfunction of rewards pathways; there might be common risk factors in reward genes for these behaviors. *DRD2 TaqI A1* allele was identified at a higher frequency in alcoholic and nonalcoholic smokers than nonsmoking controls[14]. Smoking and obesity related to overeating might serve as confounding factors when association is analyzed between reward genes and alcohol dependence.

The Nur-related receptor 1 (*Nurr1*), *NR4A2*, is a transcription factor in the orphan nuclear receptor family[15-17], and is important for development of dopaminergic neurons. Ablation of *Nurr1* leads to agenesis of midbrain dopaminergic neurons as demonstrated by an absence of dopaminergic cell markers including tyrosine hydroxylase, as well as a loss of striatal dopamine neurotransmitter[18]. *Nurr1* knockout mice failed to develop ventral mesencephalic dopaminergic neurons and died within 12-48 h[19]. In addition, *Nurr1* increases expression of the human dopamine transporter gene in the mature brain; whereas other members of the nerve growth factor-induced clone B subfamily of nuclear receptors have lesser or even no effects[19]. Expression of *Nurr1* continues in mature dopaminergic neurons during adulthood, suggesting that *Nurr1* is also required for normal function of mature dopaminergic neurons[20]. Thus, *Nurr1* is an upstream signaling molecule for regulating the dopamine pathway and plays a broad-spectrum role in brain development.

Nurr1 involvement in the regulation of the dopaminergic system makes it a good candidate to study neuropsychiatric disorders. Several polymorphisms in the gene have been identified: *-469delG*, *M97V*, *H103R*, *DY122*, *-2922(C)2-3*, *IVS6+17* approximately *+18insG* and *EX8+657(CA)9-10*[21-24]. Among these polymorphisms, *-2922(C)2-3*, *IVS6+17* approximately *+18insG* and *EX8+657* variants are common (frequency of minor alleles > 15%) in both Caucasian and Asian populations. Two variants (*-291Tdel* and *-245T→G*) of *Nurr1* are associated with Parkinson's disease[25]. However, another study failed to identify any of the described variants in Parkinson's patients or controls[26]. In the regulatory region of the *Nurr1* gene, at least five polymorphic sites [*P1(A/T*, reference SNP number rs1462374), *P2(G/C)*, *P3(C/G)*, *P4(C/A/G)*, *P5(del C)*] have been identified, with no significant association between the genotype or allele frequency of these variants and schizophrenia or Parkinson's disease. In addition, *P2(G/C)*, *P3(C/G)*, and *P5(del C)* are in linkage disequilibrium (LD) with each other[27]. Further investigation of common *Nurr1* variants [*-2922(C)2-3*, *IVS6+17* approximately *+18insG*, *EX8+657(CA) 9-10*] has not supported their pathogenic role for schizophrenia among Japanese individuals[28]. There are two coding synonymous polymorphism sites in exon 3 (rs16840266) and exon 5 (rs61748236), and three coding nonsynonymous polymorphism sites in exon 3 (rs36083712 and rs35100271) and exon 8 (rs61729997); some other rare missense mutations have been studied in psychiatric disorders[22], but none of them has been found to be associated with alcohol addiction.

The role of *Nurr1* in alcohol dependence was studied in Japanese individuals[29], and polymorphisms including *-2922(C) 2-3* and *EX8 +657(CA)9-10* were examined. They have reported that the genotypic distribution of these two polymorphisms was in Hardy-Weinberg equilibrium in the controls and alcoholics. The allele frequency of the *(C) 2* and *(CA) 9* and allele in the alcoholics was similar to that in the controls. Significant LD between these two polymorphisms was observed in both controls ($P = 0.007$) and alcoholics ($P < 0.0001$), but the LD was much stronger in alcoholics than in controls (D' = 0.84 *vs* D' = 0.30). There was a significant difference in haplotype distributions between the alcohol dependence and control groups. The haplotypic association was not based on an increased frequency of a specific haplotype, but rather based on stronger LD in alcoholics. This finding implies that more recent ancestral chromosome sharing by the alcoholics than by the controls, and that the *Nurr1* locus is one of the genome regions that contribute to alcohol dependence.

Nurr1 is involved in the regulation of the dopaminergic system, which is the primary neurotransmitter in the reward pathway. The dopamine signaling can be activated by alcohol. Thus, we hypothesize the presence of an association between the polymorphism of the *Nurr1* gene and alcohol dependence. We selected three alleles in the regulatory region of the *Nurr1* gene that include *-2922(C)2-3*, *-1345(G/C)* and *-1198(C/G)* (promoter P2 and P3 in reference literature from Andrea Carmine) as well as coding synonymous *Ex+132(G/T/A/C*, rs16840266) and coding nonsynonymous *Ex+715(T/-*, rs35100271) in exon 3. These alleles are supposed to contribute to the function of *Nurr1* and may have an impact on alcohol dependence in Mexican Americans.

MATERIALS AND METHODS

Participants

All participants in this project were enrolled from unrelated Mexican Americans who lived in Los Angeles County, CA. The study cohorts included 374 alcoholics and 346 nonalcoholics, and the ratio of cases to controls was about 1.08. The groups were sex- and age-matched. All subjects provided informed consent for their participation in this study. This study was approved by the University of Kansas Medical Center Human Subjects Committee. All participants were investigated, diagnosed, and assigned based on the Diagnostic and Statistical Manual of Mental Disorder, Fourth Edition (DSM-IV). The alcoholic participants were interviewed by the Semi-Structured Assessment for the Genetics of Alcohol dependence II in English or Spanish[30]. The inclusion criteria for alcoholic participants included: (1) the ability to give informed consent; (2) between 21 and 76 years of age; (3) no less than three of four biological grandparents of Mexican heritage; (4) fluency in either Spanish or English; (5) no current use of other substances (except tobacco and caffeine), or history of such use within the past 6 mo; (6) no current or past diagnosis of mental illness such as schizophrenia, schizophrenia disorder, schizoaffective disorder, schizotypal disorder, major depression, bipolar disorder, or Parkinson's disease; and (7) no other clinical unacceptable disease based on physical examinations. The inclusion criteria for nonalcoholic participants included: (1) no current or history of diagnosis of DSM-IV alcohol dependence or alcohol abuse; and (2) no other clinical unacceptable disease based on physical examinations. The interview information also covered body weight, height, smoking status, tea and coffee intake, as well as marriage, and education status. Body mass index (BMI) equals body weight in kilograms divided by height in square meters (kg/m^2). Smoking status was defined as having smoked one or more cigarettes per day during the past 30 d[31].

Genotyping

Participants' peripheral blood samples were collected into tubes containing K_2-EDTA. Genomic DNA was extracted with GeneCatcher gDNA Blood kits (Invitrogen, Carlsbad, CA, United States). The primers used for PCR were: *-2922(C)2-3*, forward: 5'-AAAAGGGGATGAACCGGGTAGG-3', reverse: 5'-TTTTCCGAAAGAGGTGTGACCT-3'; *-1345(G/C)* and *-1198(C/G)*, forward: 5'-ATCCCGAATAGTTCCACGGAG-3', reverse: 5'-CACGAGTTTTAAGGGAATAAATCG-3'; *Ex3+132(G/T/A/C)* and *Ex3+715(T/-)*, forward: 5'-GCTGAGTGTGTTATCACCTGTTT-3', reverse: 5'-GCTGAGTGTGTTATCACCTGTTT-3'.

PCR amplification was carried out in a 25-μL reaction mix containing 100 ng genomic DNA, 1× Go Taq Flexi buffer, 0.2 μmol/L each primer, 0.2 mmol/L dNTP, 2.0 mmol/L $MgCl_2$, 0.2 mmol/L dGTP, and 1 U GoTaq DNA polymerase. The thermal cycling conditions were set at 95 ℃ for 5 min, then 35 cycles for 30 s at 95 ℃, 30 s at 50 ℃ (for the three alleles in the regulatory region) or 55 ℃ (for the two alleles in exon 3), and 60 s at 72 ℃, with a final extension step of 10 min at 72 ℃. PCR products were examined on a 2% agarose gel.

The *-2922(C)2-3* PCR products were digested with *Sau96I*, which generated 176- and 158-bp fragments for wild-type alleles. Positive and negative digestion controls were set in each PCR and each agarose gel to ensure correct digest results. Retest was needed when results could not be read clearly. Other genotyping was studied by sequencing. All samples were sequenced in two directions. Results where two direction sequences did not match each other were excluded. Sequencing results were read by Finch TV (1.4 Version, Geospiza, Inc.) graphical viewer.

Statistical analysis

Statistical analyses were performed with SPSS Version 15.0 software (SPSS Inc., Chicago, IL, United States). Two-sided analyses were conducted and $P < 0.05$ was used as the significance threshold. Pearson's χ^2 tests were used to compare sex, BMI, smoking, genotype, and allele distribution between alcoholics and their controls.

Table 1 Genotype and allele frequency of the *-2922(C)2-3* and *-1198(C/G)* allele in Mexican American alcoholics and nonalcoholics *n* (%)

Groups	*-2922(C)2-3* allele					*-1198(C/G)* allele				
	Genotype			Allele frequency	Total	Genotype			Allele frequency	Total
	(C)2/(C)2	*(C)2/(C)3*	*(C)3/(C)3*	*(C)3*		*C/C*	*C/G*	*G/G*	*C*	
Non-alcoholic	119 (34.4)	132 (38.2)	95 (27.5)	322 (46.5)	346	76 (23.5)	146 (46.1)	98 (30.3)	298 (46.6)	320
Alcoholic	87 (23.3)	192 (51.3)	95 (25.4)[a]	382 (51.1)	374	48 (13.9)	176 (50.9)	122 (35.3)[a]	272 (39.3)[b]	346

[a]$P = 0.007$; [b]$P = 0.005$ *vs* nonalcoholic.

RESULTS

Population characteristics

There were 374 alcoholics and 346 nonalcoholic controls. The alcoholic group had 299 (79.9%) men and 75 (20.1%) women, and the nonalcoholic control group had 260 (75.1%) men and 86 (24.9%) women. The number of young (≤ 30 years), middle-aged (30-60 years), and old (> 60 years) participants in the control group was 93 (26.9%), 245 (70.8%), and 8 (2.3%), respectively, and 99 (26.5%), 266 (71.1%), and 9 (2.4%) in the alcoholic group. The mean ± SD age of the alcoholics and controls was 38.3 ± 10.5 years (range: 21-76 years) and 37.3 ± 10.4 years (range: 19-65 years), respectively. No significant differences were found between the two cohorts regarding sex or age distribution with Pearson's χ^2 test.

BMI is the measurement of choice to determine obesity, and the clinical diagnosis of obesity is BMI ≥ 30 kg/m^2. The BMI was not significantly different between the alcoholic and nonalcoholic groups [BMI < 30 kg/m^2, 198 (57.6%) *vs* 213 (63.6%); BMI ≥ 30 kg/m^2, 146 (42.4%) *vs* 122 (36.4%), $P = 0.108$]. However, the smoker distribution was significantly different between the alcoholic and nonalcoholic groups [non-smoker, 197 (52.7%) *vs* 282 (82.7%); smoker, 177 (47.3%) *vs* 59 (17.3%), $P = 0.000$]. The alcoholic group had more smokers than the control group had.

Genotypes of -2922(C)2-3, -1345(G/C), -1198(C/G), Ex3+132(G/T/A/C) and Ex3+715(T/-) in Mexican Americans

The genotypes of *-2922(C)2-3*, *-1345(G/C)*, *-1198(C/G)*, *Ex3+132(G/T/A/C)* and *Ex3+715(T/-)* were studied in Mexican American alcoholics and controls. The *-2922(C)2-3* and *-1198(C/G)* alleles were polymorphic in Mexican Americans, but *-1345(G/C)*, *Ex3+132(G/T/A/C)* and *Ex3+715(T/-)* were shown to be present in the studied population (*G/G*, *G/G*, and *T/T*, respectively). These results are consistent with reports which come from Swedish[27], Japanese[29] and Canadian[32] populations with neurological disorders. This is believed to be the first research reporting the absence of polymorphisms in Mexican Americans at the three genomic sites mentioned above.

The genotypes of *-2922(C)2-3* and *-1198(C/G)* as well as their minor allele frequencies are shown in Table 1. The Hardy-Weinberg equilibrium *P* values of *-2922(C)2-3* and *-1198(C/G)* in alcoholics were 0.6914 and 0.2751, respectively, and the *P* values for these two alleles in nonalcoholic controls were 0.00002 and 0.1854. The genotype distribution of the *-2922(C)2-3* allele was significantly different between alcoholics and nonalcoholics ($P = 0.001$); however, there was no significant difference at the allelic level. The genotype as well as the allele frequency of the *-1198(C/G)* allele was significantly different between alcoholics and nonalcoholics ($P = 0.005$ and $P = 0.007$), suggesting the importance of sequence variations in the regulatory region of the *Nurr1* gene in differentiating these two cohorts. In addition, the *-2922(C)2-3* and *-1198(C/G)* alleles showed a strong LD (D' = 0.88) in the alcoholic population, but not in the nonalcoholic population.

Association of -2922(C)2-3 and -1198(C/G) with alcohol dependence after controlling for confounding effect of smoking and obesity

Alcoholics and nonalcoholic controls were stratified according to the smoking and obesity status. When obese individuals were excluded, a significant difference was found at both the genotypic and allelic level between non-obese alcoholics and non-obese controls, for the *-2922(C)2-3* polymorphism (Table 2, $P = 0.000$ and $P = 0.049$) and *-1198(C/G)* polymorphism (Table 3, $P = 0.008$ and $P = 0.032$). When smokers were excluded, between alcoholics and controls, a significant difference was found only at the genotypic level for the *-2922(C)2-3* polymorphism (Table 2, $P = 0.037$), but only at the allelic level for the *-1198(C/G)* polymorphism (Table 3, $P = 0.034$).

DISCUSSION

Hispanics are one of the fastest growing ethnic groups in the United States and were expected to become the largest minority group by the year 2010[33]. About two-thirds of Hispanic population is of Mexican American or Mexican origin. These population demographics have shown evidence of a serious problem of alcohol dependence with a prevalence rate of heavy drinking in Mexican American men that is three times higher than in non-Hispanic male populations[34]. Excessive alcohol drinking carries a high risk of developing various types of chronic diseases. Alcohol-related problems in this population, such as alcoholic liver disease, malignant neoplasms, psychiatric conditions, neurological impairment, and cardiovascular disease show a significantly higher

Table 2 Genotype and allele frequency of the *-2922(C)2-3* allele in Mexican American alcoholics and non-alcoholics controlling for confounding effect of smokers and obesity *n* (%)

Groups	Genotype			Allele frequency
	(C)2/(C)2	*(C)2/(C)3*	*(C)3/(C)3*	*(C)3*
Non-alcoholic				
BMI < 30 kg/m^2 (213)	73 (34.3)	78 (36.6)	62 (29.1)	202 (47.4)
BMI ≥ 30 kg/m^2 (122)	41 (33.6)	48 (39.3)	33 (27.0)	114 (46.7)
Alcoholic				
BMI < 30 kg/m^2 (198)	37 (18.7)	107 (54.0)	54 (27.3)[a]	215 (54.3)[b]
BMI ≥ 30 kg/m^2 (146)	37 (21.5)	74 (50.7)	35 (25.9)	144 (49.3)
Non-alcoholic				
Non-smokers (282)	100 (35.5)	101 (35.8)	81 (28.7)	263 (46.6)
Smokers (59)	17 (28.8)	29 (49.2)	13 (22.0)	55 (46.6)
Alcoholic				
Non-smokers (197)	49 (24.9)	88 (44.7)	60 (30.5)[c]	208 (52.8)
Smokers (177)	38 (21.5)	104 (58.8)	35 (19.8)[d]	174 (49.2)

[a]*P* = 0.000 *vs* nonalcoholic body mass index (BMI) < 30 kg/m^2; [b]*P* = 0.049 *vs* nonalcoholic BMI < 30 kg/m^2; [c]*P* = 0.037 *vs* non-alcoholic non-smokers; [d]*P* = 0.016 *vs* alcoholic non-smokers.

Table 3 Genotype and allele frequency of the *-1198(C/G)* allele in Mexican American alcoholics and non-alcoholics controlling for confounding effect of smoking and obesity *n* (%)

Groups	Genotype			Allele frequency
	C/C	*C/G*	*G/G*	*C*
Non-alcoholic				
BMI < 30 kg/m^2 (199)	54 (27.1)	81 (40.7)	64 (32.2)	189 (47.5)
BMI ≥ 30 kg/m^2 (115)	21 (18.3)	62 (53.9)	32 (27.8)	104 (45.2)
Alcoholic				
BMI < 30 kg/m^2 (181)	26 (14.4)	92 (50.8)	63 (34.8)[a]	144 (39.8)[b]
BMI ≥ 30 kg/m^2 (136)	18 (13.2)	73 (53.7)	45 (33.1)	109 (40.1)
Non-alcoholic				
Non-smokers (265)	61 (23.0)	122 (46.0)	82 (30.9)	244 (46.0)
Smokers (55)	15 (18.3)	24 (53.9)	16 (27.8)	54 (49.1)
Alcoholic				
Non-smokers (185)	27 (14.6)	90 (48.6)	68 (36.8)	144 (38.9)[c]
Smokers (161)	21 (13.0)	86 (53.4)	54 (33.5)[d]	128 (39.7)

[a]*P* = 0.008 *vs* nonalcoholic body mass index (BMI) < 30 kg/m^2; [b]*P* = 0.032 *vs* nonalcoholic BMI < 30 kg/m^2; [c]*P* = 0.034 *vs* nonalcoholic non-smokers; [d]*P* = 0.05 *vs* non-alcoholic smokers.

incidence than when compared with those from other ethnic backgrounds[35]. Apart from environmental factors, using family, twin pair, half sibling, and adoption studies, alcohol dependence has been proved to be a polygenic disorder[1-4].

Candidate gene associations approach has been widely used to explore related genetic factors. We have previously studied genes involved in both alcohol metabolism and reward pathways in Mexican American alcoholics and have established associations between certain phenotypes of alcohol dependence and the polymorphism of these genes[9-13,36,37]. We showed that most Mexican Americans carry the *ADH1B*1* (95%) and *ALDH2*1* (99.4%) genes. Thus, the *ADH1* and *ALDH2* genotypes do not distinguish those more prone to alcohol drinking from those who are not[9]. Our data also showed the importance of polymorphism of *DRD2 (-141C Del/Ins)* and *5-HTTLPR* in contribution to alcohol dependence[9-12]. Other components of the dopamine pathway have also been associated with alcohol dependence. *DRD4 VNTR* genotypes without the 7-repeat allele have been found to be risk factors for alcohol dependence in Mexican Americans[13]. In the current study, to the best of our knowledge, we reported for the first time a significant association between the polymorphisms in the promoter region of the *Nurr1* gene and alcohol dependence in Mexican Americans. Our findings indicated that polymorphisms of *-2922(C)2-3* and *-1198(C/G)* are associated with alcohol dependence in the Mexican American population, even when the confounding effects of smoking and obesity were controlled, suggesting the reliability of our findings. The frequency of the *-1198 G* allele was significantly higher in alcoholics than in controls, suggesting that the *G* allele of *-1198(C/G)* might potentially have a pathogenic effect on alcohol drinking. The functional significance of these two polymorphisms still needs to be explored. Taken together, since *Nurr1* is upstream of the dopamine pathway, our data indicate the importance of *Nurr1*/dopamine signaling in alcohol abuse in this important minority group.

Smoking and obesity might represent significant confounding factors in the relationship between risk factors and alcohol dependence. Alcohol and constituents of tobacco are potent inducers of *CYP2E1*. When smokers were excluded from both the control and alcoholic groups, the **5B* allele of the *CYP2E1* gene was significantly associated with alcohol dependence[38]. Similar findings were observed for the *DRD2 -141C Ins/Del* allele[10]. In the present studied population, 47.3% of alcoholics and 17.3% of nonalcoholics were smokers. The distribution of smokers and nonsmokers in alcoholic and nonalcoholic groups showed a significant difference. When all participants were considered, distribution of both *-2922(C)2-3* and *-1198(C/G)* polymorphisms showed significant differences between the alcoholic and nonalcoholic groups. When smokers were excluded, the association of alcohol dependence with these two polymorphisms remained. When obesity was considered, *-2922(C)2-3* and *-1198(C/G)* polymorphisms were associated with alcohol dependence in the non-obese groups. Associations of these two polymorphisms and alcohol dependence are consistently found in the nonsmoking and non-obese group, therefore, this increases the reliability of our findings.

In conclusion, the polymorphism of *-2922(C)2-3* and *-1198(C/G)* in the regulatory region of the *Nurr1* gene were shown to be associated with alcohol dependence in Mexican Americans. The functional significance of these two polymorphisms still needs to be studied. Alcohol dependence is a multifactorial disease, therefore, it is also important to study the interaction of *Nurr1* with other components of the dopamine signaling pathway in contributing to alcohol dependence, which might lead to effective treatment strategies.

COMMENTS

Background

It is widely accepted that genetic factors play an important role in the development of alcohol dependence. Family clustering surveys have shown that alcohol dependence rates are higher among persons who are biologically related to an alcoholic. Nur-related receptor 1 (*Nurr1*) is a transcription factor in the orphan nuclear receptor family that can regulate dopamine neurotransmission and influence the expression of genes important for human brain development.

Research frontiers

Nurr1 regulates the dopaminergic system. Several polymorphisms in the gene have been identified. Two variants (*-291Tdel* and *-245T→G*) of *Nurr1* are associated with Parkinson's disease. The *Nurr1* locus is located in the genome region that contributes to alcohol dependence. Thus, the authors hypothesize the presence of an association between polymorphism of the *Nurr1* gene and alcohol dependence.

Innovations and breakthroughs

Several polymorphisms of *Nurr1* have been identified: *-469delG*, *M97V*, *H103R*, *DY122*, *-2922(C)2-3*, *IVS6+17* approximately *+18insG* and *EX8+657(CA)9-10*. *-2922(C)2-3*, *IVS6+17* approximately *+18insG* and *EX8+657* variants are common in both Caucasian and Asian populations. Two variants (*-291Tdel* and *-245T→G*) of *Nurr1* are associated with Parkinson's disease. Further investigation of common *Nurr1* variants did not support their pathogenic role, and none was found to be associated with alcohol addiction. In the present study, the authors showed that genotype distribution of *-2922(C) 2-3* and *-1198(C/G)* was significantly different between nonalcoholic and alcoholic Mexican Americans. The *-1198C* frequency was found to be significantly higher in nonalcoholics than that in alcoholics. However, the *-1345(G/C)*, *Ex3+132(G/T/A/C)*, and *Ex3+715(T/-)* alleles were not polymorphic in Mexican Americans; all the studied Mexican Americans had *G/G*, *G/G and T/T* genotype for these three alleles, respectively.

Applications

This study suggested that polymorphisms in the regulatory region of the *Nurr1* gene are implicated in pathogenesis of alcohol dependence of Mexican Americans. The *Nurr1*/dopamine signaling pathway might be important for the development of alcohol dependence in Mexican Americans.

Terminology

NR4A2 is a transcription factor in the orphan nuclear receptor family, and is important for development of dopaminergic neurons. *Nurr1* is an upstream signaling molecule for regulating the dopamine pathway and plays a broad-spectrum role in brain development.

Peer review

This is a good descriptive study in which the authors analyzed the polymorphisms of the *Nurr1* gene in 374 alcoholic and 346 nonalcoholic Mexican Americans. They found that the genotype distributions of *-2922(C) 2-3* and *-1198(C/G)* were different between alcoholics and non-alcoholics. The presented data suggest that the *Nurr1*/dopamine signaling pathway might be important for the development of alcohol dependence in Mexican Americans.

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BRIEF ARTICLE

Antifibrotic effect of N-acetyl-seryl-aspartyl-lysyl-proline on bile duct ligation induced liver fibrosis in rats

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Abstract

AIM: To investigate the preventive effect of N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) on bile duct ligation (BDL)-induced liver fibrosis in rats.

METHODS: Liver fibrosis in rats was induced by BDL and AcSDKP was infused subcutaneously for 2 wk *via* a osmotic minipump (Alzet 2ML4) immediately after BDL operation. After scarifying, serum and liver specimens were collected. Hematoxylin and eosin staining, Sirius red staining, enzyme linked immunosorbent assay, Western blot or real-time polymerase chain reaction were used to determinate liver functions, histological alterations, collagen deposition, mRNA expression of markers for fibroblasts, transforming growth factor-β1 (TGF-β1) and bone morphogenetic protein-7 (BMP-7).

RESULTS: When compared to model rats, chronic exogenous AcSDKP infusion suppressed profibrogenic TGF-β1 signaling, α-smooth muscle actin positivity (α-SMA), fibroblast specific protein-1 (FSP-1) staining and collagen gene expression. Col Ⅰ, Col Ⅲ, matrix metalloproteinase-2, tissue inhibitors of metalloproteinase-1 and tissue inhibitors of metalloproteinase-2 mRNA expressions were all significantly downregulated by AcSDKP infusion (2.02 ± 1.10 *vs* 14.16 ± 6.50, 2.02 ± 0.45 *vs* 10.00 ± 3.35, 2.91 ± 0.30 *vs* 7.83 ± 1.10, 4.64 ± 1.25 *vs* 18.52 ± 7.61, 0.46 ± 0.16 *vs* 0.34 ± 0.12, respectively, $P < 0.05$). Chronic exogenous AcSDKP infusion attenuated BDL-induced liver injury, inflammation and fibrosis. BDL caused a remarkable increase in alanine transaminase, aspartate transaminase, total bilirubin, and prothrombin time, all of which were reduced by AcSDKP infusion. Mast cells, collagen accumulation, α-SMA, TGF-β1, FSP-1 and BMP-7 increased. The histological appearance of liver specimens was also improved.

CONCLUSION: Infusion of exogenous AcSDKP attenuated BDL-induced fibrosis in the rat liver. Preservation of AcSDKP may be a useful therapeutic approach in the management of liver fibrosis.

Key words: N-acetyl-seryl-aspartyl-lysyl-proline; Liver fibrosis; Transformating growth factor-β1; α-smooth mucle actin; Bone morphological protein-7; Fibroblast specific protein-1; Epithelial-mesenchymal transition

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INTRODUCTION

N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) is an endogenous tetrapeptide normally present in the plasma and organs of humans and experimental animals[1-3]. It is released locally in tissues from its precursor thymosin-β4 (Tβ4) most likely by prolyl oligopeptidase (POP), a serine proteinase found in mammalian tissues[4,5]. AcSDKP is cleaved to an inactive form by the NH_2-terminal catalytic domain of angiotensin converting enzyme (ACE)[6].

Originally described as a natural inbibitor of hematopoietic stem cell proliferation, AcSDKP is now recognized as a critical negative regulator for extracellular matrix (ECM) accumulation in organs under both physiological and pathological conditions. Decreased basal levels of endogenous AcSDKP by ACE over expression or by POP inhibitors promote cardiac fibrosis and/or glomerulosclerosis[7,8]. Exogenous AcSDKP infusion reduces collagen deposition in rats heart and/or kidney under hypertensive and ischemic conditions[9]. AcSDKP also mediates the antifibrogenic effect of ACE inhibitors in the heart[10]. The mechanism of action of AcSDKP includes suppression of inflammation, ECM-producing cell proliferation, collagen production, and more importantly transforming growth factor-β1 (TGF-β1) signaling[7-9,11,12].

Indeed, these key cellular and molecular mechanisms are critical in regulating ECM accumulation in multiple organs, in particular the liver[13]. Moreover, ACE inhibition is beneficial in several liver fibrosis models where there is increased ACE activity and potentially excessive AcSDKP degradation, Tβ4 and significant POP activity are present in the liver, where AcSDKP is produced locally, and may play a role in the regulation of hepatic cell responses *in vivo*[14,15].

Our previous studies had revealed that AcSDKP ameliorated carbon tetrachloride (CCl_4)-induced liver fibrosis and liver functions in the rat liver. The current study was aimed to investigate the preventive effect of AcSDKP on bile duct ligation (BDL)-induced liver fibrosis in rats. The potential mechanisms involved were also examined.

We explored the effects of AcSDKP on liver fibrosis by infusion of exogenous AcSDKP into the BDL rat models. Our results demonstrate that exogenous AcSDKP preserves basal levels of AcSDKP in the liver and significantly reduces the development of liver fibrosis in this model. Based on these findings, we propose that AcSDKP plays an important role in attenuating liver fibrosis. The underlying mechanisms may involve decreased production of profibrotic cytokines and reduced collagen expression and accumulation.

MATERIALS AND METHODS

Materials

BDL-induced rat liver fibrosis models: all animal handling and experimental procedures were approved by the Animal Care and Use Committee of the Shanghai Jiaotong Uinversity School of Medicine. Male Sprague-Dawley rats (200-250 g) were obtained from the Shanghai Experimental Animal Center (Shanghai, China). The rat model of liver fibrosis was induced by BDL. Upon sacrifice, blood was collected and serum and/or plasma were obtained. Liver tissue was either fixed in 10% neutral buffered formalin, frozen in optimal cutting temperature, or snap frozen in liquid nitrogen and stored at -80 ℃.

Experiment: AcSDKP-infused BDL-treated rats and the BDL model were established as above (n = 8-10). AcSDKP-infused BDL-treated rats were infused with AcSDKP at 800 μg/kg per day through a subcutaneous osmotic minipump (Alza Corp, Palo Alto, CA) beginning simultaneously with BDL. Rats were sacrificed at 2 wk. This dosage was used because it increased plasma AcSDKP to a concentration similar to that induced by captopril (100 μg/kg per day, 3- to 5-fold-change), without any adverse effect on the circulatory system[9].

Serum assays

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, and albumin in serum and prothrombin time in plasma were measured using an automated analyzer.

Histological analysis

Formalin-fixed paraffin sections of the liver were stained with hematoxylin and esosin for pathological analysis or Sirius red for collagen. Collagen was quantified with Image Quant 5.1 software as previously described[16]. Positive cells were enumerated in 10 randomly selected fields at 400 × magnification.

Gene expression

Total RNA was extracted from livers using Trizol and was reverse-transcribed using an iscript cDNA synthesis kit. Real-time polymerase chain reaction (PCR) was performed on an iCycler system using the SYBR green Master Mix. Primer specificity was confirmed by sequencing PCR products. β-actin was the internal control. Data were presented according to the $\Delta\Delta C_t$ method.

Western blot

Frozen liver tissue was homogenized in ice-cold RIPA buffer containing protease and phosphatase inhibitors. A full list of antibodies is available in Supplemental data. Western blot was performed as previously described[17]. Bands were quantified by Scion Image 4.0.3. The loading control was tubulin.

Hydroxyproline content

Hydroxyproline content in liver tissue was determined as previously described[18].

Statistical analysis

Data are expressed as means ± SE. Comparisons were performed using analysis of variance. Least significant difference procedure analyses were performed when > 2 groups were present. $P < 0.05$ was considered statistically significant.

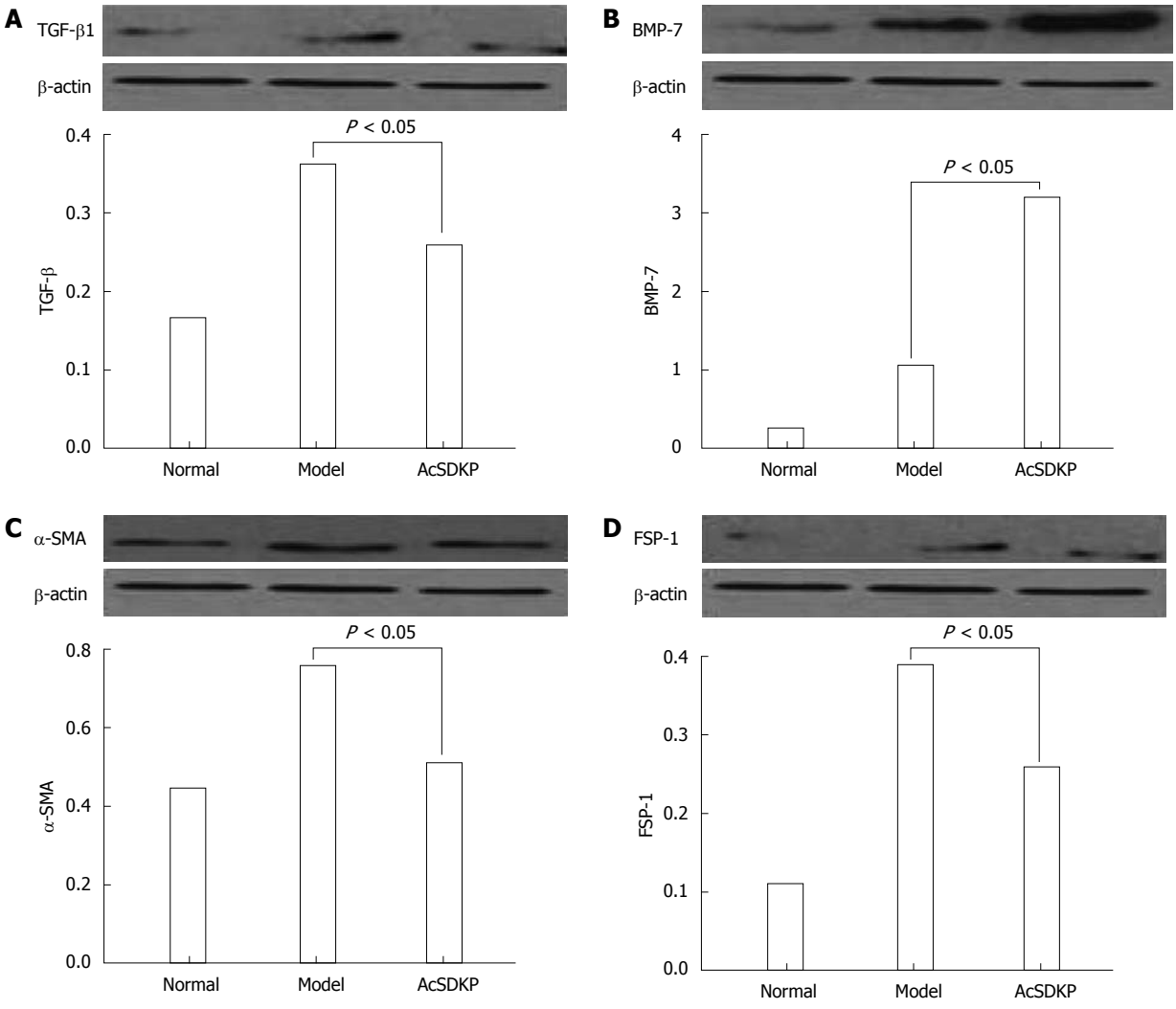

Figure 1 Western blotting and quantitive analysis. A: Transforming growth factor-β1 (TGF-β1); B: Bone morphogenetic protein-7 (BMP-7); C: α-smooth muscle actin positivity (α-SMA); D: Fibroblast specific protein 1 (FSP-1).

RESULTS

Chronic exogenous AcSDKP infusion suppressed profibrogenic TGF-β1 signaling, α-SMA, eibroblast specific protein-1 and bone morphogenetic protein-7 staining and collagen gene expression

When compared to model rats, TGF-β1 was significantly downregulated in AcSDKP-infused BDL-treated rats (Figure 1A). In contrast, bone morphogenetic protein-7 (BMP-7) staining in the liver of BDL-treated rats was increased by AcSDKP (Figure 1B). α-SMA, fibroblast specific protein-1 (FSP-1), collagen I, collagen Ⅲ, tissue inhibitor of metalloproteinase-1 and 2 mRNA all were downregulated by AcSDKP infusion (Figure 1C and D). Collagen Ⅰ, collagen Ⅲ, matrix metalloproteinases-2, tissue inhibitors of metalloproteinase-1 and tissue inhibitors of metalloproteinase-2 mRNA expressions were all significantly downregulated by AcSDKP infusion (2.02 ± 1.10 *vs* 14.16 ± 6.50, 2.02 ± 0.45 *vs* 10.00 ± 3.35, 2.91 ± 0.30 *vs* 7.83 ± 1.10, 4.64 ± 1.25 *vs* 18.52 ± 7.61, 0.46 ± 0.16 *vs* 0.34 ± 0.12, respectively, $P < 0.05$). Matrix metalloproteinase-2 expression was increased in BDL-treated rats but suppressed by AcSDKP.

Chronic exogenous AcSDKP infusion attenuated BDL-induced liver injury, inflammation and fibrosis

BDL caused a remarkable increase in ALT, AST, total bilirubin, and prothrombin time, all of which were reduced by AcSDKP infusion (Table 1). The histological appearance of liver specimens was also improved (Figure 2A-C). Marked collagen accumulation was observed in AcSDKP-infused BDL-treated *vs* model rats, which was attenuated by AcSDKP infusion (Figure 2D-F). The reduction in total collagen was further confirmed by decreased hydroxyproline content. When compared to model rats, hyaluronic acid, ammonia terminal procollagen β peptide and hydroxyproline were all significantly decreased by AcSDKP infusion (127.4 ± 31.8 *vs* 267.2 ± 99.4, 6.9 ± 0.5 *vs* 35.2 ± 4.3, 162.3 ± 42.4 *vs* 398.2 ± 60.4, respectively, $P < 0.05$). Total mast cells decreased in AcSDKP *vs* model BDL-treated rats (Figure 2G-I).

DISCUSSION

Here, we demonstrate that in the liver, chronic exogenous AcSDKP infusion preserves basal levels of AcSDKP and attenuates BDL-induced fibrosis. This is supported by

Table 1 Comparision of liver functions

Group	*n*	ALT (IU/L)	AST (IU/L)	TBIL (μmol/L)	AKP (IU/L)	PT (S)	ALB (g/L)
Normal	10	71.0 ± 32.8	146.0 ± 36.7	1.0 ± 0.8	221.8 ± 96.5	9.3 ± 1.6	38.1 ± 0.9
Model	8	66.9 ± 46.7	472.1 ± 236[a]	56.4 ± 53.9[a]	299.1 ± 37.4[a]	16.6 ± 4.6[a]	29.7 ± 9.5[a]
BDL + AcSDK	8	70.0 ± 34.9	245.7 ± 92.8[c]	6.3 ± 3.3[c]	265.4 ± 77.5	11.8 ± 1.0[c]	34.0 ± 4.9

[a]$P < 0.05$ *vs* normal; [c]$P < 0.05$ *vs* model. BDL: Bile duct ligation; AcSDK: N-acetyl-seryl-aspartyl-lysyl-proline; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin; AKP: Alkaline phosphatase; PT: Prothrombin time; ALB: Albumin.

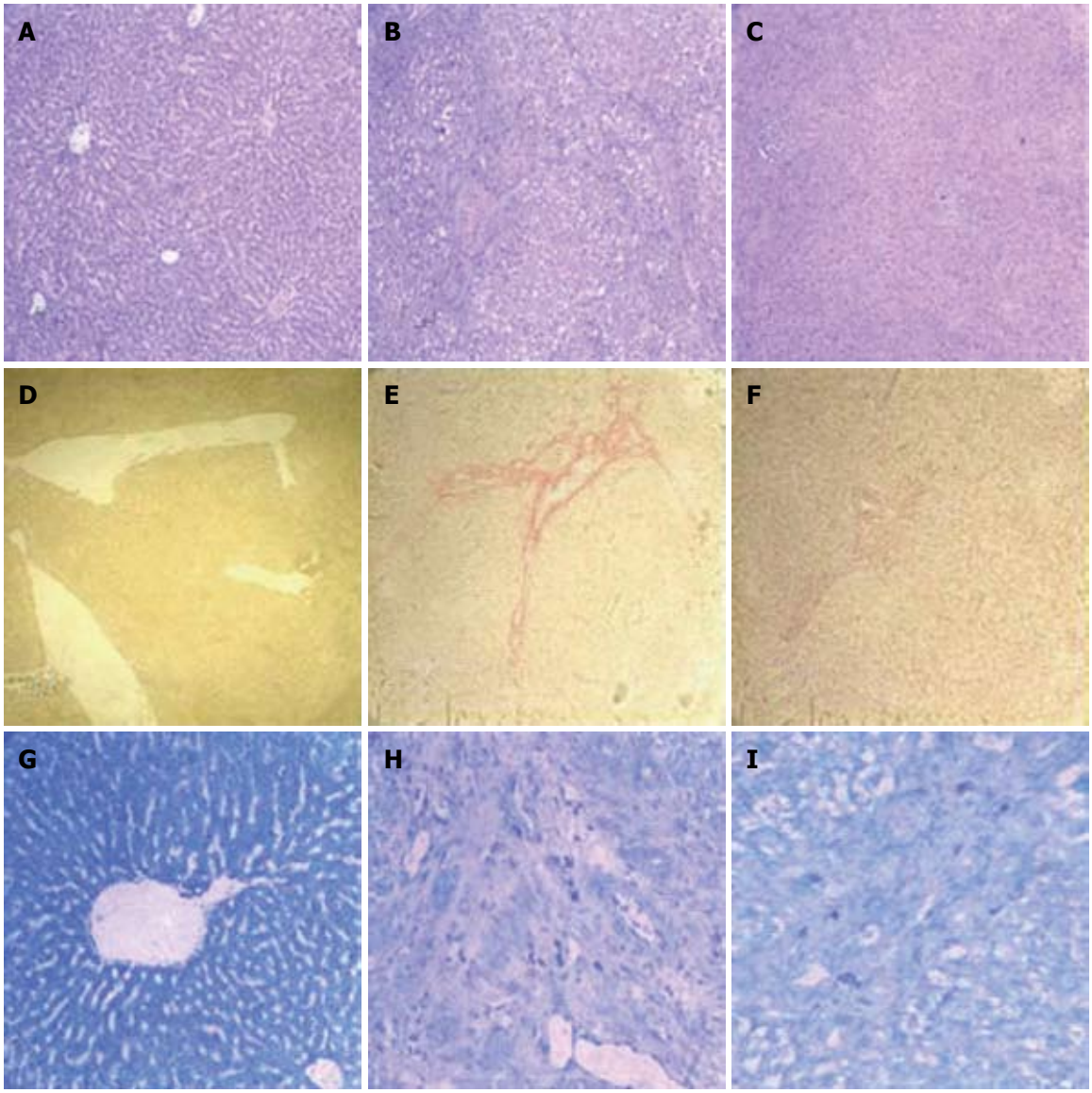

Figure 2 Hematoxylin and eosin, Sirius red, Giemsa staining for liver tissues in each group. A: Normal, hematoxylin and eosin (HE) (× 100); B: Model, HE (× 100); C: N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP), HE (× 100); D: Normal, Sirius red (× 100); E: Model, Sirius red (×100); F: AcSDKP, Sirius red (× 100); G: Normal, Giemsa (× 200); H: Model, Giemsa (× 200); I: AcSDKP, Giemsa (× 200).

other studies showing an important role of AcSDKP in preventing heart[19,20] and kidney[21,22] fibrosis at basal concentrations. Our previous studies had also revealed that AcSDKP ameliorated CCl_4-induced liver fibrosis and liver functions in rats.

Attenuation of liver fibrosis by AcSDKP is associated with suppressed inflammation and TGF-β signaling. Our results show that AcSDKP suppressed mast cells infiltration, TGF-β1 signaling and myofibroblasts *in vivo*.

Recent evidence suggests that epithelial-to-mesenchymal transition (EMT) may also contribute to liver fibrogenesis[23]. TGF-β1 is still generally considered to be the main positive regulator of EMT and ECM accumulation[23]. Indeed, our results show that AcSDKP suppressed TGF-β signaling and reduced the EMT markers α-SMA and FSP-1 *in vivo*[24,25]. In addition, AcSDKP increased

BMP-7. BMP-7 counteracts the effects of TGF-β1 and is a prototypical negative regulator of EMT. Nevertheless, a more sophisticated study is required to fully elucidate the possible role of AcSDKP-induced inhibition of EMT in the attenuation of liver fibrosis.

There are some limitations in this study. We did not include a group of control rats infused with AcSDKP primarily because exogenous infusion of AcSDKP restored the peptide levels to control levels and this dose has been shown to have no adverse effects systemically. Secondly, the cellular mechanisms of AcSDKP action were not fully elucidated in our current study. The presence of an AcSDKP receptor on cells has been suggested[26]. We speculate that AcSDKP may directly affect liver cells by binding and activating its receptor on the cell surface, resulting in suppression of certain profibrogenic intracellular signaling pathways. Further studies to clone the receptor or develop specific receptor antagonists will enable full characterization of the cellular mechanisms involved in the antifibrotic effects of AcSDKP *in vivo* and *in vitro*.

In summary, this study shows that chronic exogenous AcSDKP infusion preserves basal levels of AcSDKP and attenuates liver fibrosis induced by BDL in rats. Our study strongly suggests a significant role for AcSDKP in the development of liver fibrosis and potentiates the usefulness of this tetrapeptide in the prevention of this disease. Additional studies are needed to gain further insight into the biological effect of AcSDKP in the liver and further studies are ultimately warranted in the human.

COMMENTS

Background

N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) is an endogenous tetrapeptide *in vivo* which has antifibrogenic effects on the heart, the lung and the kidney. The authors' previous studies had revealed that AcSDKP ameliorated carbon tetrachloride-induced liver fibrosis and liver functions in the rat liver.

Research frontiers

Originally described as a natural inhibitor of hematopoietic stem cell proliferation, AcSDKP is now recognized as a critical negative regulator for extracellular matrix accumulation in organs under both physiological and pathological conditions.

Innovations and breakthroughs

This is the first study to investigate the preventive effect of endogenous AcSDKP in bile duct ligation (BDL)-induced fibrosis in the rat liver and the potential mechanisms involved were also examined. The results strongly suggest a significant role for AcSDKP in the development of liver fibrosis and potentiates the usefulness of this tetrapeptide in the prevention of this disease.

Applications

Preservation of AcSDKP may be a useful therapeutic approach in the management of liver fibrosis.

Peer review

This is a good descriptive study in which authors analyze the preventive effect of AcSDKP on BDL-induced liver fibrosis in rats. The results are interesting and suggest that infusion of exogenous AcSDKP attenuated BDL-induced fibrosis in the rat liver. Preservation of AcSDKP may be a useful therapeutic approach in the management of liver fibrosis.

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BRIEF ARTICLE

Minilaparotomy to rectal cancer has higher overall survival rate and earlier short-term recovery

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Abstract

AIM: To report our experience using mini-laparotomy for the resection of rectal cancer using the total mesorectal excision (TME) technique.

METHODS: Consecutive patients with rectal cancer who underwent anal-colorectal surgery at the authors' hospital between March 2001 and June 2009 were included. In total, 1415 patients were included in the study. The cases were divided into two surgical procedure groups (traditional open laparotomy or mini-laparotomy). The mini-laparotomy group was defined as having an incision length ≤ 12 cm. Every patient underwent the TME technique with a standard operation performed by the same clinical team. The multimodal preoperative evaluation system and postoperative fast track were used. To assess the short-term outcomes, data on the postoperative complications and recovery functions of these cases were collected and analysed. The study included a plan for patient follow-up, to obtain the long-term outcomes related to 5-year survival and local recurrence.

RESULTS: The mini-laparotomy group had 410 patients, and 1015 cases underwent traditional laparotomy. There were no differences in baseline characteristics between the two surgical procedure groups. The overall 5-year survival rate was not different between the mini-laparotomy and traditional laparotomy groups (80.6% *vs* 79.4%, $P = 0.333$), nor was the 5-year local recurrence (1.4% *vs* 1.5%, $P = 0.544$). However, 1-year mortality was decreased in the mini-laparotomy group compared with the traditional laparotomy group (0% *vs* 4.2%, $P < 0.0001$). Overall 1-year survival rates were 100% for Stage Ⅰ, 98.4% for Stage Ⅱ, 97.1% for Stage Ⅲ, and 86.6% for Stage Ⅳ. Local recurrence did not differ between the surgical groups at 1 or 5 years. Local recurrence at 1 year was 0.5% (2 cases) for mini-laparotomy and 0.5% (5 cases) for traditional laparotomy ($P = 0.670$). Local recurrence at 5 years was 1.5% (6 cases) for mini-laparotomy and 1.4% (14 cases) for traditional laparotomy ($P = 0.544$). Days to first ambulation (3.2 ± 0.8 d *vs* 3.9 ± 2.3 d, $P = 0.000$) and passing of gas (3.5 ± 1.1 d *vs* 4.3 ± 1.8 d, $P = 0.000$), length of hospital stay (6.4 ± 1.5 d *vs* 9.7 ± 2.2 d, $P = 0.000$), anastomotic leakage (0.5% *vs* 4.8%, $P = 0.000$), and intestinal obstruction (2.2% *vs* 7.3%, $P = 0.000$) were decreased in the mini-laparotomy group compared with the traditional laparotomy group. The results for other postoperative recovery function indicators, such as days to oral feeding and defecation, were similar, as were the results for immediate postoperative complications, including the physiologic and operative severity score for the enumeration of mortality and morbidity score.

CONCLUSION: Mini-laparotomy, as conducted in a single-centre series with experienced TME surgeons, is a safe and effective new approach for minimally invasive rectal cancer surgery. Further evaluation is required to evaluate the use of this approach in a larger patient sample and by other surgical teams.

Key words: Rectal neoplasm; Mini-laparotomy; Survival; Total mesorectal excision

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INTRODUCTION

Laparoscopic surgery has become popular throughout the world[1-3]. This surgical method enhances early postoperative recovery with less pain, less analgesic use, an earlier return of gastrointestinal function, and fewer wound and pulmonary complications. Total mesorectal excision (TME) is acknowledged worldwide as the preferred technique for surgical resection of rectal cancer[4-6]. Multiple studies have reported marked reductions in local recurrence with the TME technique, including single-centre, multiple-centre, and population studies[7,8]. The laparoscopic approach to TME resection of rectal cancer is currently being evaluated in multicentre randomised trials[9-12].

A less-recognised surgical technique aimed at improving postoperative recovery is mini-laparotomy. With this technique, surgical dissection is performed under direct vision, as in open surgery; laparoscopic equipment and training is not required. Early experience with mini-laparotomy has been reported from a few medical centres in case series of colon and rectal resection[13-15]. Mini-laparotomy has been developed as a techniques based on the advanced recognition of more information about pelvic anatomy and the dissection of subtle perirectal structures in laparotomy[16,17].

In view of these circumstances, our surgical centre has performed mini-laparotomies for rectal cancer for approximately 8 years. The aim of this study is to report our experience using mini-laparotomy for the resection of rectal cancer using the TME technique. Furthermore, we aim to compare the oncologic findings and the postoperative recovery indexes of mini-laparotomy and traditional laparotomy, thus providing more evidence to help surgeons select an operating procedure.

MATERIALS AND METHODS

Included cases

This study is registered as an International Clinical Trial (ChiCTR-TRC-09000618) to compare TME resection of rectal cancer using traditional open laparotomy *vs* mini-laparotomy. This is a retrospective analysis of consecutive patients with rectal cancer observed at the Anal-colorectal Surgery Ward in West China Hospital of Sichuan University between March 2001 and June 2009. The inclusion criteria were as follows: (1) diagnosis of rectal cancer; (2) no previous history of lower abdominal operations or pelvic operations; (3) possibility of curative resection; and (4) intestinal continuity was restored by anastomosis. The exclusion criteria were (1) curative resection was not achieved; (2) resections without anastomosis (APR and Hartmann); and (3) actively exciting the study. All of the enrolled patients provided informed consent, which included information about (1) the different kinds of treatment available for their cancer; (2) the benefit of different operation procedures; and (3) their doctor's recommendation. Ultimately, the choice of surgical technique was left to the patient. The database from the anal-colorectal surgery of West China Hospital in Sichuan University provided the research data[18]. If any data required for the study were missing, the patient was excluded. Most of the patients who were excluded for this reason were missing data related to pathology and surgical baselines; 5-year survival and local recurrence; and the first time of aerofluxus, defecation, ambulation, oral feeding during the recovery phase. Ultimately, 1415 patients were included in the study.

A multimodal preoperative evaluation system was used to assess the preoperative clinical cancer stage[19]. Clinical Stages Ⅲ and Ⅳ patients were treated with neoadjuvant and adjuvant chemotherapy consisting of FOLFOX-4 (Oxaliplatin 85 mg/m^2 ivgtt 2 h, 1 d; LV 200 mg/m^2 ivgtt 2 h, 1-2 d; 5-Fu 400 mg/m^2 *iv*, 1-2 d). Perioperative radiation is not used at our centre.

Surgery was performed by traditional open laparotomy as the standard procedure. Mini-laparotomy was performed on an ad-hoc basis, with increasing frequency in the latter years of this study. No patient within the mini-laparotomy group was converted to a traditional laparotomy.

Short-term perioperative data were obtained in all cases. Long-term follow-up data were available from 7 to 103 mo. The follow-up methods used included telephone follow-up, outpatient department follow-up and follow-up letters. Follow-up data were obtained in 96.3% of cases (1362/1415).

Operation and clinical management procedures

TME and pelvic autonomic nerve preservation were performed in all cases in accordance with the Colorectal Surgery Guideline of West China Hospital of Sichuan University[20]. The surgery and perioperative management were performed by the same clinical team for both the traditional open laparotomy and mini-laparotomy groups.

A vertical incision was used for all cases. The traditional laparotomy incision extended from the pubis to above the umbilicus with a length of 13 to 18 cm, as in Figure 1. The mini-laparotomy incision extended from

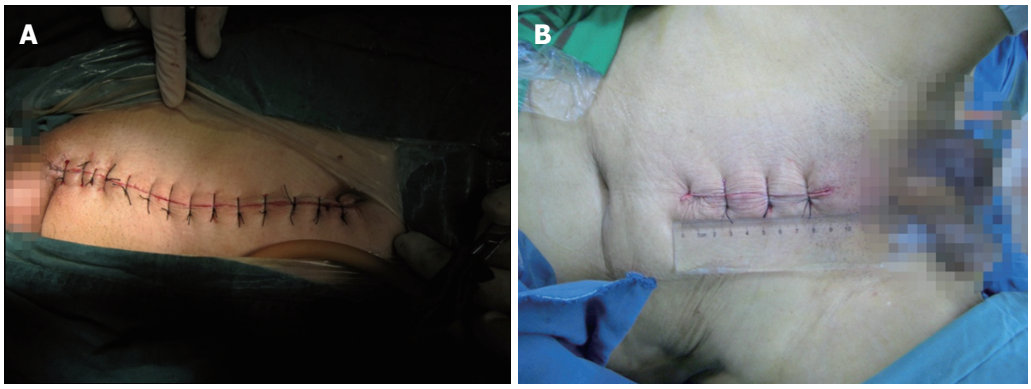

Figure 1 Mini-laparotomy (A) and traditional laparotomy (B).

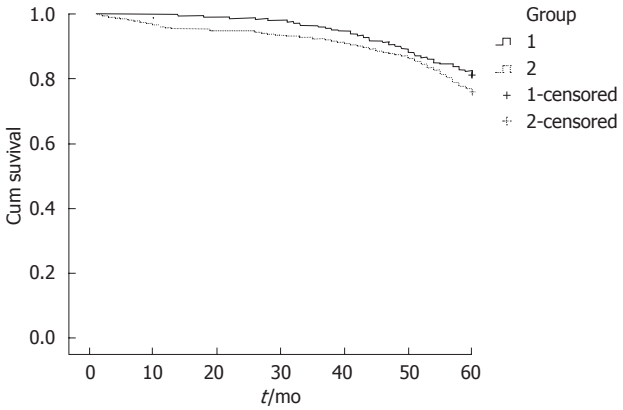

Figure 2 Survival rates of the mini-laparotomy (1) and traditional laparotomy groups (2).

the pubis for a length of 12 cm or less. For the traditional laparotomy, a fixed self-retaining retractor was used on the incision, and a moveable curved retractor was used for dynamic exposure during dissection. In mini-laparotomy, two curved retractors were used in dynamic exposure during dissection without a fixed abdominal wound retractor. Dissection was performed using electrocautery and an ultrasonic knife in both open laparotomy and mini-laparotomy. In both mini-laparotomy and traditional laparotomy, the splenic flexure was not mobilised. The superior rectal artery was ligated just below the bifurcation of the inferior mesenteric artery with clearing of the superior rectal artery lymph nodes.

A post-operative fast-track protocol was used[21,22] with early ambulation. Discharge criteria included eating a normal diet, normal ambulation, no fever, and no post-operative complications.

Definition of outcome

The primary outcome variables are 5-year survival and local recurrence. Local recurrence was classified as intro-/peri-anastomotic and pelvic recurrence. Secondary outcomes are immediate postoperative complications, including physiologic and operative severity score for the enumeration of mortality and morbidity (POSSUM) score[23], and recovery functions (the time of the first aerofluxus, defecation, ambulation, and oral feeding during the recovery phase). In addition, the patients' postoperative complications were recorded as important information about secondary outcomes, which included gastric retention, incision infection, pulmonary infection, anastomosis leakage, and intestinal obstruction.

Statistical analysis

The mini-laparotomy and traditional laparotomy data were compared and analysed with *t* tests, and the count data used the χ^2 test or Fisher's exact probability test. Local recurrence and overall survival were assessed using Kaplan-Meier survival curve analysis. The data are expressed as means ± SD. The significance level was 0.05. Statistical tests were performed using SPSS 15.0.

RESULTS

Baseline characteristics

The characteristics of the 1415 included patients (410 mini-laparotomy cases and 1005 traditional laparotomy cases) are presented in Table 1. There were no significant difference in the baseline data for the two groups ($P > 0.05$). The surgical and pathological findings are presented in Table 2, and there were no significant differences between the two surgical groups ($P > 0.05$).

Overall survival and local recurrence

Overall 5-year survival did not differ between the mini-laparotomy and traditional laparotomy groups (80.6% *vs* 79.4%, $P = 0.333$; Figure 2). The 5-year survival rates for the different clinical stages were also similar. One-year mortality was decreased in the mini-laparotomy group compared to the traditional laparotomy group (0% *vs* 4.2%, $P < 0.0001$). The overall 1-year survival rates in the traditional group were 100% for Stage Ⅰ, 98.4% for Stage Ⅱ, 97.1% for Stage Ⅲ, and 86.6% for Stage Ⅳ. Local recurrence did note differ between surgical groups at 1 or 5 years. Local recurrence at 1 year was 0.5% (2 cases) for the mini-laparotomy group and 0.5% (5 cases) for the traditional laparotomy group ($P = 0.670$). Local recurrence at 5 years was 1.5% (6 cases) for the mini-laparotomy group and 1.4% (14 cases) for the traditional laparotomy group ($P = 0.544$).

Table 1 Baseline characteristics of the mini-laparotomy and traditional laparotomy groups

	Minilaparotomy (n = 410)	Traditional laparotomy (n = 1005)	P value
Gender			0.125
Male	273 (66.6)	635 (63.2)	
Female	137 (33.4)	370 (36.8)	
Age (yr)	61.2 ± 12.1	57.8 ± 12.4	0.385
BMI (kg/m^2)	21.3 ± 3.0	22.0 ± 2.9	0.331
Distance to dentate line (cm)	8.2 ± 3.2	7.2 ± 4.0	0.118

BMI: Body mass index.

Table 2 Surgical and pathological findings for the mini-laparotomy and traditional laparotomy groups

	Minilaparotomy	Traditional laparotomy	P value
TNM stage			0.838
Stage Ⅰ	47 (11.5)	119 (11.8)	
Stage Ⅱ	127 (31.0)	320 (31.8)	
Stage Ⅲ	151 (36.8)	379 (37.7)	
Stage Ⅳ	85 (20.7)	187 (18.6)	
Differentiation			0.579
Good	105 (25.6)	240 (23.9)	
Moderate	172 (42.0)	411 (40.9)	
Poor	133 (32.4)	354 (35.2)	
Histologic types			0.277
Adenocarcinoma	337	811	
Mucinous adenocacinoma	69	164	
Squamous carcinoma	4	30	
Operation types			0.640
High anterior resection	20 (4.9)	38 (3.8)	
Low anterior resection	124 (30.2)	284 (28.3)	
Ultralow anterior resection	189 (46.1)	482 (48.0)	
Colo-anal anastomosis	77 (18.8)	201 (20.0)	
Volume of bleeding (mL)	78.5 ± 30.0	80.8 ± 28.5	0.940
Operation time (min)	115.5 ± 35.8	114.6 ± 33.4	0.217
Lymph node counts	12.4	12.7	0.796
Proximal margin of distance (cm)	3.5	3.3	0.105
Distal margin of distance (cm)	7.0	6.9	0.780

TNM: Tumor-node-metastasis. Data are presented as n (%) or mean ± SD.

Short-term recovery and postoperative complications

The data regarding postoperative recovery functions (time of first aerofluxus, defecation, ambulation and oral feeding), length of hospital stay, and immediate postoperative complications, including POSSUM score, are shown in Table 3. Days to first ambulation and aerofluxus and hospital length of stay were reduced for the mini-laparotomy group compared with the traditional laparotomy group. Days to tolerating full oral diet and first defecation were similar between surgical groups. POSSUM scores predicting mortality and morbidity were not different between the surgical groups.

Gastric retention and wound and pulmonary infection were not different between the surgical groups. Anastomotic leakage and intestinal obstruction were decreased in the mini-laparotomy group compared with the traditional laparotomy group. Other postoperative complications,

Table 3 Postoperative recovery of the two groups

	Minilaparotomy group	Traditional laparotomy group	P value
Recovery			
Aerofluxus (d)	3.5 ± 1.1	4.3 ± 1.8	0.000
Oral feeding (d)	4.1 ± 1.2	4.6 ± 1.2	0.628
Defecation (d)	5.0 ± 1.4	5.4 ± 1.5	0.370
Ambulation (d)	3.2 ± 0.8	3.9 ± 2.3	0.000
Hospital stay (d)	6.4 ± 1.5	9.7 ± 2.2	0.000
POSSUM scores (%)			
Predictive mortality	28.1	26.8	0.738
Predictive morbidity	5.3	5.0	0.844
Complications			
Gastric retention	8 (2.0)	18 (1.8)	0.494
Incision infection	6 (1.5)	15 (1.5)	0.592
Pulmonary infection	5 (1.2)	14 (1.4)	0.513
Anastomosis leakage	2 (0.5)	48 (4.8)	0.000
Intestinal obstruction	9 (2.2)	73 (7.3)	0.000
Other	13 (3.2)	35 (3.5)	0.456

POSSUM: Physiologic and operative severity score for the enumeration of mortality and morbidity.

Table 4 Colorectal cancer surgery study results regarding local recurrence and survival rates (%)

Another preference	Local recurrence	Survival rate
Our study	1.4	79.7
Andreoni *et al*[30]	8.2	71.0
Law *et al*[31]	9.6	66.5
Jung *et al*[7]	8.0	62.0

including urinary retention (2 cases *vs* 6 cases), urinary tract infection (0 case *vs* 2 cases), anastomotic bleeding (1 case *vs* 2 cases), intra-abdominal haemorrhage (0 case *vs* 1 case), wound dehiscence (1 case *vs* 2 cases), sexual dysfunction (1 case *vs* 3 cases), deep vein thrombosis (0 case *vs* 0 case), cardiocerebral vascular accident (0 case *vs* 0 case), psychosis (2 cases *vs* 4 cases), liver dysfunction (1 case *vs* 2 cases), and unknown fever (5 cases *vs* 13 cases) also did not differ between the two surgical groups.

DISCUSSION

The overall 5-year survival and local recurrence rates were not different between the mini-laparotomy and traditional laparotomy groups. The local recurrence and survival rates for mini-laparotomy (1.4% and 79.7%) compare favourably to those reported for traditional laparotomy, as shown in Table 4.

In our study, the results confirmed that the mini-laparotomy and traditional laparotomy groups had similar overall 5-year survival and local recurrence rates, but the minilaparotomy group experienced faster postoperative recovery and fewer complications. It may be that mini-laparotomy surgery is the safer and more effective operation for rectal cancer.

An improved understanding of pelvic anatomy has enabled the switch to a mini-laparotomy approach to rec-

tal cancer resection. Whereas others have reported on the use of mini-laparotomy mostly for colon cancer, we are among the first to report on the use of mini-laparotomy for the resection of rectal cancer[8,9,12]. There is a learning curve for the mini-laparotomy approach. However, we speculate that the learning curve is lower than that of laparoscopic proctectomy because standard instrumentation, direct vision and tactile feedback are maintained for mini-laparotomy, but not for laparoscopy. We have used mini-laparotomy for rectal resection in Chinese patients with a body mass index (BMI) of 21.3 kg/m^2 as the Chinese population is generally thinner than North American and European populations[24]; however, we also treat patients with BMIs > 27 using mini-laparotomy. Additionally, although splenic flexure mobilisation was not required for any patient in this sample, it is likely that the surgeon would be disadvantaged by the suprapubic mini-laparotomy approach if splenic flexure mobilisation was required to perform a low tension-free anastomosis. Mini-laparotomy was not associated with an increase in operative blood loss or operation time. In fact, mini-laparotomy was associated with the successful resection of all levels of rectal cancer.

Like laparoscopic colorectal resection[25], mini-laparotomy showed advantages in decreasing the postoperative length of hospital stay and was associated with shorter times to ambulation and aerofluxus[1,11]. Mini-laparotomy was associated with significantly decreased anastomotic leaking, a finding for which we have no ready explanation other than increased experience over the time of the study. The anastomotic leak rate of 0.5% compares favourably to that reported in other studies[26-28]. In addition, mini-laparotomy was associated with decreased postoperative intestinal obstruction, which may have resulted from less peritoneal manipulation and a shorter incision length. A Japanese study suggested that the mini-laparotomy approach in colorectal cancer would result in a reduced inflammatory response[29]. Furthermore, mortality at 1 year was significantly increased in the traditional laparotomy group compared with the mini-laparotomy group.

Although postoperative management was intended to be similar for mini-laparotomy and traditional laparotomy patients, as indicated by the time before achieving a full oral diet and defecation, traditional laparotomy patients were slower to ambulate. Bias in favour of the mini-laparotomy group is likely, as this procedure has gained favour in our hands with our increasing experience over the time of this study. A randomised study design is indicated to minimise this bias. The study was a single-centre series with experienced TME surgeons and a patient sample with a relatively small BMI. Because our hospital is a medical centre in southwestern China, our patients are primarily advanced cancer cases, and we have more experienced surgeons and more advanced surgical instruments than other small-to-medium sized local hospitals. Consequently, the study and results had an obvious selection bias. Further evaluation is required to evaluate the use of this approach in a larger patient sample and by other surgical teams, and our conclusions are not definitive.

In conclusion, we have shown that mini-laparotomy is a safe and effective new approach for minimally invasive rectal cancer surgery. This was a single-centre series with experienced TME surgeons and a patient sample with a relatively low BMI. Further evaluation is required to evaluate the use of this approach in a larger patient sample and by other surgical teams.

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COMMENTS

Background

Total mesorectal excision (TME) is acknowledged worldwide as the preferred technique for surgical resection of rectal cancer. The laparoscopic approach to TME resection of rectal cancer is currently being evaluated in multicentre randomised trials. A less-recognised surgical technique aimed at improving postoperative recovery is mini-laparotomy. With this technique, surgical dissection is performed under direct vision, as in open surgery; laparoscopic equipment and training is not required. Early experience with mini-laparotomy has been reported from a few medical centres in case series of colon and rectal resection. Mini-laparotomy has been developed as a techniques based on the advanced recognition of more information about pelvic anatomy and the dissection of subtle perirectal structures in laparotomy.

Research frontiers

An improved understanding of pelvic anatomy has enabled the switch to a mini-laparotomy approach to rectal cancer resection. Other studies have reported on the use of mini-laparotomy mostly for colon cancer, but for rectal cancer, it is still in blank. Like laparoscopic colorectal resection, mini-laparotomy showed advantages in decreasing the postoperative length of hospital stay and was associated with shorter times to ambulation and aerofluxus in past studies

Innovations and breakthroughs

Whereas others have reported on the use of mini-laparotomy mostly for colon cancer, the authors are among the first to report on the use of mini-laparotomy for the resection of rectal cancer. There is a learning curve for the mini-laparotomy approach. However, the authors speculate that the learning curve is lower than that of laparoscopic proctectomy because standard instrumentation, direct vision and tactile feedback are maintained for mini-laparotomy, but not for laparoscopy. Mini-laparotomy was associated with significantly decreased anastomotic leaking, a finding for which the authors have no ready explanation other than increased experience over the time of the study. The anastomotic leak rate of 0.5% compares favourably to that reported in other studies. In addition, mini-laparotomy was associated with decreased postoperative intestinal obstruction, which may have resulted from less peritoneal manipulation and a shorter incision length. Furthermore, mortality at 1 year was significantly increased in the traditional laparotomy group compared with the mini-laparotomy group.

Applications

The study results suggest that mini-laparotomy is a safe and effective new approach for minimally invasive rectal cancer surgery.

Terminology

Minilaparotomy is minimal invasive laparotomy for abdominal operations. It is different from laparoscopic operations, that minilaparotomy will have only one mini-incision, but not other holes from laparoscopic way. Minilaparotomy would be more better outcomes than traditional laparotomy.

Peer review

This is a good descriptive study conducted in a single-centre series with experienced TME surgeons, and mini-laparotomy is proved a safe and effective new approach for minimally invasive rectal cancer surgery. Minilaparotomy will be the future choice for rectal cancer care.

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BRIEF ARTICLE

Evaluation of a new method for placing nasojejunal feeding tubes

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Abstract

AIM: To compare fluoroscopic, endoscopic and guide wire assistance with ultraslim gastroscopy for placement of nasojejunal feeding tubes.

METHODS: The information regarding nasojejunal tube placement procedures was retrieved using the gastrointestinal tract database at Tongji Hospital affiliated to Tongji Medical College. Records from 81 patients who underwent nasojejunal tubes placement by different techniques between 2004 and 2011 were reviewed for procedure success and tube-related outcomes.

RESULTS: Nasojejunal feeding tubes were successfully placed in 78 (96.3%) of 81 patients. The success rate by fluoroscopy was 92% (23 of 25), by endoscopic technique 96.3% (26 of 27), and by guide wire assistance (whether *via* transnasal or transoral insertion) 100% (23/23, 6/6). The average time for successful placement was 14.9 ± 2.9 min for fluoroscopic placement, 14.8 ± 4.9 min for endoscopic placement, 11.1 ± 2.2 min for guide wire assistance with transnasal gastroscopic placement, and 14.7 ± 1.2 min for transoral gastroscopic placement. Statistically, the duration for the third method was significantly different ($P < 0.05$) compared with the other three methods. Transnasal placement over a guidewire was significantly faster ($P < 0.05$) than any of the other approaches.

CONCLUSION: Guide wire assistance with transnasal insertion of nasojejunal feeding tubes represents a safe, quick and effective method for providing enteral nutrition.

Key words: Enteral nutrition; Nasojejunal feeding tube; Guide wire assistance; Fluoroscopy; Endoscopy

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INTRODUCTION

Enteral nutrition (EN) not only provides energy support as with parenteral nutrition, but also maintains the functional intestinal barrier, significantly reducing the incidence of infection and organ failure, shortens hospital

stays, and lowers treatment costs[1-4]. EN has therefore become an important nutritional therapy[5]. A nasogastric (NG) tube is often associated with some problems of large gastric residual volumes, reflux and vomiting, while a nasojejunal (NJ) tube and prokinetic agents are useful for circumventing the problems associated with upper gastrointestinal intolerance of NG feeding[6]. NJ feeding tubes positioned beyond the ligament of Treitz's have been shown to allow early attainment of caloric needs and a reduction in tube-feeding aspiration events in patients with gastric feeding intolerance[7,8]. There are presently several methods for placement of NJ feeding tubes[9-11]. Previously, we would use fluoroscopic placement under direct endoscopic visualization instead of using NJ tubes.

Recently, we have applied an ultrathin transnasal endoscope which afforded us a higher success rate and a shortened procedure time. Herein we evaluated the usefulness and safety of this new method compared with the other two traditional methods.

MATERIALS AND METHODS

Patients

This is a retrospective study involving the patients who were treated with enteral feeding from January 2004 through September 2011 at our hospital. Written informed consent was obtained from all patients or their representatives. The Ethics Committee of Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology, approved the study protocol. All subjects were $\geqslant$ 18 years of age. The technique, success rate, procedure duration, and complications were recorded for each patient. Patient sex, age and diseases were also recorded. According to the placement methods, the patients were divided into three groups as described below. A 130-cm long polyurethane nasoenteral feeding tube with a front-end opening (Flocare, Nutricia, Netherlands) was used in each case. All NG tubes were removed before the start of the procedure, as they might have interfered with placement of both the feeding tube and endoscope.

Fluoroscopic technique

The feeding tube was placed by one skilled doctor. Some gastroenterologists were also involved in the fluoroscopy. Additional sedation was not required for fluoroscopic tube placement. A portable C-arm fluoroscope was positioned over the supine patient's abdomen. The timing of the procedure began when the feeding tube entered the nares. When the tube was advanced to 50-55 cm, its position was evaluated by intermittent fluoroscopy. The operators rotated the feeding tube to facilitate passage to the pylorus. Fluoroscopy was used intermittently or continuously as needed. When the tip of the feeding tube was beyond the pylorus, it was gently advanced as far as possible. Placement of the tube beyond the third portion of the duodenum was preferred. Finally, a fluoroscopic print was obtained after 10-15 mL of meglumine diatrizoate was injected into the feeding tube.

Endoscopic technique

All feeding tubes were placed at the Endoscopy Center by one skilled endoscopist. The posterior oropharynx was anesthetized with topical 4% Xylocaine. The timing of the procedure began when the feeding tube entered the nares. The lubricated feeding tube was inserted into the stomach and advanced until resistance was encountered (usually 55-60 cm), and the wire stylet was left in place. At this point, a standard forward-viewing endoscope (Olympus GIF 240 or 260, Olympus Corporation, New York, NY, United States) was placed into the esophagus and then the stomach. The stomach was insufflated with air, and the feeding tube usually traveled along the greater curvature of the stomach. The feeding tube was advanced to the nasopharynx. The distal 10-20 cm of the feeding tube was grasped by the biopsy forceps, and then the tip of the catheter was directed into the pylorus under endoscopic visualization. The feeding tube was then advanced at the nasopharynx, its distal 10-20 cm grasped with biopsy forceps and the tip directed through the pylorus under direct vision. When the feeding tube was observed in a good position, the endoscope was carefully withdrawn with the feeding tube secured by the forceps, which was advanced along with the withdrawal of the endoscope. When the forceps could no longer advance, the endoscope movement was stopped and the forceps was gently pulled back to the end of the lens. Then the forceps was again used to grasp the feeding tube and the above process was repeated until the endoscope was removed completely from the throat. The wire stylet was removed and a fluoroscopic print was obtained after 10-15 mL meglumine diatrizoate was injected into the feeding tube to confirm placement of the feeding tube into the second or third portion of the duodenum.

Guide wire technique

Topical Xylocaine was sprayed into the nose and retropharynx in conscious patients. The tip of the ultraslim transnasal endoscope with an outer diameter of 5.0 mm (Olympus XP-260N, Olympus Corporation, New York city, NY) was then passed under direct vision into one of the nasal passages. Extreme care was taken to avoid traumatizing the mucous membranes. After the endoscope arrived at the third portion of the duodenum, a 260-cm long guide wire with a soft tip (Zebra Exchange Guidewire, Boston Scientific, United States) was inserted along the endoscopic biopsy channel. Using a pull-push technique, the endoscope was slowly withdrawn while the wire was simultaneously threaded forward, so that the wire stayed in a fixed position in the intestine. Before exiting the stomach, the path made by the wire was studied and adjusted to ensure that there were no coils or loops within the gastric body. After withdrawal of the endoscope, an open-ended feeding tube was lubricated and passed over the guide wire, ensuring that the wire remained taut and

Table 1 Patient characteristics

Patient characteristics	Fluoroscopic placement	Endoscopic placement	Guide wire placement
Age (yr), mean ± SD	54.4 ± 9.9	55.8 ± 9.7	56.2 ± 9.5
Gender			
Men	15	14	16
Women	10	13	13
Primary diagnoses			
Pancreatitis	25	14	8
Postoperative gastric cancer	0	6	10
Postoperative esophageal cancer	0	4	6
Abdominal injury	0	1	2
Pancreatic cancer after Whipple surgery	0	1	1
Thoracic esophageal fistula	0	1	1
Gastric perforation	0	0	1

in place. Care was taken not to over-advance the tubes because this often results in coiling in the stomach and loss of duodenal access. Finally, using the adjacent naris, the endoscope was reintroduced into the proximal stomach to check final placement. In most cases, the hub of the wider gastric aspiration tube was too short and had to be advanced gently into the antrum visually, making sure that the tube remained straight along the greater curvature and that the jejunal extension slid further through the pylorus. With an assistant securing the feeding tube to prevent displacement, the endoscope was then eased back into the apex of the body to check the final position before exiting the esophagus. Transnasal endoscopy was not feasible in patients with congestion or stenosis of the nasal passageway. Conventional per-oral endoscopy was used to place the guide wire, which consequently ended up emerging from the mouth. The wire was then redirected through the nose by nasopharyngo-oral cannulation with a small 2-mm internal diameter flexible tube, allowing final placement of the NJ feeding tube.

Statistical analysis

All data were presented as the mean ± SD. The SPSS 15.0 software package (SPSS, Inc., United States) was used for all statistical analyses. Differences between and among outcome groups were determined using the χ^2 test. Significance was determined at P <0.05.

RESULTS

Patient characteristics

Demographic data of the included patients are shown in Table 1. The mean age was 55.5 years (range: 24-70 years). There were 45 men and 36 women. Common primary diagnoses were pancreatitis, postoperative gastric cancer, postoperative esophageal cancer, abdominal injury, pancreatic cancer after Whipple surgery, thoracic esophageal fistula and gastric perforation. All patients demonstrated either high gastric residuals on attempted NG feeding or a physiologic requirement for postduodenal enteral feedings (i.e., pancreatitis), or they were believed to be at high risk for gastric aspiration.

Table 2 Outcome data of the patients

Variables	Fluoroscopic placement	Endoscopic placement	Guide wire placement	
			Transnasal	Transoral
Time to complete procedure (min)	14.9 ± 5.8	14.8 ± 4.9	11.1 ± 2.2[a]	14.7 ± 1.2
Successful placement	23/25 (92)	26/27 (96.3)	23/23 (100)	6/6 (100)
Complications	0/25 (0)	4/27 (14.8)	0/23 (0)	0/6 (0)

Data are presented as mean ± SD or *n/N* (%). [a]P < 0.05 *vs* the other three groups.

Patient outcomes

Outcome data of the patients are shown in Table 2. NJ feeding tubes were successfully placed in 78 of 81 (96.3%) patients. The success rate by fluoroscopy was 92% (23 of 25), by endoscopic technique was 96.3% (26 of 27), and by guide wire was 100% with either transnasal endoscopy or transoral endoscopy. Significant differences between the guide wire assistance with transnasal ultraslim endoscopy and the other three groups were noted in placement duration, whereas there were no significant differences among the other three treatment groups. No significant differences among all the groups were noted in the success or complication rate. No complications were reported from fluoroscopic placement. There were four instances of epistaxis related to replacement of the NG tube after endoscopic placement. All cases of epistaxis resolved without intervention. There was no death related to either procedure.

DISCUSSION

It is well known that malnutrition of critically ill patients is associated with poorer clinical outcomes, and early, sufficient nutritional support can significantly improve the outcomes of the patients[12-16]. EN support is indicated for patients who are unable to take foods orally but have normal intestinal function[17,18], such as those with severe acute pancreatitis, cerebrovascular accidents, traumatic brain injury, *etc*. EN can be delivered through NG tube or NJ tube. The complications of upper gastrointestinal intolerance to EN has been reported to occur in 31%-46% of the patients with NG feeding, some prokinetic agents such as metoclopramide and erythromycin were used to enhance gastric motility and tolerance of enteral feeding. Whether it should be reserved for those patients who are at high risk of upper gastrointestinal intolerance or have already experienced it while receiving NG feeding, requires further studies. Moreover, the optimal dose remains unknown. NJ feeding leads to fewer gastrointestinal complications, largely by reducing gastric residual volumes. So placement of a NJ feeding tube to provide energy support or medication, is increasingly used as a standard clinical practice for many patients[19-21].

But how to place the NJ feeding tube quickly and safely remains an important technique for doctors. The approaches of placing NJ tubes include placement at

surgery, under fluoroscopic or ultrasound-guidance, at endoscopy and blind introduction at the bedside with or without prokinetic administration. The Cathlocator™ is a novel device that permits real time localization of the end of feeding tubes through generating a low energy electromagnetic field from a coil incorporated in the tip of a modified enteral feeding tube connected by wires to a proximal interface. Previously, we would use fluoroscopic placement and/or under direct endoscopic visualization to place NJ tubes. Recently, we have used an ultrathin transnasal endoscope that afforded us a greater success and a shortened procedure time. We evaluated three common methods used to place NJ feeding tubes.

Many studies reported that fluoroscopic guidance in the placement of NJ feeding tubes had a success rate of > 84%[22-24], and endoscopic placement presented a success rate ranging from 90% to 100%[25,26], which are consistent with our outcome. However, fluoroscopic placement exposed patients and doctors to varying doses of radiation. Endoscopic placement procedures are often time consuming, technically cumbersome, and require a significant learning curve[27]. As a result, most gastroenterologists and surgical endoscopists are not satisfied with the current techniques of endoscopic placement. We therefore described a new method to place NJ tubes through guide wire assistance with ultraslim gastroscopy.

Our experience with 29 consecutive guide wire placements of feeding tubes showed that the technique was successful in most patients. Before the operation, we asked the patient whether he/she had received nasal surgery before, and whether accompanied by associated diseases, such as severe bending septum, nasal polyps, severe rhinitis, often epistaxis and other diseases. Six patients who had the aforementioned diseases and subsequently changed to transoral insertion also had the tube placed smoothly in the correct position. In 6 patients with the above complaints, where the assembly was inserted using the transoral route, this did not impair smooth passage of the feeding tube into the correct position. No complications were reported from these methods. Transnasal insertion possessed many advantages compared with other methods. Firstly, the total success rate in the feeding tube placement was high, up to 100%. The operative point was the retropulsion of the feeding tube from the small intestine to the stomach when the endoscope or guide wire was withdrawn. It is not easy to place the guide wire at or beyond the Treitz's ligament using a common endoscope. However, with the transnasal ultraslim endoscope, it became less difficult. Moreover, before inserting the feeding tube along the guide wire, it is very important to lubricate the inner lumen of the feeding tube with paraffin in advance. It not only makes the procedure of withdrawing the guide wire easier, but also avoids pulling out the feeding tube. Secondly, the procedure required less time. In 23 cases with successful one-time transnasal tube placement, the average time required from endoscopic transnasal insertion to the complete removal of the guide wire was only 11.1 ± 2.2 min. Thirdly, the procedure was safe and produced few complications.

In conclusion, our experience showed that the technique of placing NJ feeding tubes with the transnasal ultrathin endoscope is quick, effective and safe.

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COMMENTS

Background

Enteral nutrition (EN) not only provides energy support as with parenteral nutrition, but also maintains the functional intestinal barrier, significantly reducing the incidence of infection and organ failure, shortening hospital stays, and lowering treatment costs. Placement of a nasojejunal (NJ) feeding tube with the aim of providing metabolic support or medication, is increasingly used as a standard clinical practice for many patients.

Research frontiers

NJ feeding tubes positioned beyond the ligament of Treitz's have been shown to allow early attainment of caloric needs and a reduction in tube-feeding aspiration events in patients with gastric feeding intolerance. There are presently several methods for placement of NJ feeding tubes.

Innovations and breakthroughs

The authors used guide wire assistance to place NJ tubes, using an ultrathin transnasal endoscope that afforded them a higher success rate and a shortened procedure time. The authors evaluated the usefulness and safety of the new method compared with the other two traditional methods.

Applications

The technique of placing NJ feeding tubes with the transnasal ultrathin endoscope is quick, effective and safe, which can be applied in clinical practice.

Terminology

Guide wire technology means through guide wire to place NJ feeding tubes. Guide wire was placed beyond the ligament of Treitz's under the ultraslim transnasal endoscope.

Peer review

This article directly compared guide wire method with other two old methods in the duration, success and complications, providing sufficient evidence to prove the superiority of the guide wire method.

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CASE REPORT

Origin of celiac disease: How old are predisposing haplotypes?

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Abstract

We recently presented the case of a first century AD young woman, found in the archaeological site of Cosa, showing clinical signs of malnutrition, such as short height, osteoporosis, dental enamel hypoplasia and cribra orbitalia, indirect sign of anemia, all strongly suggestive for celiac disease (CD). However, whether these findings were actually associated to CD was not shown based on genetic parameters. To investigate her human leukocyte antigen (HLA) class Ⅱ polymorphism, we extracted DNA from a bone sample and a tooth and genotyped HLA using three HLA-tagging single nucleotide polymorphisms for DQ8, DQ2.2 and DQ2.5, specifically associated to CD. She displayed HLA DQ 2.5, the haplotype associated to the highest risk of CD. This is the first report showing the presence of a HLA haplotype compatible for CD in archaeological specimens.

Key words: Celiac disease; Human leukocyte antigen haplotype; Ancient DNA; Single nucleotide polymorphisms; Malabsorption

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Gasbarrini G, Rickards O, Martínez-Labarga C, Pacciani E, Chilleri F, Laterza L, Marangi G, Scaldaferri F, Gasbarrini A. Origin of celiac disease: How old are predisposing haplotypes? *World J Gastroenterol* 2012; 18(37): 5300-5304 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i37/5300.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i37.5300

INTRODUCTION

In 2008, we were involved in the "case of Cosa", a skeleton of a young woman dating to the first century AD, found in the archaeological site of Cosa, southwest of Tuscany, Italy (Figure 1)[1]. Based on the physical anthropology description[2], she was a 18-20 year -old woman, dead in physical impairment, showing signs of failure to thrive and malnutrition, all signs of typical celiac disease (CD), in particular, she was slightly built and moderately short for her age (140 cm in height), with clear signs of bone fragility and osteoporosis. She showed on her orbital roof a pathological sign, the "cribra orbitalia" (yet published)[1], a bone porosity also well distinguished in the bone of the skull vault. This condition is generally linked to bone marrow hypertrophy following anemic conditions, such as iron deficient chronic anemia. This

excessive porosity could be also found in the external surface of the bones, in particular in the skull vault (Figure 2A). Furthermore she showed the bone marrow reactive hypertrophy associated to bone atrophy (Figure 2B). Although teeth structure and number were normal, she presented basal dental enamel hypoplasia (Figure 2C), a marker of nutritional or infectious stress. Measuring in the femur the angle between the neck and the diaphysis, it appears larger than normal adult angle (135° *vs* 125°), consistent with a diagnosis of coxa valga, typical of the hip subluxation, due to congenital dysplasia. This diagnosis is supported by the flattening of the postero-superior part of the acetabular cavity. All these signs, taken together, strongly suggested an advanced state of chronic malnutrition, consistent with a typical form of CD[3-5]. From the beginning, a poor availability of food was excluded as several signs indicated that she was member of a wealthy family, as suggested by the jewels she wore and by the overall quality of her tomb. Based on ethologic data[6], we considered that her diet was variegated and probably rich in wheat, and consequently in gluten. We hypothesized that the young woman suffered from CD. Moreover, her death was caused by severe malnutrition or by a complication of it.

The most ancient case of CD ever described was reported by Areteus of Cappadocia in the 1st-2nd centuries AD[7], indicating that CD could have a old origin. However the appearance of the predisposing haplotypes in humans as well as the origin of CD is still unknown.

Genetic susceptibility is a crucial step in the pathogenesis of CD, as demonstrated by studies on monozygotic twins, that show disease concordance rate of 75% compared with 11% in dyzigotic twins[8]. Furthermore siblings have an increased risk of CD, of which about 40% depends on human leukocyte antigen (HLA) genes[9]. The role of HLA class Ⅱ molecules as the major genetic risk factor for CD is well known, even if the genetic effect attributable to HLA is only 54%[10]. It has been reported that over 90% of CD patients express HLA-DQ2 heterodimer while the others express HLA-DQ8[11]. In Europe only fewer than 0.5% of celiac patients express neither DQ2 nor DQ8[12], making HLA genotyping a marker with a very powerful negative predictive value, excluding the diagnosis in people who do not have CD risk HLA haplotype. Until present no information were available on CD genetics in ancient time, since DNA analysis of human remains is technically difficult.

CASE REPORT

In order to confirm the clinical hypothesis that the young woman was actually suffering from CD, we extracted DNA from parts of the skeleton and studied HLA polymorphisms known to be involved in susceptibility to CD. In particular, we analyzed DQ2 (encoded by the *DQA1* 05* and *DQB1*02* alleles) and DQ8 (encoded by the *DQA1*03* and *DQB1*0302* alleles) HLA heterodimers[13,14].

Ancient DNA analysis of human remains is particularly challenging, therefore every attempt was made to ensure the generation of authentic and meaningful data following the strictest available criteria[15-19]. To extract the genetic material, a bone sample and the left mandibular third molar (Figure 2D) were chosen since they are the best sources of DNA. Bones and teeth consist of hard material that contains small hollow spaces with single cells that are less affected by diagenetic processes and by natural contamination (microorganisms, fungus), and modern contaminations are likely to be removed prior to extraction. Bone and tooth sample were firstly brushed and irradiated for 1 h under ultraviolet (UV) light. Afterwards, the entire surface was removed by using dental drills, and the samples were cut into smaller pieces with drill. The samples were again UV-irradiated for 45 min, grounded to fine powder and stored until use at 4 ℃. Only commercially certified DNA/RNA-free consumables were utilized and all tools and containers used were sterile and DNA-free. All workers wear gloves, safety masks, disposable coveralls, plus particular shoes. Every item entering is extensively washed with bleach and subsequently UV-irradiated.

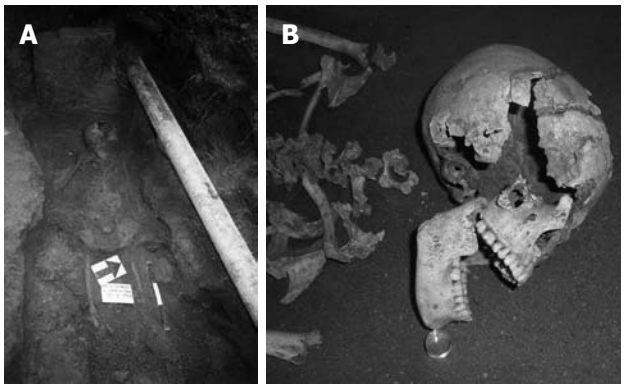

Figure 1 A particular of the skeleton of Cosa in the original site. A: The original site: The ancient remains of Cosa in the original site where they were found; B: The young girl: A particular of the skeleton of the young girl of Cosa.

As first step, we studied the preservation of bone collagen, one of the best indicator of bone preservation and therefore of DNA survival[16]. Collagen was extracted from a small bone fragment following the procedure reported in Craig *et al*[20]. The results obtained indicated the good state of preservation of the collagen. In fact, collagen yield expressed as weight percentage was 1.065% and the ratio C/N was in the range between 3.25 and 3.34 as expected when organic substances are saved from decay[21]. Based on these results, DNA extraction was performed in a laboratory physically isolated from all other laboratories which offers all the state-of-the-art facilities for aDNA studies[17,22,23]. They consist of a contamination resistant facility, which are maintained at positive pressure, frequently cleaned with HCl, NaClO and DNAzap™, UV and high-efficiency particulate arresting filtered, and have restricted access, designed to minimize the possibility of contamination with extant human DNA[23]. The laboratory has consecutive rooms, every room is fitted with UV-C light sources (254 nm) that can be switched on and off from outside the re-

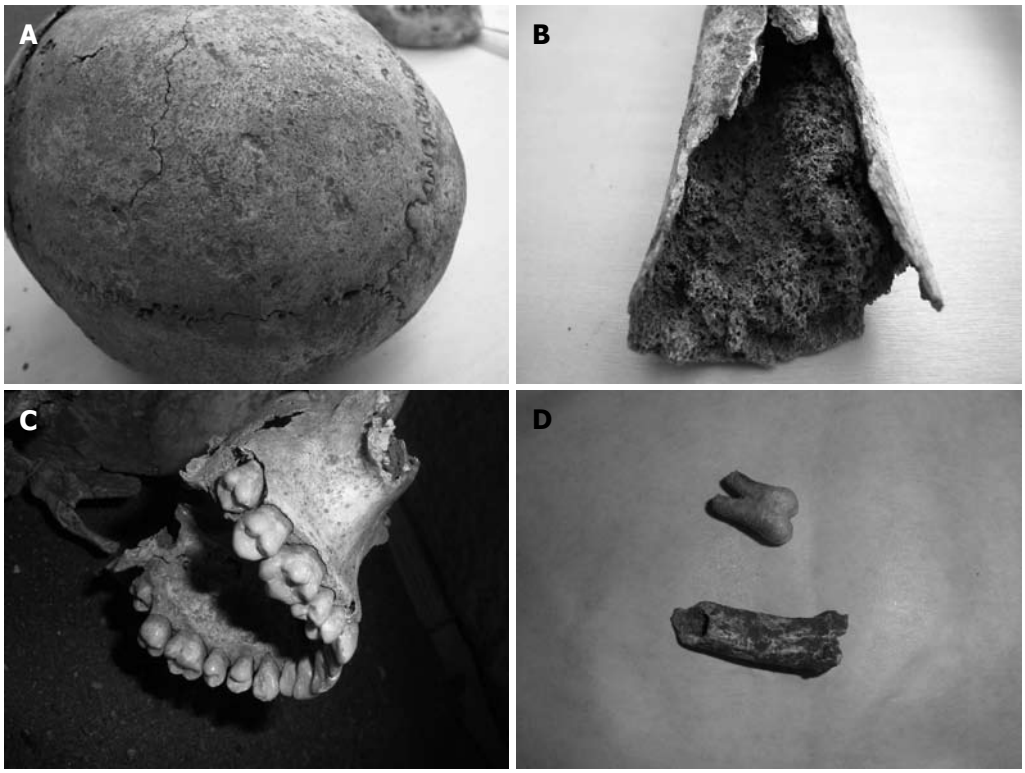

Figure 2 Research on the skeleton of the young girl. A: Skull vault showing excessive porosity; B: A particular of the femur showing cortical atrophy and spongiosum bone hypertrophy, indirect sign of bone marrow hypertrophy, well preserved in 2000 years; C: A detail of teeth, showing basal dental enamel hypoplasia; D: DNA extraction: A tooth and a sample of bone from which DNA was extracted.

Table 1 Primers and length of polymerase chain reaction fragments analyzed for predicted single nucleotide polymorphism

SNPs	Forward primer	Reverse primer	Length fragment (bp)
rs7454108	ACTATTATTTCTCCAAGTTCTGACTTCCCT	GCCAAGTTGGAATAAGCCCACTATA	155
rs7775228	AGGAAAGGAACTATCTGGGTATGGA	TGCAAAGCCCCTTTATCATTATCCT	80
rs2187668	GTGAGGTGACACATATGAGGCAG	GGCTGAATGCCTTCAACAATCATTT	74

SNPs: Single nucleotide polymorphism.

spective lab. The first room has an entry area for changing into suitable clean room clothing. The second room has bench space for handling sampling with fine scale for weighing of samples, a dentist drill for cutting and drilling samples as well as mortars and pestles for griding samples. Another two independent labs with hoods (with internal UV-C sources and biosafety cabinets) are used for DNA extraction and one other for polymerase chain reaction (PCR) setup.

Briefly five hundred milligrams of powder were digested in a proteinase K lysis buffer and DNA was extracted through silica-based spin columns[24]. At least two independent DNA extractions were performed on bone and teeth respectively; mock-extraction controls were carried out identically to those on the samples.

Three HLA-tagging single nucleotide polymorphism (SNPs) were genotyped in order to capture the DQ8, DQ2.2 and DQ2.5 HLA types as reported by Monsuur *et al*[25], using TaqMan chemistry and the on demand assays by Applied Biosystems (Foster City, CA, United States, www.appliedbiosystems.com: Assay IDC_29817179_10dbSNP ID rs7454108; Assay IDC_29315313_10dbSNP ID rs7775228; and Assay IDC_ 58662585_10dbSNP ID rs2187668). Negative controls for amplification (PCR without template DNA) were set up simultaneously to detect contamination and at least 4 independent amplifications of each fragment were performed.

TaqMan® single nucleotide polymorphism (SNP) genotyping assays provide optimized assays for genotyping SNPs and make it easy to perform SNP genotyping discrimination studies. Samples were amplified and genotyped using the manufacturer's instructions on an ABI Prism 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA, United States). All SNPs were typed using the standard amplification protocol as supplied by Applied Biosystems (hold 10 min, at 95 ℃, and 40 PCR cycles with denature 15 s, at 92 ℃ and anneal/extend 1 min, at 60 ℃).

Moreover, in order to confirm the RT-PCR results, we amplified and sequenced the three predictive fragments.

The list of primers designed for the experiment and the length of each PCR fragment analyzed are reported in Table 1. PCR amplification was performed in 25 μL reaction containing 2 μL DNA extract, with a final concentration of 1XPCR Gold Buffer Ⅱ, 2.5 mmol $MgCl_2$, 1 mmol dNTPs, 100 nmol primers, 0.1 mg/mL bovine serum albumin, 1 U AmpliTaq Gold (Applied Biosystems). The PCR reaction was run for 35 cycles at 94° for 30 s, 60 ℃ for 30 s and 72 ℃ for 30 s, with a first denaturation step (94 ℃ for 5 min), and a final extension (72 ℃ for 10 min).

PCR products were visualized by gel electrophoresis on a 1.5% agarose gel stained with GelStar (Cambrex, Rockland, ME, United States). Positive amplification products were purified through ExoSap-IT (USB Affymetrix, Santa Clara, CA) according to manufacturer's specifications. Afterwards, they were labeled with fluorescent dyes, purified by the ethanol precipitation technique and submitted to sequencing reaction in an ABI Prism 3100 Avant (Applied Biosystems, Foster City, CA) following the recommended sequencing kit protocols. Sequences were verified through complete overlapping of forward and reverse strands.

Genetic results were independently reproduced multiple times and all sequences were confirmed by at least two different amplified products in order to identify possible contamination.

The young girl turned out to be homozygous FAM for rs7454108, homozygous FAM for rs7775228, and homozygous VIC for rs2187668. The result is compatible with DQ2.5 homozygous genotype which is associated with higher risk of CD. This finding supports on molecular basis our hypothesis that the skeleton found in the site of Cosa suffered from CD.

Finally, to verify the endogenous nature of aDNA and track down any possible modern contaminations, molecular sex and mtDNA characterizations were performed.

Sex determination was carried out by amplification of a segment of the X-Y homologous amelogenin gene using the primer system amelogenin A/B as described by Mannucci *et al*[26]. This method is usually applied for typing samples of a very degraded nature, since short X and Y-specific products of 106 and 112 bp, respectively are generated from a single primer pair. The result were resolved by 12% Acrylamide electrophoresis. Molecular data confirmed the morphological and morphometric sex diagnosis of being female.

Mitochondrial DNA (mtDNA) typing[27] was performed also on the DNAs of all molecular anthropologists and archaeologists who handled the ancient sample. All the extant sequences differed from the girl "Cosa" consensus mtDNA sequence (16270T, 16362C, 73G, 150T and 263G) excluding modern DNA contamination. The ancient haplotype was certainly phylogenetically assigned to U5b2b1a haplo-group following the classification proposed by van Oven *et al*[28]. This haplo-group is European specific and its PAML (Phylogenetic Analysis by Maximum Likelihood) based age estimate is 9325.2 ± 3443.5 years[29-31].

DISCUSSION

This is the first report of HLA typing in ancient remains and it could be considered a very intriguing result, although it does not allow us to diagnose definitively CD. The presence of CD associated-HLA is a necessary condition, although not sufficient to develop the disease. It fact, although about 30%-35% of the actual general population express CD associated HLA genotypes, it has been estimated that, only 2%-5% of risk gene carriers develop the disease[32]. The risk increases further in homozygous for DQ2.5 (*HLA-DQA1*05- DQB1*02*) as shown by a recent study in United States population[12] and by another study exploring relative risks for CD in European population[33]. Another study, on sibs and parents of Italian celiac children, shows that a DQ 2.5 homozigous sib had a risk of 28% of developing CD[34]. Recent advances indicate that other genetic factors may play a role in determining which HLA compatible people could develop CD[35], particularly genes involved in T-cell regulation and inflammation, but they have not been considered for this paper. It has been reported that these genes are contributing for 3%-4% in the risk of CD, together with environmental factors, like early introduction of gluten in infants diet, early infection with enteropathic viruses or the presence of a changed bacterial flora[32].

In the case of "Cosa", even without precise understanding of how environmental factors impacted the girl's life, HLA typing provide us a precious information. The presence of HLA-DQ2.5, in combination with the phenotypic observations, increases the likelihood that the young girl of Cosa suffered from CD and that CD existed already 2000 years ago, like Areteus hypothesized on clinical bases. Our results, strengthen the idea that CD was born a long time ago, walking along together with humans for a long stretch of their history, perhaps even since wheat feeding was introduced.

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CASE REPORT

Biliary tract schwannoma: A rare cause of obstructive jaundice in a young patient

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Abstract

Schwannoma is a tumor derived from Schwann cells which usually arises in the upper extremities, trunk, head and neck, retroperitoneum, mediastinum, pelvis, and peritoneum. However, it can arise in the gastrointestinal tract, including biliary tract. We present a 24-year-old male patient with obstructive jaundice, whose investigation with computed tomography abdomen showed focal wall thickening in the common hepatic duct, difficult to differentiate with hilar adenocarcinoma. He was diagnosed intraoperatively schwannoma of common bile duct and treated with local resection. The patient recovered well without signs of recurrence of the lesion after 12 mo. We also reviewed the common bile duct schwannoma related in the literature and evaluated the difficulty in pre and intraoperative differential diagnosis with adenocarcinoma hilar. Resection is the treatment of choice for such cases and the tumor did not recur in any of the resected cases.

Key words: Schwannoma; Neurilemoma; Biliary tract; Hilar adenocarcinoma; Computadorized tomography

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INTRODUCTION

Although it is considered to be a rare tumor, adenocarcinomas are the most common malignant neoplasms of extrahepatic bile ducts[1,2]. However, other non-epithelial tumors can develop, whether they are malignant, such as lymphomas and neuroendocrine cancer[3], or benign, such as adenomas, lipomas, fibromas and schwannomas[4,5], which sometimes appear similar to hilar adenocarcinomas (cholangiocarcinomas).

The goal of this case study was to present a rare case of common bile duct schwannoma that simulated a hilar adenocarcinoma. It was diagnosed during surgery, which allowed only a local resection.

CASE REPORT

A 24-year-old male patient was clinically diagnosed with

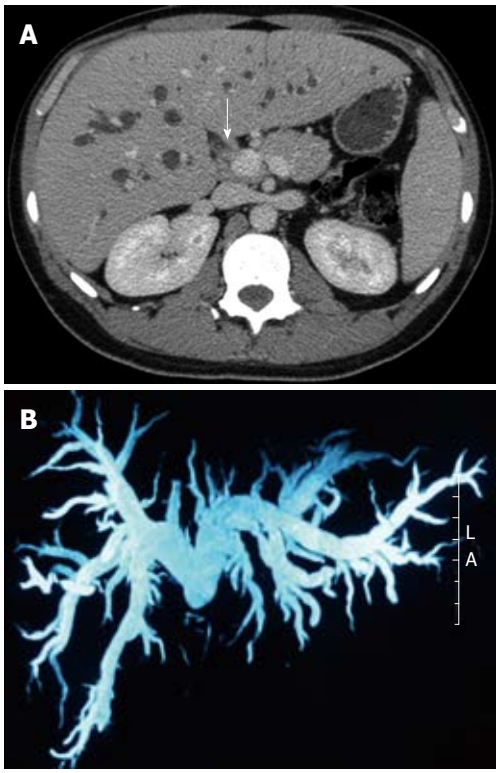

Figure 1 Large dilatation close to hepatic duct. A: Stenosis and thickening of the common hepatic duct (arrow) with upstream biliary dilation; B: Magnetic resonance cholangiography showing large dilatation close to the lesion in the common hepatic duct. A: Ahead; L: Left.

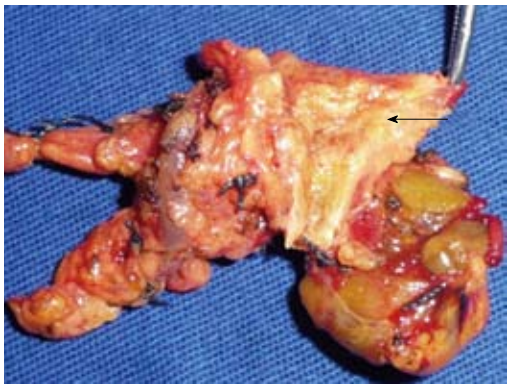

Figure 2 Hepatocholedochal tumor opened longitudinally with the affected area (arrow).

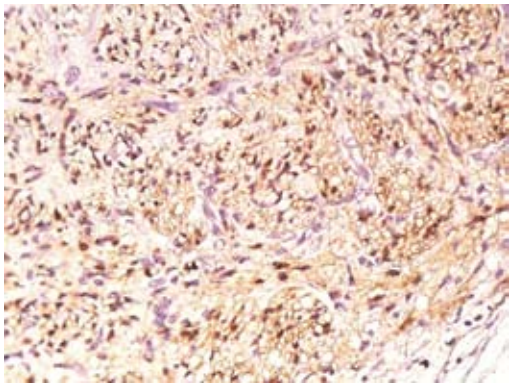

Figure 3 Immunohistochemical staining for the S-100 protein (40 ×).

obstructive jaundice, had 2 mo of epigastric pain and vomiting, and lost 15% of his body weight. The patient had a history of smoking and social drinking. A physical examination confirmed that the patient had jaundice with a flaccid, painless abdomen and palpable liver 4 cm below the right costal margin. There were no other relevant findings.

The laboratory studies revealed: total bilirubin 23.8 mg/dL; direct bilirubin 22.9; serum alkaline phosphatase 298 IU/L; and serum gamma-glutamyl transpeptidase 1052 IU/L; serum asparate aminotransferase 121 IU/L; serum alanine aminotransferase 249 IU/L; and carbohydrate antigen 19-9 was above normal range, 62.7 U/mL. Others laboratory tests were normal.

Imaging studies began with abdominal ultrasound sonography test, which revealed increased liver volume and dilation of the intra-and extrahepatic bile ducts up to the hepatic hilum.

A computed tomography scan of the abdomen showed focal wall thickening in the common hepatic duct 1.5 cm from the hepatic duct confluence. As a result, the diameter of the lumen was reduced, and there was upstream dilation (Figure 1A). The vascular structures and adjacent fat planes were preserved.

A magnetic resonance cholangiography showed biliary tract dilation with abrupt obstruction in the common hepatic duct (Figure 1B).

We opted for surgical treatment without biliary intervention for decompression or endobiliary biopsy, with a preoperative diagnosis of hilar adenocarcinoma. In the cavity, we discovered that the liver was enlarged and appeared to be cholestatic. There was a 1-cm nodular lesion that was palpable in the common hepatic duct; additionally, the proximal bile duct was dilated. A cholecystectomy dissection was performed from the common bile duct above the pancreas to the nodular lesion in the common hepatic duct. During the dissection of the affected area, we observed that the tumor was well defined and regular, with no signs of having infiltrated adjacent tissues (Figure 2). We performed frozen sections, which showed a mesenchymal tumor with free margins in the hepatic duct. In view of these findings, we opted for local resection and Roux-en-Y hepaticojejunostomy.

The patient recovered and was discharged in good condition on the 7th postoperative day. A pathological examination showed a 0.8-cm lesion in the common hepatic duct, which was diagnosed as benign schwannoma with free margins in the extrahepatic bile duct. The diagnosis was made through microscopic examination and immunohistochemistry (Figure 3). The patient was reexamined 12 mo after surgery and was in excellent general health with no signs of recurrence.

DISCUSSION

Benign schwannoma, which is also known as neurile-

Table 1 Cases studies of biliary schwannoma in the literature

Observations/references	Age (yr)	Sex	Signals/ symptoms	Initial diagnosis	Location of tumor	Preoperative tissue acquisition
Oden *et al*[17]	40	F	Abdominal pain + obstructive jaundice	Choledocholithiasis	Commom bile duct	No
Whisnant *et al*[18]	15	F	Abdominal pain + weight loss + obstructive jaundice		Distal portion of the commom bil duct	No
Complicated by liver abscess, treated with drainage/Balart *et al*[19]	56	F	Abdominal pain + obstructive jaundice	Cholangiocarcinoma or extrinsic compression of the bile duct	Commom hepatic duct	No
Jakobs *et al*[20]	37	M	Abdominal pain + obstructive jaundice	Intra-ductal benign tumor	Commom hepatic duct	Yes
Honjo *et al*[13]	48	F	Obstructive jaundice	Benign non-epithelial tumor	Commom bile duct	Yes (transpapillary brush citology, non-diagnostic)
Otani *et al*[21]	59	F	Abdominal pain		Remnant bile duct (pancreatic portion)	No
Park *et al*[22]	53	F	Asymptomatic		Porta hepatis	No
Vyas *et al*[23]	29	F	Abdominal pain + obstructive jaundice		Commom bile duct	Yes (non-diagnostic)
Kamani *et al*[24]	39	F	Jaundice + weight loss	Klatskin tumor	Proximal portion of the commom hepatic duct	No
Fenoglio *et al*[16]	41	F	Obstructive jaundice + weight loss		Middle segment of the commom bile duct	No
Jung *et al*[4]	64	F	Asymptomatic		Proximal portion of the commom bile duct	No
Madhusudhan *et al*[5]	46	M	Obstructive jaundice	Variable polypoid cholangiocarcinoma	Intrahepatic bile duct	Yes
Kulkarni *et al*[7]	38	M	Abdominal pain + weight loss + jaundice		Commom bile duct/ porta hepatis	No
Patient has von Recklinghausen's disease/De Sena *et al*[25]	58	F	Obstructive jaundice	Biliary schwannoma	Extrahepatic bile duct	No
Previous malignant melanoma/Panait *et al*[26]	54	F	Gastroesophageal reflux symptoms	Recurrent metastatic melanoma	Porta hepatis	Yes (non-diagnostic)

F: Female; M: Male.

moma, is a tumor derived from Schwann cells, which form the inner portion of the peripheral nerve sheath[6]. Theoretically, the tumor can affect any organ or nerve trunk, except the optic and olfactory nerves, which lack Schwann cells[7]. The most common locations are the upper extremities, trunk, head and neck, retroperitoneum, mediastinum, pelvis, and peritoneum[8].

Schwannomas in peripheral nerves can be associated with neurofibromatosis type 2, while many schwannomas can occasionally be associated with neurofibromatosis type 1[5,9].

Schwannomas in the digestive tract are relatively rare. These tumors are most common in the stomach followed by the colon/rectum and esophagus[10-12]. Additionally, schwannomas can develop in the biliary tract because there is an abundant network of sympathetic and parasympathetic nerve fibers along the wall of the gallbladder and bile ducts, but these cases are extremely rare[4].

Diagnostic imaging tests do not allow preoperative diagnoses of biliary schwannoma because the findings are similar to those observed in most common lesions, particularly central cholangiocarcinoma. The most relevant finding in our case was that the preoperative examinations revealed a well-defined ductal injury with no signs of adjacent structural involvement or distant metastases[5].

A schwannoma is usually a macroscopically encapsulated solid globular or ovoid tumor. Degenerative cystic changes are occasionally observed within the tumor. Microscopically, the tumor has two components: a hypercellular component with areas of spindle cells forming palisades (Antoni type A) and a myxoid component containing cuboidal cells with clear cytoplasms (Antoni type B)[13].

We found 15 case studies of extrahepatic biliary schwannomas in the literature, which are shown in Table 1. Of these cases, the ages of the patients ranged from 15 to 64 years (with an average age of 44 years), and the patients were predominantly female (12/15). The most common symptom was jaundice (11/15 patients) followed by abdominal pain (7/15) and weight loss (4/15); two patients were asymptomatic. A preoperative diagnosis of hilar adenocarcinoma occurred in 3/15 patients. The lesion was resected in all but one case in which resection was not conducted because of the extensive involvement of the tumor[5]. In this case, endoscopic prosthesis was suggested, but the patient refused the procedure with clinical follow-up. The tumor did not recur in any of the resection cases.

Immunohistochemical analysis is necessary to distinguish schwannomas from neurofibromas, gastrointestinal stromal tumors and leiomyomas. Schwannomas are

strongly positive for vimentin and S100 protein and are negative for muscle cell markers and CD117 (kit), which are found in smooth muscle and gastrointestinal stromal tumors. The CD34 antigen is expressed by a distinct cell population in peripheral nerves, nerve sheath tumors, and related lesions. This antigen is also a useful parameter for the immunohistochemical diagnosis of gastrointestinal stromal tumors. Schwannomas in the digestive tract are usually negative for CD34, although Hou *et al*[14] identified 3 gastrointestinal schwannomas with CD34-positive spindle cells in 33 analyzed cases. Our patient was positive for both the S100 protein, as was expected, and CD34, as in the 3 cases described by Hou *et al*[14].

While there may be preoperative suspicion, the diagnosis of schwannoma requires intraoperative and histopathological confirmation[15]. Despite the possible complications, resection is the treatment of choice for such cases[16]. Schwannomas in the digestive tract have an excellent prognosis after surgical resection, as do schwannomas in other locations. To date, there is no evidence suggesting that these tumors are potentially malignant[4]. In keeping with these findings, our patient remains asymptomatic after 12 mo of postoperative follow-up.

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CASE REPORT

An endoluminal aortic prosthesis infection presenting as pneumoaorta and aortoduodenal fistula

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Abstract

Herein, we present a case of pneumoaorta and aortoduodenal fistula (ADF) caused by an endoluminal aortic prosthesis infection. An 82-year-old man underwent endovascular aneurysm repair with a stent graft to exclude a 5.1-cm abdominal aortic aneurysm. Three months after the index procedure, the patient was taken to the emergency department at a medical university hospital. He presented with a 2-d history of bloody diarrhea. An endoluminal aortic stent graft infection was diagnosed, and an ADF was identified. The patient died of septic shock despite emergency surgery and intensive care. When encountered, stent graft infections require appropriate antibiotics and graft explantation. The diagnosis of an ADF is important, and surgery remains the most effective management if septic shock presents despite conservative treatment.

Key words: Aortoduodenal fistula; Endovascular aneurysm repair; Infection; Stent graft; Shock

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INTRODUCTION

The use of stent grafts to treat abdominal aortic aneurysms (AAAs) has recently become more widely used[1-3]. Stent graft infection rates associated with use of the endovascular technique appear to be lower than those for conventional open repair (range: 0.3%-0.4%)[1,2] as demonstrated by Ducasse *et al*[3], who reported a frequency of infection of 0.43%. However, the incidence of infection resulting from the implantation of an endoluminal aortic prosthesis has been reported to be as high as 6%[4,5]. Although prophylactic antibiotics are routinely prescribed prior to an operation, the incidence of infection and possible sequelae remain difficult to predict. Surgical intervention with complete stent graft removal may provide the best outcome for patients with an infection[3]. The overall treatment strategy can be optimized with the early detection of an endovascular stent graft infection. Herein, we present the case of an elderly male patient with an

endovascular stent graft infection who ultimately died of septic shock despite intensive care.

CASE REPORT

An 82-year-old male patient with type 2 diabetes initially presented with a bulging mass in the abdomen and localized pain. The patient underwent endovascular aneurysm repair with a stent graft (Zenith; Cook, Bloomington, IN) to exclude a 5.1-cm AAA in October 2010 (Figure 1A). The patient recovered without incident post-surgery and was discharged. Although the post-surgical protocol calls for an abdominal computed tomography (CT) scan at one month, the patient did not return to the cardiothoracic surgery clinic following his discharge.

Three months post-surgery, the patient was taken to an emergency department at a medical university hospital reporting a 2-d history of bloody diarrhea. Upon further examination, his blood pressure was measured at 121/55 mmHg, his body temperature was 37.8 ℃, his pulse was 68/min, and his oxygen saturation was 88% as measured using a nasal cannula with 3 L/min of oxygen flow. A physical exam revealed pale conjunctiva and a distended abdomen. Furthermore, blood analysis revealed leukocytosis with a white blood cell count of 21 250 cells/mm^3 and a hemoglobin level of 9.6 g/dL. Serum chemistry was unremarkable except for mildly elevated creatinine (1.3 mg/dL) with an estimated glomerular filtration rate of 56.45 mL/min. Empiric antibiotics were prescribed, including flomoxef and vancomycin. An abdominal CT scan disclosed a fistula between the aorta and the retroperitoneal duodenum, suggesting the formation of an aortoduodenal fistula (ADF) (Figure 1B). There was circumferential fluid collection with air surrounding the stent region, suggesting the presence of necrotic tissue and associated gas formation (Figure 1B-D). Due to the occurrence of refractory shock, an emergency operation was indicated. A bilateral axillary-femoral extra-anatomic bypass was performed with 8-mm polytetrafluoroethylene grafts. In addition, a retroperitoneal abscess and an abdominal aortic aneurysmal sac necrosis were debrided. The ADF was located at the bare-metal stent supra-renal fixation point. The stent graft was removed, and the aortic stump was closed just distal to the renal artery orifices. The tear at the third portion of the duodenum was repaired using LigaSure (Tyco-Healthcare, United States). A segmental resection of the duodenum and a side-to-end duodenojejunostomy were performed. The ADF was 1.5 cm × 1.3 cm in diameter.

Blood cultures revealed a mixed growth of *Salmonella* species, *Bacteroides fragilis*, *Clostridium* species and *Gemella morbillorum*. Bacterial culture of the necrotic tissue demonstrated a mixed Salmonella and Bacteroides infection. The patient died of septic shock two days after admission despite intensive care.

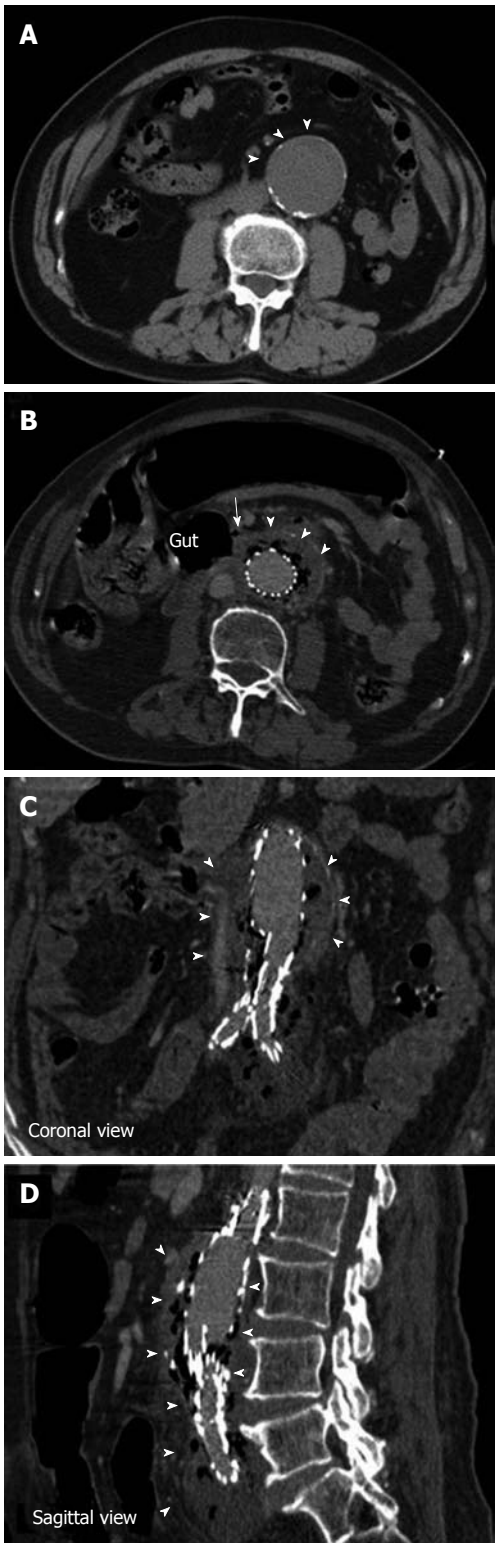

Figure 1 Abdominal computed tomography scans of the patient. A: The abdominal aortic aneurysm before stent-graft implantation (arrowheads); B: The aortoduodenal fistula (arrow); B-D: The necrotic tissue and associated gas formation (arrowheads).

DISCUSSION

In the current case, a gas-forming bacterial infection resulted in the development of a pneumoaorta, which is uncommon. In one recent report[6], a 77-year-old man was diagnosed with a stent graft infection, and his CT scan demonstrated soft-tissue thickening and air present in the right anterolateral aspect of the aneurysm sac. Ad-

ditionally, coagulase-negative staphylococci were identified in a blood culture. Another case report[7] described a stent graft infection due to *Bacteroides fragilis*. The patient's condition was successfully managed with staged extra-anatomic revascularization followed by graft excision.

ADF is a well-recognized and dangerous condition[8-15]. According to a single-center review, five patients developed an ADF between 18 d and 1 year after successful endovascular aneurysm repair[12]. ADF has also been shown to occur as late as five years after endovascular aneurysm repair[13]. The patient in this study presented with ADF and bloody stool passage three months after the index procedure. ADFs and aortic aneurysms can be caused by biliary stent-induced retroperitoneal perforation[14]. However, the current patient did not report any prior gastrointestinal (GI) procedures, such as biliary stenting. The ADF etiology might have included infection and endoleak[12], but an endoleak was not observed on the CT scan. An ADF might further act as a connecting route between the GI tract and the aorta, causing bacterial propagation and infection-related deterioration. Therefore, the bloody stool passage was a possible sequela of the ADF formation[15].

In conclusion, prompt diagnosis and intervention are crucial for effectively treating a patient with an endovascular stent graft infection. A combination of the appropriate antibiotics and surgical repair is the best course for avoiding a fatal outcome. The most effective surgical intervention consists of a complete stent graft explantation followed by *in situ* reconstruction. Endovascular prosthesis implantation is a challenging technique for AAA, but the early recognition and detection of a possible stent infection may be more critical.

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CASE REPORT

Penicillium marneffei chylous ascites in acquired immune deficiency syndrome: A case report

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Abstract

Penicillium marneffei (*P. marneffei*) infection usually occurs with skin, bone marrow, lung or hepatic involvement. However, no cases of *P. marneffei* infection with chylous ascites have been reported thus far. In this report, we describe the first case of acquired immune deficiency syndrome (AIDS) which has been complicated by a *P. marneffei* infection causing chylous ascites. We describe the details of the case, with an emphasis on treatment regimen. This patient was treated with amphotericin B for 3 mo, while receiving concomitant therapy with an efavirenz-containing antiretroviral regimen, but cultures in ascitic fluid were persistently positive for *P. marneffei*. The infection resolved after treatment with high-dose voriconazole (400 mg every 12 h) for 3 mo. *P. marneffei* should be considered in the differential diagnosis of chylous ascites in human immunodeficiency virus patients. High-dose voriconazole is an effective, well-tolerated and convenient option for the treatment of systemic infections with *P. marneffei* in AIDS patients on an efavirenz-containing antiretroviral regimen.

Key words: Chylous ascites; *Penicillium marneffei*; Acquired immune deficiency syndrome; Voriconazole; Efavirenz; Fungal sepsis

INTRODUCTION

Penicillium marneffei (*P. marneffei*) infection now represents one of the most common acquired immune deficiency syndrome (AIDS)-defining opportunistic infections in endemic areas of Southeast Asia[1-3]. The infection is associated with a high mortality rate if timely treatment with appropriate antifungal drugs is not administered[2,4]. *P. marneffei* usually occurs with skin, bone marrow, lung or hepatic involvement; however, no cases of *P. marneffei* infection with chylous ascites have yet been reported. In this report, we describe the first case of AIDS which has been complicated by a *P. marneffei* infection causing chylous ascites. We describe the details of the case with emphasis on treatment regimen.

CASE REPORT

A 47-year-old man, a native of Yunnan province, southwest China, infected with human immunodeficiency virus (HIV) who had a CD4 cell count of 66 cells/μL had a 1-year history of intermittent fever and a 6-mo history of abdominal distension. Culture of blood and ascitic fluid

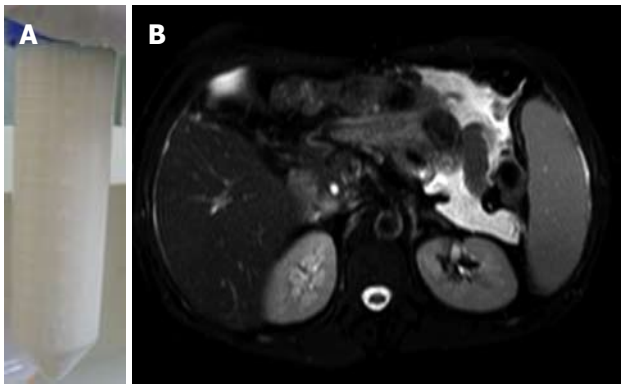

Figure 1 The auxiliary examination for the patient. A: The ascitic fluid had a milky appearance; B: Abdominal magnetic resonance imaging showed hepatosplenomegaly with ascites.

revealed *P. marneffei*. He was diagnosed with AIDS and *P. marneffei* infection. He had received antiretroviral therapy (ART) with stavudine (30 mg twice daily), lamivudine (300 mg once daily) and efavirenz (600 mg daily before bedtime). He was treated with amphotericin B 25 mg/d which was continued for 3 mo, but his condition did not improve. Persistent abdominal distension remained, so paracentesis was performed periodically to relieve the symptoms. Cultures in ascitic fluid were persistently positive for *P. marneffei*. He came to our hospital for further management. Examination revealed the presence of shifting dullness and paracentesis confirmed diagnosis of chylous ascites. Total serum protein, albumin, total cholesterol and triacylglycerol were 65.80 g/L, 37.70 g/L, 3.92 mmol/L and 0.95 mmol/L, respectively. The ascitic fluid had a milky appearance (Figure 1A) and showed 270 white blood cells/μL with 60% multinucleated cells and 40% lymphocytes, triglyceride 13.59 mmol/L (normal range < 1.70 mmol/L), and total protein 31.5 g/L. No malignant cells were found on pathology. Chest computer tomography scan showed no abnormalities while abdominal magnetic resonance imaging showed hepatosplenomegaly with ascites (Figure 1B). Treatment with oral voriconazole (400 mg every 12 h) was started and was continued for 3 mo. He continued on ART (stavudine, lamivudine and efavirenz), but he was recommended to take a lower dose of efavirenz (300 mg once daily before bedtime). In order to monitor efficacy and side effects, we performed therapeutic drug concentration monitoring (TDM) of both voriconazole and efavirenz. The results showed that the peak and trough concentrations of voriconazole were 2.31 mg/L and 1.42 mg/L, respectively and the peak and trough concentrations of efavirenz were 3720 ng/mL and 2680 ng/mL, respectively. While on voriconazole, he improved. All disease-related clinical symptoms and signs gradually disappeared. Fungal cultures of chylous ascites became negative after receiving 2 wk of voriconazole treatment. The values of laboratory tests including electrolytes, renal function and transaminases were within normal ranges during treatment. The infection resolved after treatment with voriconazole for 3 mo and was discontinued when recovery was thought to be achieved. He was recommended to take a normal dose of efavirenz (600 mg once daily) after discontinuation of voriconazole. Six months after initiation of ART, his CD4 cell count rose to 110 cells/μL. He refused to receive secondary prophylaxis with antifungal drugs. No relapse was found in an 8-mo follow-up.

DISCUSSION

Prior to the epidemic of AIDS, penicilliosis was a rare event. The incidence of this fungal infection has increased markedly during the past few years, paralleling the incidence of HIV infection[1]. In China, *P. marneffei* infection is endemic in Guangdong, Guangxi, Yunnan, HongKong and Taiwan[5-7]. In recent years, with the increase of HIV infection, cases have been reported from non-endemic regions[3,8].

Chylous ascites is a rare complication of AIDS. It is caused by the leakage of chyle into the peritoneum due to rupture or obstruction of the thoracic duct. *P. marneffei* is a systemic fungal infection which usually occurs with skin, bone marrow, lung or hepatic involvement. This patient is the first case of AIDS which has been complicated by a *P. marneffei* infection causing chylous ascites. This case suggests that *P. marneffei* should be an important differential diagnosis in chylous ascites in HIV patients, especially in visitors to, or residents of, endemic areas for *P. marneffei*.

The recommended treatment for *P. marneffei* is amphotericin B for 2 wk, followed by oral itraconazole for a subsequent duration of 10 wk[9]. ART should be administered simultaneously to improve outcome[9], but avoiding adverse drug interactions between antifungals and antivirals is also important as these can complicate therapy. This patient had failed to respond to initial amphotericin B treatment. *P. marneffei* is highly susceptible to miconazole, itraconazole, ketoconazole, and 5-flucytosine. Amphotericin B has intermediate antifungal activity[9] which means some patients may not respond to treatment with it. Up to now, alternative treatment options for penicilliosis have not been established. A small case series[10] reported good outcomes with voriconazole in AIDS patients with systemic *P. marneffei* infections, but none of the patients in that study received ART simultaneously. Efavirenz is a mixed inducer and inhibitor of CYP3A4, 2C9 and 2C19. Concomitant use of itraconazole and efavirenz may result in subtherapeutic levels of itraconazole[9], so use of an alternate antifungal treatment may be necessary. Alternatively efavirenz can be replaced with a noninducing class of antiretrovirals. The limited number of antiviral drugs limits the choice of treatment of AIDS in China. We chose voriconazole as the alternative treatment option for penicilliosis. Voriconazole is metabolized primarily by CYP2C19, as well as CYP2C9 and CYP3A4[11]. Voriconazole is also known to inhibit these enzymes[11], and the manufacturer reports an extensive list of drugs that interact with voriconazole. Efavirenz reduces the plasma concentration of voriconazole which increases the plasma concentration of efavirenz,

thus dose adjustments for voriconazole and efavirenz may be needed if concomitant use of these two agents is necessary[9]. It is recommended that clinicians increase voriconazole to 400 mg every 12 h and decrease efavirenz to 300 mg once daily before bedtime[9]. Therefore, we increased the voriconazole dose to 400 mg twice daily and decreased the efavirenz dose to 300 mg once daily before bedtime.

A relationship between progression of fungal infection and voriconazole concentrations was demonstrated in several studies[12-14]. They showed that monitoring voriconazole concentration and adjusting dosage to attain an appropriate plasma concentration is necessary to ensure antifungal effect and to avoid toxicity. TDM represents an important process to optimize the outcome of immunocompromised patients receiving triazoles[15]. The patient was treated with amphotericin B followed by 3 mo of high-dose voriconazole therapy, which resulted in a clinical cure. Treatment with high-dose voriconazole was well tolerated, with no discontinuations caused by drug-related adverse events. The patient also received ART containing low-dose efavirenz, without any relapse of *P. marneffei* infection. Treatment with low-dose efavirenz in this patient was also effective, with clinically significant increases in his CD4 counts. The results of this study suggest that high-dose voriconazole is an effective, well-tolerated, and convenient option for the treatment of systemic infections with *P. marneffei* in AIDS patients on efavirenz-containing ART. There may be a role for voriconazole and efavirenz serum concentration monitoring to ensure therapeutic efficacy when the drugs are used concomitantly.

All patients who successfully complete treatment for penicilliosis should receive oral itraconazole as maintenance therapy to prevent relapse[9]. Discontinuation of secondary prophylaxis is recommended for AIDS patients who receive ART and have CD4 count > 100 cells/μL for > 6 mo [9]. This patient did not receive secondary prophylaxis with antifungal drugs. He had a CD4 count > 100 cells/μL 6 mo after initiation of ART. During the follow-up, no relapse of the fungal infection was observed.

In conclusion, this case report indicates that penicilliosis marneffei should be considered in the differential diagnosis of chylous ascites in HIV patients. High-dose voriconazole is an effective, well-tolerated, and convenient option for the treatment of penicilliosis marneffei in AIDS patients on efavirenz-containing ART. Further research into alternative treatment options for penicilliosis in AIDS patients is required.

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Vague relationship between alcohol consumption and metabolic syndrome in nonobese people

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Abstract

Fatty liver, including non-alcoholic fatty liver disease, is closely associated with metabolic syndrome (MS). Thus, the presence of fatty liver without MS in some conditions may be clinically important. Many studies have shown that compared with no or occasional alcohol intake, moderate alcohol consumption is associated with lower prevalence rates of hypertension and type 2 diabetes, and lower levels of circulating C-reactive protein, a valuable marker for MS and insulin resistance. Considering these findings, light to moderate alcohol consumption has theoretical benefits on fatty liver and MS. Fatty liver, including non-alcoholic fatty liver disease, may be more clinically important than MS, particularly in non-obese individuals, because fatty liver can develop before MS in several conditions, such as regular alcohol consumers. Furthermore, most of the currently used MS criteria are unable to detect "true MS" because of variations in multiple factors such as age, height, medications, and complications.

Key words: Alcohol consumption; Non-alcoholic fatty liver disease; Metabolic syndrome; Adult treatment panel Ⅲ

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TO THE EDITOR

We read the recent article by Hamaguchi *et al*[1] with much interest. The authors showed in a cross-sectional study that the effects of alcohol consumption differed between metabolic syndrome (MS) and fatty liver, and that light to moderate alcohol consumption had a favorable effect on fatty liver, but not on MS, in Japanese men and women. Habitual alcohol consumption, which generally impairs fatty acid oxidation and stimulates lipogenesis in the liver[2,3], substantially influences morbidity, all-cause mortality, and cardiovascular mortality rates[4,5]. Thus, the findings reported by Hamaguchi *et al*[1] are impressive in terms of public health and scientific interest. As described by the authors, fatty liver, including non-alcoholic fatty liver disease (NAFLD), is closely associated with MS and is a hepatic manifestation of MS or insulin resistance. This is based on the concept that MS is a leading cause for fatty liver in the cause-effect relationship.

In addition, many studies have shown that moderate alcohol consumption is associated with a low incidence of adverse health outcomes associated with hypertension[5] and type 2 diabetes[6], and with lower levels of cir-

culating C-reactive protein (CRP)[7,8], an important marker for MS, obesity, and insulin resistance[9,10]. The association between moderate alcohol consumption and lower CRP was independent of body mass index (BMI)[9,10] and alcohol-related effects on lipids[10].

Considering these findings, light to moderate alcohol consumption is hypothesized to have beneficial effects on fatty liver and MS. Plausible explanations for the lack of a beneficial effect of light to moderate alcohol consumption on the risk of MS include the characteristics of the subjects studied, the criteria used to define MS, or food patterns specific for light to moderate alcohol consumers. Of particular note, NAFLD also occurs, albeit relatively infrequently, in normal weight people who commonly have metabolic abnormalities and insulin resistance[11,12]. Most of the subjects in the study by Hamaguchi *et al*[1] were of normal weight (mean BMI 23.2-23.5 kg/m^2 for men and 20.9-21.4 kg/m^2 for women), in whom the prevalence of MS is likely to be reduced when waist circumference is dichotomized according to the criteria for MS with a fixed threshold. High waist circumference is necessary for the diagnosis of MS according to the International Diabetes Federation (IDF) criteria for MS, but not the adult treatment panel (ATP)-Ⅲ criteria for MS. This may help to explain the lower prevalence of people fulfilling the IDF criteria than the ATP-Ⅲ criteria in that study. By contrast, fatty liver can be diagnosed using ultrasound techniques irrespective of manufacturer-specific criteria, which likely resulted in the unexpected finding that more than half of the subjects with fatty liver did not have MS.

Furthermore, although the authors claimed in the discussion that there was no significant association between alcohol consumption and BMI > 25.0 kg/m^2, the prevalence of BMI > 25.0 kg/m^2 was actually lower, particularly in females with light to moderate alcohol consumption. This suggests that the observed association might be mediated by lower BMI, which is also affected by dietary patterns and nutrient intake in such alcohol consumers[13,14]. Sub-analyses of subjects divided into specific groups (e.g., overweight/obese groups) or analyses controlling for BMI, waist circumference, and dietary patterns (as assessed by food frequency questionnaires, for example) are needed to clarify whether the observed association is independent of obesity, MS criteria, or dietary patterns.

Taken together, the results of the study by Hamaguchi *et al*[1] provide meaningful insight into the etiological differences in metabolic abnormalities between liver and visceral adipose tissue, in which lipolysis is reduced by acetate produced in the liver[15]. In addition, the results indicate that fatty liver, including NAFLD, may be clinically more important than MS, particularly in non-obese people, because fatty liver can develop before MS in some conditions and that MS criteria are often unable to detect "true MS". The authors propose that fatty liver without MS is an important disease in the general population. Similarly, Stefan *et al*[16] proposed that fatty liver may be more important than visceral fat for the discrimination of benign obesity that is not accompanied by insulin resistance.

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Cambridge, United Kingdom

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Canadian Digestive Diseases Week 2012
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Acknowledgments

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ic 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.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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