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Hepatitis C virus-associated pruritus: Etiopathogenesis and therapeutic strategies

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Abstract

In addition to its contributing role in the development of chronic liver diseases, chronic hepatitis C virus (HCV) infection is associated with extrahepatic manifestations, particularly, cutaneous-based disorders including those with pruritus as a symptom. Pruritus is frequently associated with the development of chronic liver diseases such as cholestasis and chronic viral infection, and the accumulation of bile acids in patients' sera and tissues as a consequence of liver damage is considered the main cause of pruritus. In addition to their role in dietary lipid absorption, bile acids can trigger the activation of specific receptors, such as the G protein-coupled bile acid receptor (GPBA/TGR5). These types of receptors are known to play a crucial role in the modulation of the systemic actions of bile acids. TGR5 expression in primary sensory neurons triggers the activation of the transient receptor potential vanilloid 1 (TRPV1) leading to the induction of pruritus by an unknown mechanism. Although the pathologic phenomenon of pruritus is common, there is no uniformly effective therapy available. Understanding

the mechanisms regulating the occurrence of pruritus together with the conduction of large-scale clinical and evidence-based studies, may help to create a standard treatment protocol. This review focuses on the etiopathogenesis and treatment strategies of pruritus associated with chronic HCV infection.

Key words: Hepatitis C virus; Pruritus; Cholestasis; Autotoxin; Lysophosphatidic acid; PI3 kinase

Core tip: Pruritus is a frequent symptom of chronic liver diseases. Chronic hepatitis C virus (HCV) infection can cause pruritus through both direct and indirect mechanisms. The direct mechanisms include induction of pro-inflammatory cytokines and chemokines as a consequence of the chronic HCV infection. Indirect mechanisms are associated with HCV-induced cholestasis leading to the accumulation of autotaxin, which is responsible for the conversion of lysophosphatidic choline into lysophosphatidic acid. This stimulates epidermal nerve endings leading to pruritus.

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INTRODUCTION

In addition to hepatic damage, chronic hepatitis C virus (HCV) infection is associated with the development of extrahepatic manifestations, particularly, cutaneous-based disorders including pruritus[1,2]. The occurrence of pruritus is associated with skin disorders (*e.g.,* atopic dermatitis and psoriasis) as well as chronic liver disease[3,4]. Pruritus is a the common dermatologic manifestations that is recognized as an early sign of chronic HCV infection[5,6], particularly infections associated with the development of cholestasis[7]. Hepatic disorders such as cholestasis can impair the bile flow from the liver to the duodenum. Consequently, the accumulation of bile acids and bilirubin in patients' plasma and tissues can result in the development of pruritus, which is recognized as a common pathological phenomenon in patients with jaundice[8]. Thus, pruritus in patients with chronic liver disease, particularly those associated with chronic HCV infection, is thought to be a consequence of the molecular action of bile salts. In cholestatic HCV patients, the activation of signaling pathways mediating the occurrence of pruritus is attributed to the toxic compound of the accumulated bile salts both in patient sera and tissues. In addition to its occurrence in patients with chronic viral hepatitis infection, pruritus has also been recognized as a common adverse effect of the treatment of viral hepatitis[9]. Thus, the induction of pruritus in patients with chronic HCV infection is not only the consequences of the infection, but also result from the treatment[10]. Bile salts are known as potent signaling molecules in gastrointestinal organs including liver, bile ducts, and intestine[11]. The accumulation of the toxic bile compounds is thought to trigger the activation of signaling pathways mediating the induction of chronic liver disease-associated symptoms including pruritus. This review focuses on the etiopathogenesis and treatment strategies of pruritus associated with chronic HCV infection.

MECHANISMS OF HCV INFECTION AND TREATMENT- ASSOCIATED PRURITUS

Pruritus in HCV infected patients may be the results of HCV-induced mechanisms, particularly those associated with the induction of cholestasis as well as those associated with the alteration of chemokine and cytokines profile in patients with chronic HCV infection. HCV-associated cholestasis is well described in different reports on liver transplantation. Its occurrence is attributed to viral overload and the continuous suppression of host immune response as a consequence of anti- viral agents[12]. Accordingly, the mechanisms of HCV-associated pruritus are attributed to HCV-induced cholestasis and the induction of interferon-stimulated genes (ISGs) as a result of viral overload[13]. The elevated production of cytokines (*e.g.,* IL-8) and chemokines (*e.g.,* CCL2, CXCL1 and CXCL5) during the course of cholestatic hepatitis C[14-17], is expected to be the main mediators for the induction of HCV-associated pruritus.

Cholestasis like features in patients with chronic HCV infection were first described by Poulson and Christoffersen[18], Who found that the occurrence of pruritus in HCV patients is correlated with significant damage in small or medium sized bile ducts causing the formation of hepatic type lesions, which offer a favorable environment for virus propagation[19]. Bile duct lesions are recognized as a histopathological sign for chronic infection with viral hepatitis[20]. The formation of bile duct lesions is common among patients with chronic HCV infection; however, the pattern of their formation is progressive, and the mechanism is bile duct damage-independent[19]. Although they share common features, histological examination reveals that the bile duct damage related to HCV infection is significantly different than damage related to the primary biliary cirrhosis (PBC)[21,22]. To that end, both the occurrence and the outcome of HCV-associated cholestasis are expected to differ from those associated with PBC. Unlike inflammatory bile duct lesions, HCV-associated lesions are characterized

by the appearance of specific-pathological features such as swelling, vacuolization, nuclear irregularity, and stratification of the epithelial cells[23].

The frequency of the bile duct lesions associated with chronic HCV infection is more significant than that lesions induced by chronic hepatitis B virus (HBV) infection[24]. Even the induction mechanisms of pruritus in HCV-infected patients developing cholestasis differ from those implicated in the regulation of pruritus in non-cholestatic HCV infected patients[25]. Of note, the most described HCV-associated cholestasis was reported in liver transplant recipients as a consequence of the viral overload[26], however, chronic HCV infection-associated pruritus has not yet been described in detail. Although most reported data support an association between HCV-induced cholestasis and the occurrence of pruritus[27-30], the mechanisms remain unknown. The investigation of the clinicodemographic and histological features of HCV-associated cholestasis may help to address the mechanistic role of chronic HCV infection in the development of pruritus.

Although the pruritogenic mediators and their receptors have been identified, there is no consensus regarding the mechanisms regulating the etiopathogenesis of pruritus. Apart from the suspected mediators and their origin, the onset of pruritus is closely associated with the development of intrahepatic cholestasis[31]. Although hepatic disorders such as chronic infection with viral hepatitis, primary biliary cirrhosis or primary sclerosing cholangitis are considered the main cause of intrahepatic cholestasis[32,33], the elevation of bile salts and μ-opioids levels in patients with intrahepatic cholestasis is largely associated with pruritus[34]. To date, the causative link between bile salts levels and severity of pruritus has not yet been investigated. However, pruritus in patients with chronic HCV infection is thought to be a consequences of HCV-induced cholestasis[35,36].

The development of pruritus during the course of liver diseases is regulated by various pruritogenic mediators such as autotaxin (ATX)[37,38]. Thus, in addition to its role in the synthesis of lysophosphatidic acid, ATX may play a role in the regulation of vascular and neural development, wound healing, neuropathic pain, and cancer development[39,40].

Although the ATX- lysophosphatidic acid (LPA)-axis has been identified as a key mechanism in the development of chronic liver diseases, particularly those associated with pruritus, the source of serum ATX has not been determined. To that end, Ikeda *et al*[41] assumed that the elevated activity of ATX in patients' sera may be a consequence of HCV-induced chronic liver diseases. However, the contribution of ATX to the pathogenesis of hepatic fibrosis and the subsequent induction of pruritus suggests that ATX acts as a mediator in the modulation of HCV-induced liver damage leading, to the development of pruritus.

The ATX protein is encoded by the ecto-nucleotide pyrophosphatase/phosphodiesterase 2 (*ENPP2*) gene, whose expression is regulated by the transcription factors v-Jun and signal transducer and activator of transcription 3 (STAT3)[42,43]. The activation of STAT3 by HCV suggests an essential role for HCV-induced STAT3 activation in the regulation of ATX during HCV infection[44,45]. ATX also is a lysophospholipase that mediates the formation of the bioactive lipid mediators such as LPA to form lysophosphatidylcholine (LPC)[46]. Of note, LPA has been suggested to be a highly potent signaling molecule that is involved in the regulation of various cellular functions *via* mechanism mediated by a family of G-protein-coupled receptors[47]. Thus, the ubiquitous and highly expressed level of LPA1 receptor on neurons is thought to play an essential role in the modulation of pruritus during chronic liver diseases including cholestasis and viral infection[48]. The role of LPA in the promotion of pruritus has been demonstrated by the development of dose-dependent scratch in response to the injection of mice with LPA[49]. LPA is a highly potent signaling molecule with the ability to trigger the activation of the transient receptor potential cation channel subfamily V member 1 (TrpV1), known as the capsaicin receptor[50]. The regulation of LPA by PI3k, protein kinase A (PKA) and C (PKC)-dependent mechanisms has been reported[51]. Activation of PI3k, PKA and PKC in response to HCV infection was noted both *in vitro* and *in vivo*[52-54], suggesting a central role for PI3 kinase, PKA and PKC pathways in the regulation of HCV-associated pruritus. More importantly, the role of PKC in the modulation of phorbol ester-induced phosphorylation of LPA1 receptor suggests a contributing role for PKC in the modulation of HCV-associated pruritus[55]. Moreover, in addition to its role in the regulation of TRPV receptor, PI3k has been implicated in the regulation of ATX. Li *et al*[56] demonstrated the importance of the PI3K-AKT-β-catenin pathway in the induction of ATX following the stimulation of THP-1 cells with lipopolysaccharides (LPS). In addition, the involvement of the mitogen activated protein kinase (MAPK) in the regulation of ATX has been established. The activation of c-Jun-N-terminal (JNK) and p38 MAPK has been shown to be essential for the induction of ATX[56], and the activation of mitogen activated protein kinase (MAPK) signaling pathways in response to infection with HCV has been reported in several studies. These include the activation of c-jun-N-terminal kinase (JNK), p38 and extracellular regulated kinase (ERK) pathways[57,58]. Similar to its role in modulation of cholesteric pruritus, the LPA- ATX -axis seems to be essential for the initiation and progression of pruritus in patients with chronic HCV infection[59].

Under pathological conditions elevated bile salts in patient's tissues and sera such as taurourosodeoxycholate (TUDCA), glycochenodeoxycholate are able to signal the activation of ATX-LPA signaling[34], leading to the accumulation of LPA, which in turn can initiate or

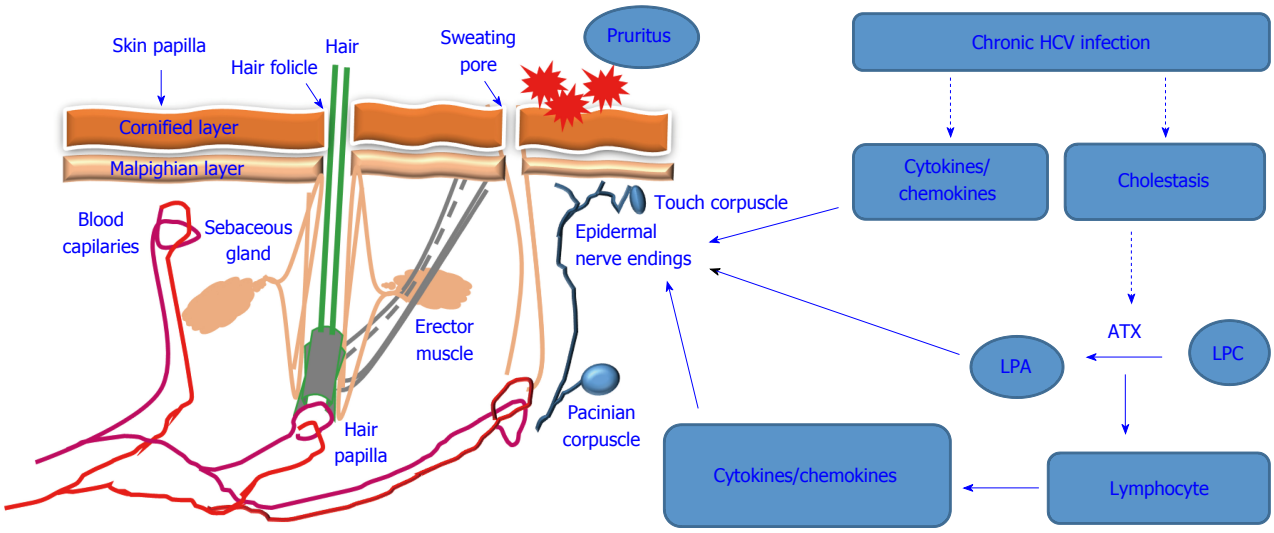

Figure 1 Proposed model for hepatitis C virus-induced pruritus. Chronic hepatitis C virus (HCV) infection can cause pruritus symptom by both direct and indirect mechanisms. The direct mechanisms include induction of pro-inflammatory cytokines and chemokines during the course of the chronic HCV infection. While the indirect mechanisms are associated with HCV-induced cholestasis leading to the accumulation of autotaxin (ATX) that is responsible for the conversion of the lysophosphatidic choline (LPC) into lysophosphatidic acid (LPA) that, in turn stimulates the epidermal nerve ending leading to the occurrence of pruritus symptom. Also, accumulated LPA can promotes the activation of lymphocytes for the production of pro-inflammatory cytokines and chemokines that, in turn stimulates the epidermal nerve ending leading the occurrence of pruritus symptom.

potentiate pruritus. The association between ATX-LAP and cholestatic pruritus was first reported by Kremer *et al*[34] who demonstrated that the intradermal injection of LPA induces itching response in mice. Thus, it is expected that the occurrence of HCV-associated pruritus is a consequence of HCV-induced cholestasis leading to the activation of the ATX-LPA signaling pathway. A proposed diagram is outlined in Figure 1 and describes the possible mechanisms that are thought to be involved in the modulation of HCV-associated pruritus.

THERAPEUTIC STRATEGIES OF HCV-ASSOCIATED PRURITUS

Pruritus is a common symptom that cannot be uniformly classified or quantified to date. As a consequence, its treatment, particularly, in patients with chronic liver disease is a challenge for clinicians and patients. Although there are similarity between pruritus occurrence in patients with cholestatic *vs* noncholestatic liver diseases, no uniformly treatment protocol has been established[60]. Current treatment strategies include topical therapies for mild and localized pruritus as well as systemic therapies for patients with severe or generalized pruritus[61,62].

Based on the fact that histamine-dependent mechanisms are responsible for the occurrence of pruritus associated with urticaria, the clinical utilization of antihistamines has been suggested as a therapeutic option for prurigo nodularis or aqua genic pruritus[63-66]. Thus, once the cause of pruritus has been identified, the implementation of the therapeutic modalities can be determined. The current guidelines suggest the application of topical substances such as capsaicin and calcineurin inhibitors, particularly in patients with chronic pruritus[64]. These substances have been approved for their effects on cutaneous neurons, where they serve as suppressors for chronic pruritus[67,68].

Substances like opioid receptor antagonists, anti-convulsants, selective serotonin re-uptake inhibitors and antidepressants have been recommended[69,70]. Although therapeutic options of pruritus are available, the lack of well-conducted, randomized, controlled studies is an obstacle for the development of an effective and uniform treatment protocol. Cholestyramine is the most recommended first line therapy for pruritus[71], while rifampicin, opiate antagonists and sertraline have been utilized as second-, third-, and fourth-line therapies, respectively[72,73].

In addition to the poor prognosis of patients with pruritus, topical and systemic therapies can offer only short term relief and most are associated with complicated adverse effects, particularly, in patients with chronic HCV infection[3].

Although antiviral therapeutics have improved in recent years, the treatment of HCV patients is associated with a marked increase in dermatological adverse effects, particularly pruritus[74]. In addition, it is difficult to distinguish between treatment- and HCV-induced pruritus in terms of causality. Even the consequences of the interference of anti-viral therapy with HCV-induced extrahepatic manifestations are not predictable. Although the treatment of HCV patients with interferon is commonly associated with local and generalized dermatological side effects, including pruritus[75], the combination of interferon with ribavirin increases the risk of pruritus occurrence[76,77]. For example, the frequency of dermatological adverse effects including pruritus associated with HCV protease

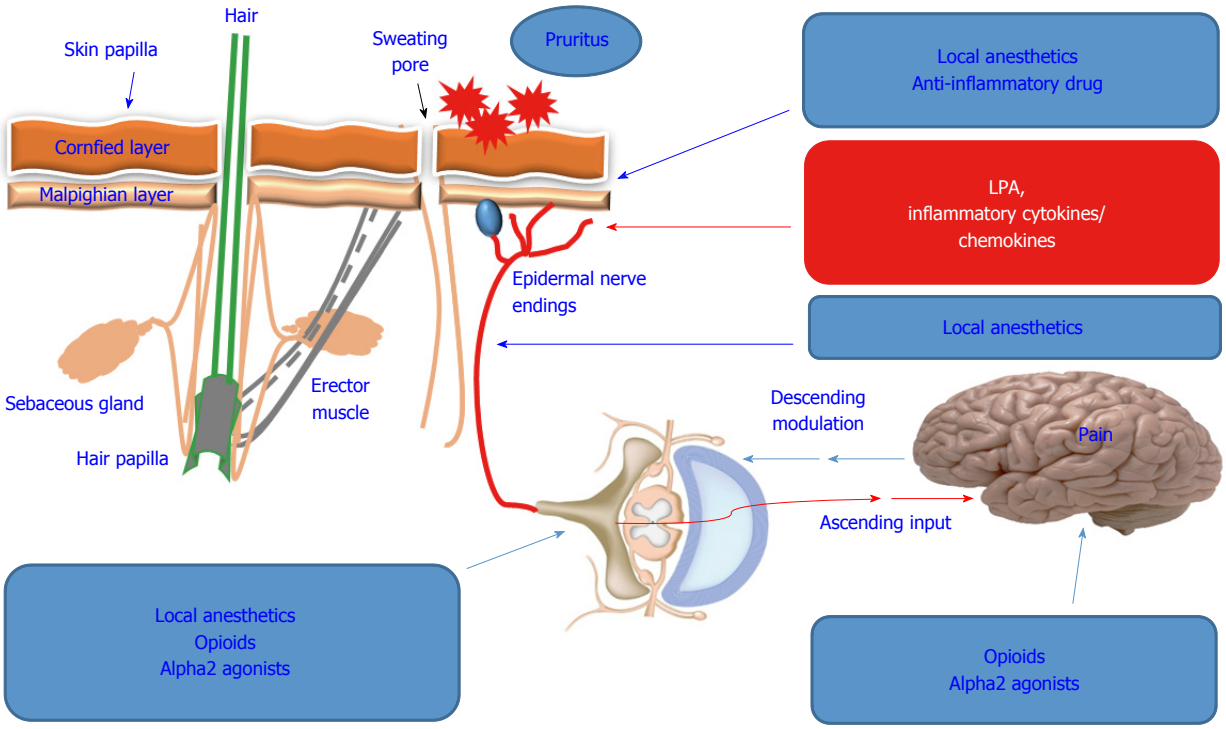

Figure 2 Overview of the treatment options of hepatitis C virus-associated pruritus. Once the underlying factors [elevated concentration of lysophosphatidic acid (LPA), pro-inflammatory cytokines, chemokines] causing pruritus have been determined, the treatment strategy can be based on the reduction of the pain or neutralization of the elevated concentration of LPA, pro-inflammatory cytokines and chemokines using local anesthetics, anti-inflammatory drug, Opioids or alpha2 agonists.

inhibitors as combinatory part of the triple therapy regimen (telaprevir/boceprevir with peginterferon/ribavirin), are higher than those associated with peg interferon/ribavirin regimen alone[78,79]. Some possible therapeutic strategies for pruritus are shown in Figure 2.

CONCLUSION

Both HCV infection and its treatment are associated with significant dermatological manifestations, particularly pruritus. In order to treat pruritus in patients with HCV infection an effective management strategy is needed to limit the severity of HCV-associated pruritus. The elevation of ATX in patients' sera may be the cause for the increase of LPA levels thought to be responsible for the simultaneous promotion of itching signaling and inhibition of the pain signaling. Thus, the investigation of the molecular mechanisms, essential for modulating the cross-talk between the activation of ATX- LPA receptor axis and the occurrence of pruritus during infection and treatment of HCV patients will drive the development of novel therapies for neglected symptoms of HCV including pruritus. Larger clinical studies will help to outline the efficacy of available anti-pruritic therapeutics.

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REVIEW

Is endoscopic ultrasound examination necessary in the management of esophageal cancer?

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Abstract

Despite substantial efforts at early diagnosis, accurate staging and advanced treatments, esophageal cancer (EC) continues to be an ominous disease worldwide. Risk factors for esophageal carcinomas include obesity, gastroesophageal reflux disease, hard-alcohol use and tobacco smoking. Five-year survival rates have improved from 5% to 20% since the 1970s, the result of advances in diagnostic staging and treatment. As the most sensitive test for locoregional staging of EC, endoscopic ultrasound (EUS) influences the development of an optimal oncologic treatment plan for a significant minority of patients with early cancers, which appropriately balances the risks and benefits of surgery, chemotherapy and radiation. EUS is costly, and may not be available at all centers. Thus, the yield of EUS needs to be thoughtfully considered for each patient. Localized intramucosal cancers occasionally require endoscopic resection (ER) for histologic staging or treatment; EUS evaluation may detect suspicious lymph nodes prior to exposing the patient to the risks of ER. Although positron emission tomography (PET) has been increasingly utilized in staging EC, it may be unnecessary for clinical staging of early, localized EC and carries the risk of false-positive metastasis (over staging). In EC patients with evidence of advanced disease, EUS or PET may be used to define the radiotherapy field. Multimodality staging with EUS, cross-sectional imaging and histopathologic analysis of ER, remains the standard-of-care in the evaluation of early esophageal cancers. Herein, published data regarding use of EUS for intramucosal, local, regional and metastatic esophageal cancers are reviewed. An algorithm to illustrate the current use of EUS at The

University of Texas MD Anderson Cancer Center is presented.

Key words: Esophageal squamous cell carcinoma; Endosonography; Echoendoscope; Esophagus cancer; Esophageal adenocarcinoma

Core tip: Endoscopic ultrasound (EUS) is not necessary or adds little in management of many cases, such as, in patients with distant metastases or following pre-operative (neoadjuvant) chemoradiotherapy. EUS is the most sensitive test to exclude local tumor invasion and regional nodal disease that would make endoscopic resection (ER) unsafe or unnecessary. Thus, for early esophageal cancer staging, EUS followed by ER and histopathologic analysis, remains the standard-of-care. For a minority of locally advanced cancers, EUS-fine-needle aspiration can define the radiotherapy field by providing tissue samples of suspicious lymph nodes that are remote from the primary tumor.

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INTRODUCTION

Dysphagia to solid food is the most common presenting symptom of patients with advanced esophageal cancer (EC). As the sixth most lethal cancer diagnosed worldwide, there are more than 450000 cases of EC diagnosed annually[1,2]. The American Cancer Society estimates 16910 cases of EC will be diagnosed in the United States in 2016[2-4]. The incidence of esophageal adenocarcinoma (EAC) has increased six-fold from 1975 to 2000, making it the most rapidly increasing cancer incidence in America[5,6]. Obesity, defined as body mass index > 30 kg/m^2, has been strongly linked to EAC, with an odds ratio of 16.2 (95%CI: 6.3-41.4) compared with the leanest persons with body mass index < 22 kg/m^2[7]. Meanwhile, the incidence of squamous cell carcinoma (SCC) in the US is declining[8].

Men are more commonly effected by EC; the median age at diagnosis is 67 and lifetime incidence is 1 in 125 (a rate 3 to 4 times higher than for women)[3,9]. Fifteen percent of EC are diagnosed in people younger than 55 years old. Additional risk factors for EC depend upon histologic subtype and include: European ancestry, gastroesophageal reflux disease, sleep apnea, and intestinal metaplasia (Barrett's esophagus) for EAC; *vs* African ancestry, tobacco smoking, distilled alcohol consumption, palmoplantar keratosis (tylosis), and Plummer-Vinson syndrome for SCC[2,4,10-13]. Less common EC (such as sarcoma, melanoma, and lymphoma) may occur, although data regarding use of endoscopic ultrasound (EUS) in these cancers are limited.

The majority of patients (about 60%) have advanced cancer when diagnosed, as early EC are frequently asymptomatic[14,15]. Five-year relative survival rates for localized, regional, and distant stages of all types of esophageal cancers are currently estimated at 40%, 21%, and 4%, respectively[3]. Overall five-year survival rates for patients with EC have improved four-fold over the past four to five decades (Figure 1)[3,9]. This substantial improvement in life expectancy likely represents advances in accurate staging and treatment by dedicated professionals with research support from cancer societies, patient groups, industry, and local and national agencies. Per the National Institutes of Health (NIH)/National Cancer Institute, resource utilization and expenditures in 2010 for EC topped $1.3 billion, which is projected to increase to $1.8 billion by 2020[16].

Since the mid-1980s, EUS has evolved to occupy an important niche in EC staging, particularly in evaluating tumor invasion and surrounding lymph nodes. According to NIH/Surveillance, Epidemiology, and End Results program data, local and regional esophageal carcinomas, which are most amenable to EUS evaluation, are found in half of the patients (Figure 2)[9]. With radial and linear endoechoscopes, the five major layers of the esophagus are visible (Figure 3) and represent: (1) the innermost superficial mucosa or squamous epithelium; (2) the deep mucosa or lamina propria; (3) the submucosa, which contains an innumerable number of lymphatics, blood vessels, nerves and mucous glands, and is the most common route of extra-esophageal cancer spread; (4) the hypoechoic muscularis propria; and (5) the hyperechoic adventitia. Cytology specimens may be obtained from suspicious nodes using fine-needle aspiration (FNA).

EC JARGON

The seventh edition of the tumor-node-metastasis (TNM) staging system, developed by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control, is the most commonly used staging system[17-19].

In general, *localized disease* refers to esophageal carcinoma, including intra-esophageal (T1-2) and penetrating cancers (T3-4, also known as, *locally advanced* cancers). *Regional disease* describes surrounding lymph node involvement (N-stages), such as celiac and thoracic lymph nodes. Together *locoregional* cancers fall into the AJCC anatomic stage/prognostic group Ⅰ-Ⅲ (so called stage Ⅰ-Ⅲ cancers; Figure 4). *Distant/metastatic disease* (M1) is identified by cancer spread to adjacent organs, distant lymph nodes (*i.e.*,

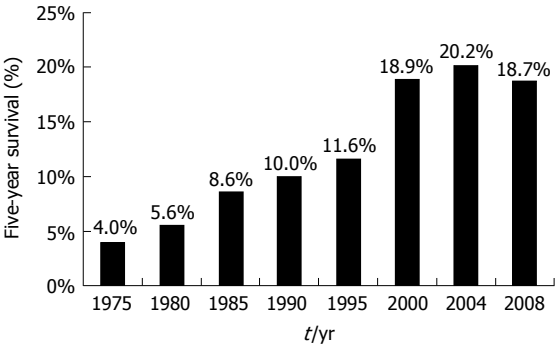


Figure 1 Five-year survival trends in esophageal cancer. Data from Surveillance, Epidemiology, and End Results Cancer Statistics Factsheets: Esophageal Cancer. National Cancer Institute. Bethesda, MD[9].

lungs or supraclavicular lymph nodes) or below the diaphragm (*i.e.*, liver or mesenteric lymph nodes); stage Ⅳ is the anatomic stage/prognostic group[20]. While the TNM components for staging EAC *vs* SCC are identical, the AJCC anatomic stage/prognostic groups differ depending on histologic type because of differing mortality rates between EAC and SCC stages.

An understanding of evolving TNM sub-stages is necessary, such as, *EUS* stage (*i.e.,* uT4), *vs clinical* stage [*i.e.*, cT4; based upon pre-surgical evaluation, including endoscopic resection (ER)], *vs postoperative* stage (*i.e.*, pT4; based upon pathologic examination of surgical specimen), *vs neoadjuvant postoperative* stage (*i.e.*, ypT4)[14,21]. Cancers involving the submucosa (T1b) are further divided into *sm1* to *sm3* stages based upon the depth of invasion[22].

LITERATURE SEARCH

A literature search was completed using Google, PubMed and Cochrane Library for combinations of "EUS" and "EC". Study titles and abstracts were screened for relevance. Then, full text publications in English were selected for in-depth review and the references were further scrutinized to identify pertinent studies.

DISCUSSION

In 1980, a group of investigators from SRI International (formerly of Stanford University) and Mayo Clinic developed the "Ultrasonic Endoscope" prototype. It was felt with planned improvements in size and design that this device "should improve the investigation of cardiac, gastrointestinal, and genitourinary diseases"[23]. In 1986, EUS was used for evaluation of lesions of the upper gastrointestinal tract by Gordon, Rifkin and Goldberg, who described the endosonographic anatomy of the upper gastrointestinal tract in 25 patients[24]. Since then, a median of 50 manuscripts per year have been indexed for PubMed on the topics of "EC" and "EUS" (total 1286, range 1-83).

Radial EUS scopes provide a circumferential view of the visceral wall and surrounding tissues, similar to axial images obtained by computed tomography (CT). Often considered easier to interpret by early users, radial EUS images are more similar to transverse/cross-sectional imaging displays. The linear array echoendoscope is commonly used for tissue acquisition *via* fine needle aspiration (FNA) or biopsy, as it allows for direct needle visualization during passes into the target abnormality[25,26].

Higher frequency EUS devices yield increased superficial anatomic resolution, but lack deeper sonographic tissue penetration, limiting regional assessment. For example, most radial and curvilinear array echoendoscopes operate at frequencies of 7.5-12 megahertz (MHz), and penetrate 3-4 cm of surrounding tissue with good resolution. Very high frequency, through the scope, EUS miniature probes (mini-probes) can readily distinguish seven layers of the esophagus with a frequency of 20-30 MHz. However, useful sound wave breadth and depth with EUS mini-probes are substantially reduced and inadequate for cancer staging.

If malignant lesions extend to the fundus or gastric cardia, or if intra-esophageal cancers are small; conventional radial or linear EUS may not accurately evaluate the depth of the lesion due to the technical difficulty in reaching or locating the lesion by the echoendoscopes. In those cases, a high frequency EUS mini-probe may be employed under endoscopic guidance to most accurately stage the tumor. For example, in distinguishing T1a *vs* T1b intramucosal lesions, high frequency mini-probes have been shown to more accurately assess depth of invasion in comparison to radial or linear EUS. The disadvantage of using an EUS mini-probe is the limited sonographic width and depth, which precludes a comprehensive survey of regional lymph nodes. Furthermore, if the lesion is large (*i.e.*, 5 cm), EUS mini-probes cannot expediently assess penetration depth of the entire lesion.

EUS FOR EC STAGING

When assessed by EUS, malignant lymph nodes classically originate near the intraluminal cancer, and appear as round, hypoechoic nodes with smooth borders that may be enlarged (> 10 mm)[27]. Per 2016 guidelines published by the National Comprehensive Cancer Network, once distant metastases from EC have been excluded, EUS should be employed for evaluation with possible FNA cytologic sampling[28]. At the time of diagnosis, a contrast-enhanced CT scan of the chest and abdomen is recommended to assess for distant metastases (*i.e.*, to liver, lung, bone or adrenals), thereby distinguishing M0 *vs* M1 stages. Following EUS, the optimal treatment regimen changes significantly based on the presence of tumor invasion into the submucosa, detection of regional lymph node malignant spread or distant malignancy. EUS is the most sensitive test for locoregional staging of EC,

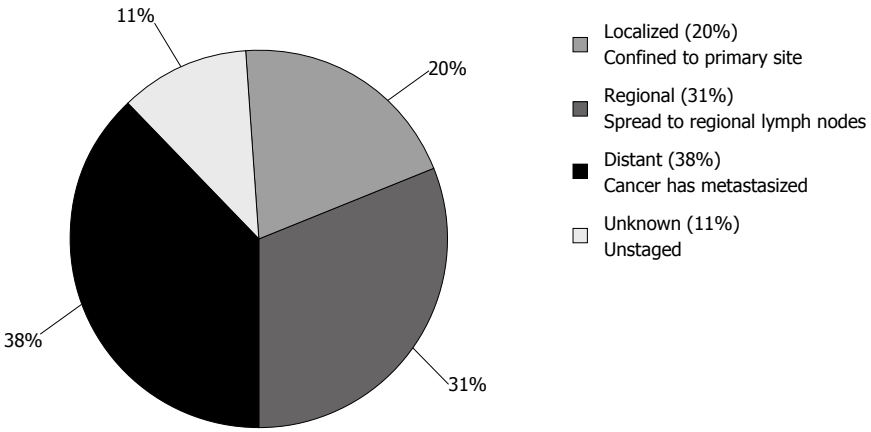


Figure 2 Esophageal cancer stages at diagnosis. Surveillance, Epidemiology, and End Results Cancer Statistics Factsheets: Esophageal Cancer. National Cancer Institute. Bethesda, MD[9].

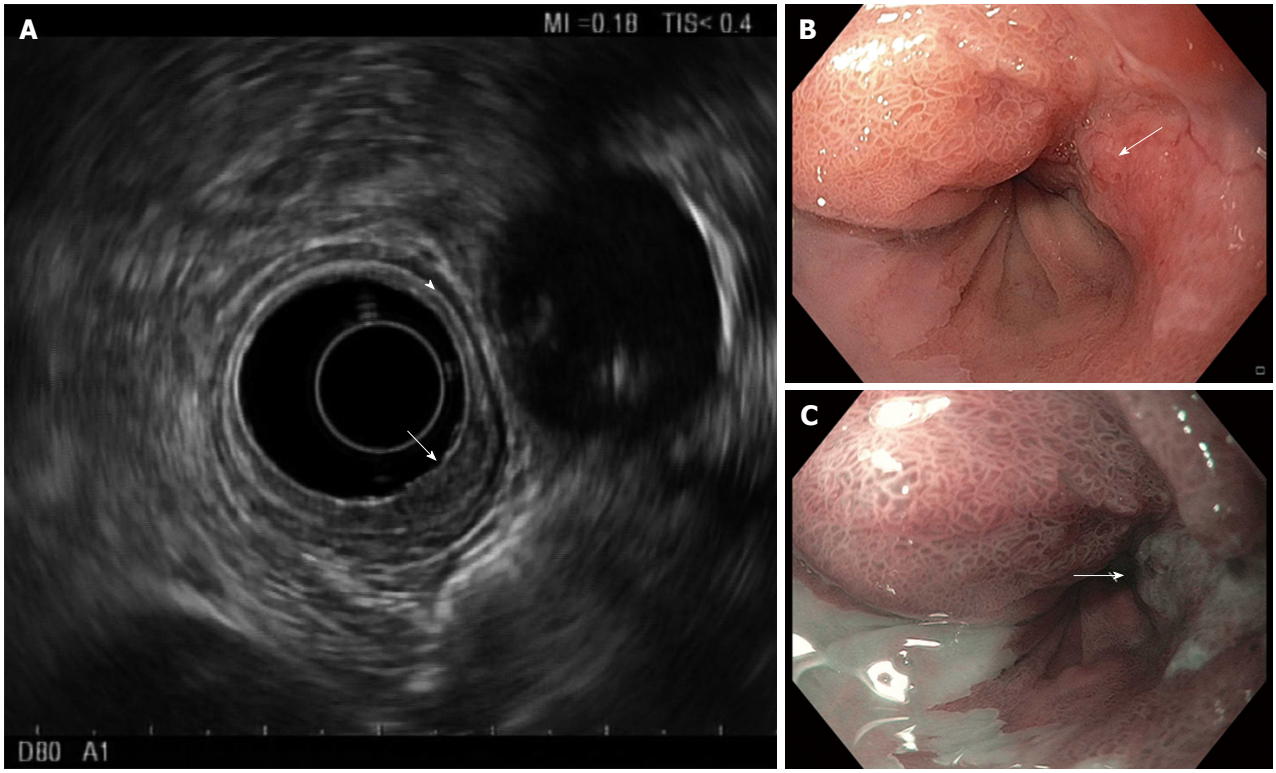


Figure 3 Endosonography of distal esophageal adenocarcinoma. A: Five layers of the esophagus are visible with standard frequency (7.5 MHz) endoscopic ultrasound. From innermost to outermost: the hyperechoic (bright) superficial mucosa, hypoechoic (dark) deep mucosa, the submucosa (arrowhead), followed by the muscularis propria (hypoechoic, very dark), and adventitia (outer echogenic layer). The T1b adenocarcinoma (arrow) causes thickening and distortion of the mucosal layers and submucosa, without invasion of the muscularis propria; B: White-light; and C: Narrow band images are presented for comparison, with arrows to mark the cancer.

and maintains a critical role in developing an accurate therapy plan[27,29-35]. EUS influences the treatment of a significant, although small portion of patients with early disease, as particular attention may be given to the depth of esophageal invasion and celiac lymph node axis, which is thought to act as a gateway for distant metastatic spread[36-38]. Current data confirm the number of malignancy-involved lymph nodes is more important for prognosis than regional anatomic location, which further substantiates EUS-FNA use[39-42]. The results of meta-analyses focused on EUS are summarized in Tables 1 and 2.

DISTANT METASTATIC EC STAGING

Detection of distant metastases is improved with the use of positron emission tomography (PET), when compared to CT and EUS[29,43,44]. Use of PET and/or CT may spare the need of performing EUS when distant metastases are detected, as evaluation of the regional

Table 1 Baseline characteristics of meta-analyses on endoscopic ultrasound in esophageal carcinoma

Ref.	Timeframe	Patients (No. studies; P/R)	EUS types (MHz)	Study criteria
Puli *et al*[52], 2008	1986-2005	2020 (25; 10/15)	NR	EUS accuracy confirmed by surgery in distal and celiac axis lymph node metastasis
van Vliet *et al*[29], 2008	1985-2005	4713 (84; NA[1])	NR	Comparison of diagnostic staging performance of EUS, CT and PET
Puli *et al*[32], 2008	1986-2005	2558 (49; 16/33)	NR	EUS studies on T and N staging confirmed by surgery
Thosani *et al*[30], 2012	1988-2008	1019 (19; 12/7)	Radial and/or mini-probe (7.5-30)	EUS in T1a *vs* T1b lesions compared to histology by EMR or surgery/excluded studies on < 15 patients, or with suspicious lymph nodes (> 1 cm)
Sun *et al*[76], 2015	1992-2013	724 (16; 10/6)	Radial, linear and/or mini-probe (5-20)	EUS staging accuracy after neoadjuvant chemotherapy. Surgery was confirmatory test in all included studies.
Qumseya *et al*[36], 2015	1994-2012	656 (11; 4/7)	Radial, linear and/or mini-probe (NR)	EUS in BE and HGD, or esophageal adenocarcinoma (EAC)/excluded studies on advanced esophageal cancer

[1]Did not report retrospective or prospective nature of studies. References[29,30,32,36,52,76]. P/R: Prospective to retrospective ratio; NR: Not reported; BE: Barrett's esophagus; HGD: High-grade dysplasia; EAC: Esophageal adenocarcinoma; EUS: Endoscopic ultrasonography; CT: Computed tomography; PET: Positron emission tomography; NA: Not applicable.

Table 2 Outcomes of meta-analyses on endoscopic ultrasound in esophageal carcinoma

Ref.	Sensitivity (95%CI)	Specificity (95%CI)	Heterogeneity	Conclusion/interpretation
Puli *et al*[52], 2008	Celiac N = 66% (62-71); M = 67% (63-72)	Celiac N = 98% (97-99); M = 98% (97-99)	Insignificant: $P > 0.10$ for all estimates	EUS has low sensitivity and utility for staging metastases to celiac lymph nodes and distant sites.
van Vliet *et al*[29], 2008	N staging: EUS = 80% (75-84); CT = 50% (41-60); PET 57% (43-70)	N staging: EUS = 70% (65-75); CT = 83% (77-89); PET = 85% (76-95)	NR	EUS, CT, and PET have distinctive roles in staging. For distant metastases, PET probably has higher sensitivity than CT. No evidence of publication bias in CT *vs* EUS studies; other analyses too small to test.
Puli *et al*[32], 2008	T1 = 82% (78-85); T4 = 92% (89-95); w/o FNA N = 85% (83-86); w/ FNA N = 97% (92-99)	T1 = 99.4% (99-100); T4 = 97% (97-98); w/o FNA N = 85% (83-86); w/ FNA N = 96% (91-98)	Insignificant: $P > 0.10$ for all estimates	EUS has excellent accuracy, with better performance in T4 over T1 disease (AUC 0.94-0.98). N staging is improved with FNA use (AUC 0.99 *vs* 0.89).
Thosani *et al*[30], 2012	T1a = 85% (82-88); T1b = 86% (82-89)	T1a = 87% (84-90); T1b = 86% (83-89)	Significant; $P < 0.05$ by χ^2	EUS has good accuracy for T1a and T1b lesions; AUC ⩾ 0.93. Technical factors can affect the diagnostic accuracy of EUS.
Sun *et al*[76], 2015	T1 = 23% (16-32); T2 = 29% (19-41); T3 = 81% (72-88); T4 = 43% (31-56); N = 69% (58-79)	T1 = 95% (93-97); T2 = 84% (77-88); T3 = 42% (33-52); T4 = 96% (94-97) N = 52% (42-62)	Significant; I^2 = 0%-75% depending on stage (table presented in article)	EUS has modest accuracy after neoadjuvant therapy; AUC for T staging ranges from 0.64 to 0.84, while AUC for N-staging was 0.64.
Qumseya *et al*[36], 2015	⩾ T1sm = 56% (47-65)	>/-T1sm = 89% (85-92)	Significant; I^2 = 82%; Q = 56, $P < 0.0001$	Advanced disease detected in 14% (95%CI: 8%-22%; $P < 0.0001$). The NNT (performing EUS) to identify 1 case of advanced disease was 7 (95%CI: 5-13). EUS significantly changes therapeutic approach.

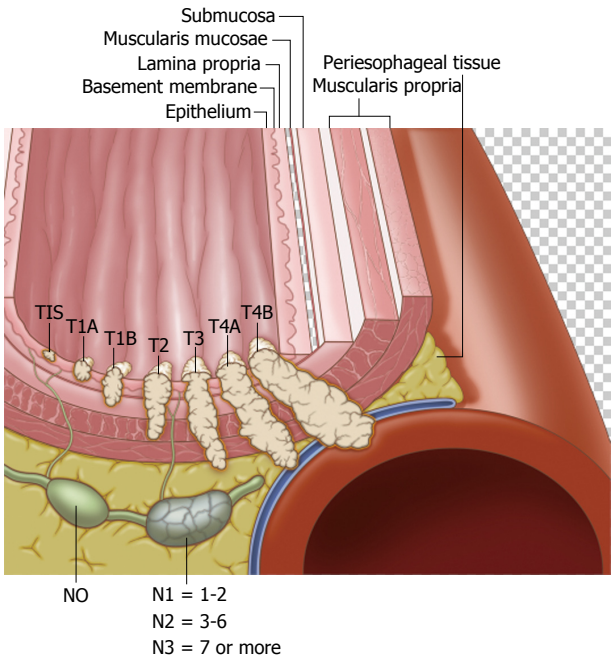
NR: Not reported; EUS: Endoscopic ultrasonography; CT: Computed tomography; PET: Positron emission tomography; AUC: Area under the curve.

lymph nodes is not necessary prior to initiation of palliative chemotherapy or chemoradiotherapy. When indicated, EUS may be used to confirm the presence distant metastases and exclude benign findings. Confirmation or exclusion of nodal involvement by EUS will help calculate the exact radiation field, especially when the lymph node is away from the primary tumor, thus minimizing radiation induced complications.

LOCOREGIONAL EC STAGING

Use of CT or PET is considered inadequate for staging celiac and mediastinal lymphadenopathy[26,29,31]. PET may not be necessary for clinical staging if distant metastatic disease is detected on CT scan. Conversely, in patients with superficial EC (T1 disease) use of PET carries risk of over-staging due to false-positive regional/distant enhancement[21].

For evaluation of regional lymph nodes, the combination of EUS and CT (EUS-CT) has been shown to be more accurate than either modality alone, and EUS-CT outperformed PET, 69% *vs* 48%, respectively. The sensitivity of combined EUS-CT was 83% *vs* 22% for PET[45]. Some data support PET scan consideration for: (1) patients with locally advanced (T2 or greater) cancers following EUS (with or without ER); (2) those with positive regional lymph nodes (N1 or greater) detected by EUS-FNA; and (3) patients in whom



Visual Art: ©2016 The University of Texas MD Anderson Cancer Center

Figure 4 Locoregional esophageal cancer staging.

complete EUS examination was not possible (*i.e.*, due to severe malignant stenoses)[29,46].

When PET scan is performed before EUS, it can provide a road map to potentially positive lymph nodes and decrease or obviate the need for stricture dilation, thus lessening the risk of esophageal perforation. One in three malignant stenoses may initially be too narrow for the EUS scope to traverse[47,48]. Incremental dilation of severe malignant strictures often is not necessary, as completion of EUS may not change treatment[49].

SUPERFICIAL EC STAGING

Intramucosal cancers (T1a) have a 6%-10% risk of metastasis, while invasion into the submucosa (T1b) increases the risk of metastasis to 19%-23%[50] In a meta-analysis including 1019 patients with T1 (superficial) esophageal cancers, Thosani *et al*[30] evaluated the diagnostic accuracy of EUS in differentiating mucosal (T1a) *vs* submucosal invasion (T1b) by EC. Nineteen international studies (12 prospective, 7 retrospective) conducted between 1988 and 2008 were included. Studies using mini-probe EUS dominated (14 mini-probe, 9 radial scopes; five studies used both) in comparing findings to the gold-standard, surgical resections of SCC and/or EAC (with or without endoscopic mucosal resection). The area under the curve for pooled sensitivity and specificity was at least 0.93 for both T1a mucosal and T1b submucosal lesions. The pooled sensitivity, specificity of EUS for T1a staging were 0.85 (95%CI: 0.82-0.88), 0.87 (95%CI: 0.84-0.90); and for T1b staging a sensitivity 0.86 (95%CI: 0.82-0.89) and specificity of 0.86 (95%CI: 0.83-0.89) were estimated. Heterogeneity was present among the studies, as the χ^2 *P* value for heterogeneity was < 0.05 for all pooled estimates.

MULTIMODAL STAGING OF LOCAL ESOPHAGEAL CANCERS

ER should be considered with EUS for staging superficial EC (T1 lesions, generally < 2 cm), which provides locoregional staging and histologic assessment of primary tumor depth and lymphovascular invasion. Due to lack of a singular near perfect test, combining EUS with ER functions as a "double check" to prevent staging errors by sonographic or histologic evaluation[51,52]. Superficial tumor invasion, which may be difficult to visualize by standard radial EUS (7.5-12 MHz) due to lower resolution, can be more accurately assessed by histology of ER specimens[51-53]. The addition of EUS to ER confers the benefit of nodal assessment with possible FNA sampling. Furthermore, EUS excludes deeper invasive cancer (T2 or deeper lesions) that would make ER unsafe and unnecessary[32,54].

For confirmed T1a cancers, ER followed by ablation of high-risk residual tissue *via* radiofrequency ablation or photodynamic therapy, offers survival rates similar to surgery[55-63]. EUS prior to ER is especially important in patients with large intraluminal tumors[64]. When EUS is combined with cross-sectional imaging, patients are considered to have completed clinical staging, thereby identifying stage T2 or T3 patients who may benefit from radical esophagectomy with extended lymphadenectomy[58,61,65,66].

ENDOSONOGRAPHY FOR ESOPHAGOGASTRIC JUNCTION CANCERS

Data regarding the utility of EUS in cancers of the EGJ is limited, and liberal use of ER has been suggested[53]. In a study by Dhupar *et al*[53] 181 patients with EGJ cancers (98% adenocarcinomas) were included that underwent EUS staging and resection (surgical or endoscopic) without neoadjuvant therapy from 1995 to 2014. The authors found that EUS accuracy at the EGJ was inferior to that of other regions of the esophagus when compared to resected specimens; with 23% under-staged and 29% over-staged by EUS. The negative effect was particularly pronounced with smaller, early EGJ cancers being more frequently over-staged.

NEOADJUVANT THERAPY PRIOR TO SURGERY

Neoadjuvant (induction) therapy may be given pre-operatively to patients with locally advanced or locoregional disease, due to improvement in survival

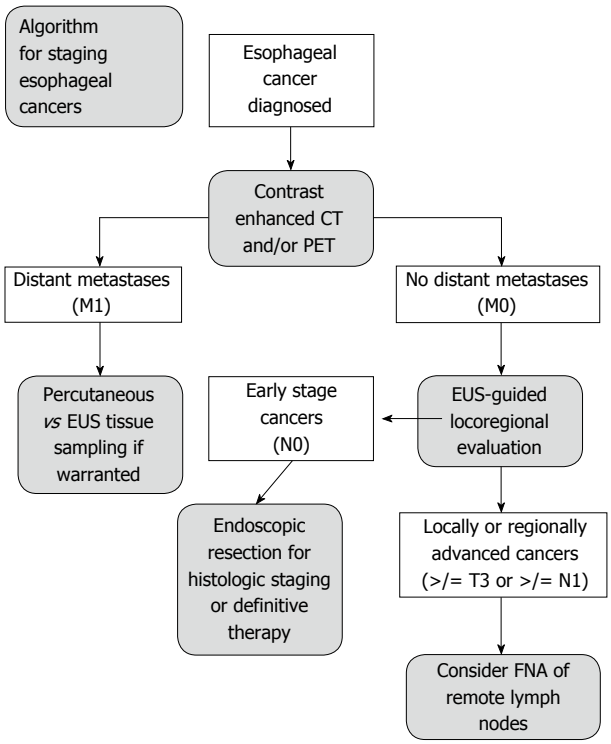


Figure 5 Algorithm for staging esophageal cancers proposed by DaVee and Lee. Esophagogastric junction cancers excluded. EUS: Endoscopic ultrasound with selective fine-needle aspiration; T, N, M: Tumor, node, and metastasis stages; CT: Computed tomography; PET: Positron emission tomography; FNA: Fine-needle aspiration.

compared to surgery alone for cancers of the esophagus and EGJ[67-73]. Data suggest the accuracy of EUS after neoadjuvant chemotherapy for locoregional cancers is subpar[35,74-76]. The reasons for lower accuracy of EUS after induction therapy are due to regional changes in response to healing and inflammation.

A meta-analysis by Sun *et al*[76] evaluated the staging accuracy of EUS for EC after preoperative chemotherapy. The authors included 724 patients (69% with adenocarcinoma) from sixteen studies (ten prospective, six retrospective) conducted between 1992 and 2013. Most procedures were performed with 7.5 and 12 MHz echoendoscopes. Pooled estimates of EUS test characteristics were used in either fixed-effects or a random-effects model, depending on study heterogeneity. EUS was most sensitive in localized staging of T3 lesions at 81% (95%CI: 72%-88%) with 42% specificity (95%CI: 33%-52%). EUS sensitivity in stages T1, T2, and T4 was poor, with T1 lesions estimated at 23% (95%CI: 16%-32%) and 95% specificity (95%CI: 93%-97%); T2 lesions at 29% (95%CI: 19%-41%) and specificity 84% (95%CI: 77%-88%), and finally T4 lesions at 43% (95%CI: 31%-56%) with specificity 96% (95%CI: 94%-97%). When assessing for regional lymph node spread, EUS had sensitivity 69% (95%CI: 58%-79%) and specificity 52% (95%CI: 42%-62%). Overall, EUS was found to be moderately accurate after neoadjuvant therapy; AUC for T staging ranged from 0.64 to 0.84, while the AUC for N-staging was 0.64. EUS accuracy did not improve with time following neoadjuvant chemotherapy in a subgroup analysis. Therefore, EUS should only be performed in specific cases after neoadjuvant therapy, such as FNA of a suspicious lymph node that would change management.

ADJUVANT THERAPY AFTER SURGERY

Postoperative (adjuvant) therapy has been shown to improve survival and reduce the risk of local recurrence, in patients with positive resection margins, or with positive lymph nodes in cancers of the esophagus or EGJ[77-81]. However, an intensified adjuvant chemo-radiation regimen found no improvement in disease-free or overall survival in patients with EGJ and gastric adenocarcinomas[82]. A recent review concluded there are no validated adjuvant treatment strategies for SCC[82]. PET may be used to evaluate for cancer response and recurrence after multimodal therapy[83]. Data regarding the utility of EUS following surgery and adjuvant chemoradiation are limited.

RADIATION THERAPY FIELD DELINEATION

Precise EC tumor measurements are important for accurate radiation targeting and treatment. PET has been found to be accurate for evaluation of tumor length in esophageal cancers[84-86]. In a retrospective study of 53 patients by Rollins *et al*[84] PET and EUS were compared to surgical pathology for measurement of tumor length. Both PET and EUS correlated significantly with resection specimen tumor length; PET (Pearson R = 0.5977, 95%CI: 0.390-0.747, $P < 0.0001$) *vs* EUS (Pearson R = 0.5365, 95%CI: 0.311-0.705, $P < 0.0001$). In a subgroup analysis, after excluding tumors with significant response to neoadjuvant chemotherapy, both PET and EUS again correlated significantly with tumor length; PET (R = 0.5651, $P = 0.0005$) *vs* EUS (R = 0.4637, $P = 0.0057$). These data suggest EUS or PET may reliably be used in evaluation of tumor length for radiotherapy field definition, and the addition of EUS to PET imaging in these cases is low-yield. However, in patients with suspicious (but not-diagnostic) lymphadenopathy EUS-FNA may further define the radiation field (Figure 5).

COST ANALYSIS

EUS has been shown to be economical in multiple studies. For initial staging, EUS was found to be the least costly strategy by Hadzijahic *et al*[87] as EUS found T4 and/or M1 disease more frequently than CT (44% *vs* 13%, $P < 0.0001$). Furthermore, in patients without metastatic disease, EUS was found to be the most cost effective EC staging modality at $13811, *vs* CT-guided FNA $14350 and surgery $13992[88]. Pretreatment EC

staging by EUS was found to save an average of $3443 per patient, by identification of stage Ⅰ and stage Ⅳ tumors, which prevented unnecessary neoadjuvant chemoradiotherapy or surgery, respectively[89]. Furthermore, selective use of FNA for suspicious lymph nodes during EUS, resulted in reduced costs compared to routine FNA[34], however the effect on patient-outcomes remains to be determined.

EMERGING ADJUNCTS TO SONOMORPHOLOGIC EVALUATION

Generally, healthy tissue is softer and more elastic than cancerous tissues. Elastography, or elasticity imaging, may be combined with ultrasound or magnetic resonance modalities and is a non-invasive method to measure the flexibility of tissues. There are many elastography techniques under investigation, such as quasistatic/strain imaging and shear wave elasticity imaging; however, all techniques rely on measuring the degree of distortion within the tissue. Much like Doppler ultrasound, which uses color to highlight flow in vessels, EUS elastography provides the operator with a colorized image displaying the variation of elasticity of tissues. Typically, when using EUS elastography, firm tissues appear blue to violet, while softer tissues appear red, yellow or green. Elastography-enhanced EUS has been shown in small studies to improve the diagnostic accuracy of regional lymph node staging in EC patients when compared to standard EUS sonomorphologic evaluation[90-92]. Currently, the role and clinical efficacy are undefined for EUS elastography in EC, although the we speculate the technique could replace FNA cytology, as it is noninvasive and possibly lower risk for the patient.

When unique contrast agents are parenterally administered, contrast-enhanced harmonic EUS (CEH-EUS) may be used to further characterize the microvascular pattern of lesions identified by standard imaging modalities[93]. In 2016, the United States Food and Drug Administration approved the use of sulfur hexafluoride lipid-type A microspheres (Lumason®) for ultrasonographic characterization of focal liver lesions. CEH-EUS has not been rigorously studied in esophageal carcinomas, but preliminary data suggest contrast-enhanced images are of limited value due to the relative avascularity of common esophageal malignancies[93,94].

Tridimensional (3D) EUS may be used alone, or with ultrasonographic contrast, to evaluate the invasion depth of tumors. The 3D images are thought to more accurately convey the relationship of cancers to nearby organs and vessels, and may reduce the operator-dependent error that is inherent to standard EUS[95].

LIMITATIONS

Studies on EUS techniques are often limited by several factors, such as changes in practice patterns, radiographic or pathologic techniques, and sonography equipment; which has considerably evolved from 1980 to the current era. Testing characteristics for EUS vary widely depending on the type of equipment used (frequency of ultrasound probe, FNA *vs* fine needle biopsy, gauge of needles, and expertise of the endosonographer, cytotechnician, and/or pathologist).

Squamous cell esophageal cancers are more common in Japan, which may contribute to variation in EUS diagnostic accuracy and practice patterns in comparison to the United States[96]. Japan Esophageal Society guidelines suggest *sm1* lesions (T1b cancers with less than 200 micrometers invasion into submucosa) may be resected endoscopically, in contrast to EC invading the middle or deep submucosa (*sm2* or *sm3* lesions)[22,96].

In interpreting meta-analyses, the biostatistical model chosen (fixed-effects *vs* random effects models) and heterogeneity (variation) among studies may confound analysis and interpretation[97]. Higher levels of heterogeneity in meta-analyses decrease confidence in drawing conclusions about the studied relationship[98,99]. Cochran's *Q* test and the χ^2 heterogeneity statistic may be used to assess for the presence of heterogeneity within a meta-analysis[100], however the I^2 quantitatively describes the degree of heterogeneity[98,99]. In example, an I^2 index of 25%, 50%, or 75% express a numerical value that may be interpreted as low, moderate, or high levels of heterogeneity among selected studies, respectively[97].

CONCLUSION

Despite modern improvements in diagnosis and treatment, EC continues to carry a high risk of morbidity and mortality, as most cases are diagnosed at advanced stages. EUS, the most sensitive test for locoregional assessment of EC, should be considered in patients without distant metastases prior to neoadjuvant (induction) chemoradiotherapy. EUS may not add additional information in some cases of locally advanced esophageal cancers, and is not routinely recommended. When suspicious lymph nodes are identified remote to the primary tumor, EUS-FNA can obtain cytology specimens to more accurately define the radiotherapy field. Aggressive efforts at early diagnosis and innovative treatments for EC are desperately needed.

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REVIEW

Current and future therapies for inherited cholestatic liver diseases

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Abstract

Familial intrahepatic cholestasis (FIC) comprises a group of rare cholestatic liver diseases associated with canalicular transport defects resulting predominantly from mutations in *ATP8B1*, *ABCB11* and *ABCB4*. Phenotypes range from benign recurrent intrahepatic cholestasis (BRIC), associated with recurrent cholestatic attacks, to progressive FIC (PFIC). Patients often suffer from severe pruritus and eventually progressive cholestasis results in liver failure. Currently, first-line treatment includes ursodeoxycholic acid in patients with ABCB4 deficiency (PFIC3) and partial biliary diversion in patients with ATP8B1 or ABCB11 deficiency (PFIC1 and PFIC2). When treatment fails, liver transplantation is needed which is associated with complications like rejection, post-transplant hepatic steatosis and recurrence of disease. Therefore, the need for more and better therapies for this group of chronic diseases remains. Here, we discuss new symptomatic treatment options like total biliary diversion, pharmacological diversion of bile acids and hepatocyte transplantation. Furthermore, we focus on emerging mutation-targeted therapeutic strategies, providing an outlook for future personalized treatment for inherited cholestatic liver diseases.

Key words: Familial intrahepatic cholestasis; Progressive familial intrahepatic cholestasis; Inherited liver disease; *ATP8B1*; *ABCB11*; *ABCB4*; Biliary diversion; Mutation-targeted therapy; Personalized treatment

Core tip: Familial intrahepatic cholestasis (FIC) is a group of autosomal recessive liver diseases charac-

terized by intrahepatic cholestasis. Phenotypes vary from only episodic disease to progressive FIC. Current therapeutic options are often insufficient to prevent progression of the disease. This review will discuss the current therapeutic regimen as well as the development of novel therapeutic strategies, focusing on surgical and pharmacological biliary diversion, hepatocyte transplantation and mutation-specific therapy.

van der Woerd WL, Houwen RHJ, van de Graaf SFJ. Current and future therapies for inherited cholestatic liver diseases. *World J Gastroenterol* 2017; 23(5): 763-775 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i5/763.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i5.763

INTRODUCTION

Familial intrahepatic cholestasis (FIC) comprises a group of rare cholestatic diseases with autosomal recessive inheritance, which are classified based on their genetic defect[1]. Three of the responsible mutated genes, namely *ATP8B1*, *ABCB11* and *ABCB4*, were identified in 1998 and the corresponding diseases are now generally referred to as ATP8B1, ABCB11 and ABCB4 deficiency[2-4]. The gene products are specific canalicular transport proteins (Figure 1). ATP8B1 is an aminophospholipid flippase that is probably important for maintaining lipid asymmetry of the plasma membrane. Disturbances in the membrane composition might interfere with the function of other transmembrane proteins including the bile salt export pump (BSEP, ABCB11) that is encoded by *ABCB11.* BSEP accounts for the active transport of bile salts into the canalicular lumen thereby generating bile flow. Dysfunction will result in accumulation of bile salts in hepatocytes with subsequent hepatocellular damage. Finally, *ABCB4* encodes the multidrug resistance protein 3 (MDR3, ABCB4), a phosphatidylcholine (PC) floppase, responsible for the biliary secretion of phospholipids. Impaired PC secretion prevents proper micelle formation resulting in the presence of potentially harmful free bile salts[5]. ABCB11 and ABCB4 are localized solely at the canalicular membrane of hepatocytes, while ATP8B1 is abundantly expressed in a wide variety of other tissues such as the small intestine, bladder, ear and pancreas.

Patients with FIC usually present in infancy with cholestasis and associated problems like pruritus and malabsorption. Progressive cholestasis eventually results in liver failure. Patients with ATP8B1 deficiency can have multiple phenotypes ranging from benign recurrent intrahepatic cholestasis (BRIC1), associated with recurrent cholestatic attacks, to the more severe form called progressive familial intrahepatic cholestasis (PFIC1)[6]. In some patients the disease may present as a clinical continuum starting with BRIC and evolving into PFIC[6]. Despite cholestasis, patients with ATP8B1 deficiency have normal serum gamma-glutamyl transferase (GGT) activity. In line with its broad expression, multiple organ systems besides the liver can be affected by the loss of function of the ATP8B1 protein. The high incidence of extrahepatic features such as diarrhea, pancreatitis and hearing loss, distinguishes ATP8B1 deficiency from the other FIC subtypes[7-9]. Like in *ATP8B1*, mutations in *ABCB11* can also result in mild (BRIC2) as well as severe cholestasis (PFIC2) and GGT activity is normal. Therefore, these two subtypes are referred to as low-GGT FIC. ABCB11 deficiency specifically is associated with the development of cholelithiasis and a considerable risk for hepatobiliary malignancies[10,11]. In contrast, mutations in *ABCB4* predispose to several hepatobiliary disorders, such as transient neonatal cholestasis, low phospholipid-associated cholelithiasis and cholangiocarcinoma, but most frequently PFIC type 3[5,12]. Serum GGT is always elevated and no extrahepatic features or association with malignancies is described.

Recently, the tight junction protein 2 gene (*TJP2*) was found to be associated with low-GGT PFIC too[13]. This disease is now referred to as PFIC type 4. The *TJP2* gene encodes tight junction protein-2, which is not a transporter but involved in the organization of epithelial and endothelial intercellular junctions that, in the liver, separate bile from plasma (Figure 1). Still, some of the patients with low-GGT FIC do not have mutations in either of these genes, indicating that other genes might be involved.

This review will discuss the current therapeutic regimen as well as the development of novel therapeutic strategies for FIC, focusing on surgical and pharmacological biliary diversion, hepatocyte transplantation and mutation-specific therapy.

CURRENT THERAPEUTIC REGIMEN

Non-surgical therapies

Medical therapy: Alongside the standard supplementation of fat-soluble vitamins to prevent deficiencies, two drugs can be used: ursodeoxycholic acid (UDCA) and rifampicin. UDCA stimulates hepatobiliary secretion of bile salts. UDCA might enhance bile flow by stimulating the impaired targeting of transport proteins such as BSEP or the conjugate export pump MRP2 (multidrug resistance-associated protein 2, encoded by *ABCC2*) to the canalicular membrane *via* activation of a complex signaling network[14,15]. Antiapoptotic effects of UDCA conjugates may also contribute to protection of hepatocytes. Treatment with UDCA is first-line therapy and effective in more than half of the patients with ABCB4 deficiency[16]. In patients with low-GGT PFIC, the response to UDCA therapy was less promising, but still resulted in improvement of serum transaminase levels and pruritus in some patients[16,17]. Since it is safe and without major side effects, treatment can be attempted in these patients, but in case of progressive liver disease, prompt surgical

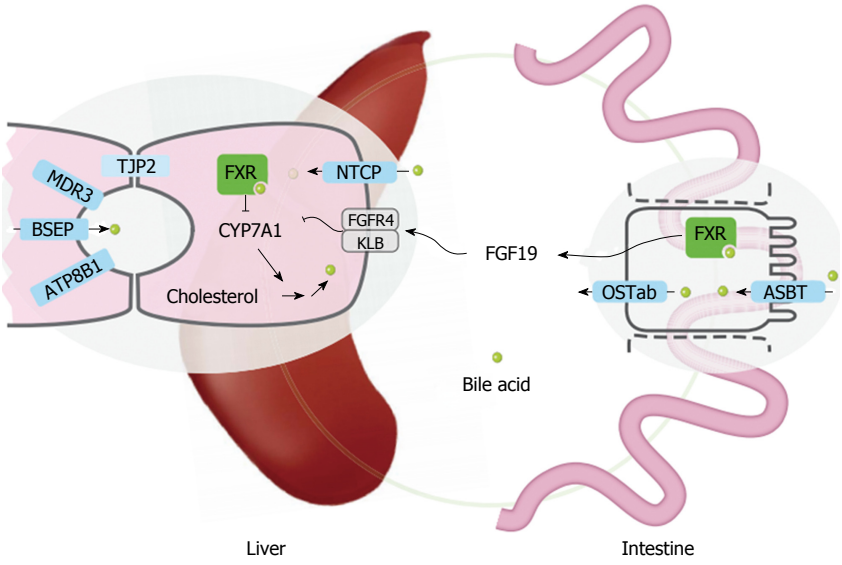

Figure 1 Overview of transporters and sensory proteins involved in hepatic bile flow (regulation). Inherited cholestatic liver diseases are a consequence of mutations in *ATP8B1*, *ABCB11* (encoding BSEP), *ABCB4* (encoding MDR3), or *TJP2*. These 4 proteins are either expressed in the canalicular membrane or at the tight junctions of the canaliculi (TJP2). NTCP mediates uptake of conjugated bile acids into hepatocytes. ASBT mediates uptake of bile acids from the lumen into the enterocyte and OSTab the bile acid export from the enterocyte into the portal circulation. FXR is a nuclear receptor for bile acids, present in multiple tissues, including liver and intestine. Activation of FXR leads to the enhanced secretion of the hormone FGF19 into the systemic circulation. FGF19 is detected by the heteromeric receptor FGFR4/βKlotho on hepatocytes leading to repression of CYP7A1, and reduced bile acid synthesis. FXR: Farnesoid X receptor; NTCP: Sodium taurocholate cotransporting polypeptide; BSEP: Bile salt export pump; ASBT: Apical sodium-dependent bile acid transporter.

intervention is needed. Rifampicin might work, at least in part, by upregulating detoxification enzymes and export pumps by mechanisms dependent of pregnane X receptor[18]. Increased excretion of bile salts in the urine through stimulation of 6-α hydroxylation could also contribute to the beneficial effect of rifampicin. In low-GGT PFIC patients, rifampicin treatment does not result in improvement of serum transaminases and bilirubin and reduces pruritus in only a few patients[16]. Recently, treatment with serotonin reuptake inhibitors was suggested for the management of refractory cholestatic pruritus in patients with PFIC[19]. Although the mechanism of action is not clear, pruritus improved upon treatment in 14 out of 20 patients with PFIC or Alagille syndrome. Since no severe adverse events were reported in this group, serotonin reuptake inhibitors like sertraline could be considered for uncontrolled pruritus.

In BRIC patients, cholestatic attacks eventually resolve spontaneously without permanent liver damage. Yet, these episodes can be long-lasting and the pruritus impairs quality of life significantly[7]. In contrast to its marginal effect in PFIC patients, rifampicin is able to reduce pruritus and even completely abort a cholestatic episode in BRIC patients. UDCA, on the other hand, is rarely effective[16].

Nasobiliary drainage: Another non-surgical intervention is nasobiliary drainage, a temporary diversion of bile established by endoscopically introducing a nasobiliary drain[20]. Due to the temporary character, this therapy is especially useful to abort long-term cholestatic episodes in BRIC patients. The risk of procedure-related pancreatitis needs to be considered.

Surgical therapies

Partial biliary diversion: The purpose of partial biliary diversion (PBD) is to reduce the enterohepatic circulation of bile salts, thereby diminishing the accumulation of these compounds and preventing hepatic injury. Currently, PBD is therapy of choice in all non-cirrhotic children with low-GGT PFIC when permanent cholestasis and/or intractable pruritus is present. This intervention was introduced in 1988, as partial external biliary diversion (PEBD) by cholecystojejunocutaneostomy[21]. In up to 85% of the non-cirrhotic patients, PEBD is successful in improving pruritus as well as biochemical parameters of cholestasis such as serum bile acids, liver enzymes and bilirubin. Also long-term results indicate that PEBD might delay or even reverse hepatic injury[22,23]. However, PEBD is associated with the disadvantage of a permanent stoma[24,25]. Therefore, alternative methods have been explored preventing the presence of a stoma, including ileal exclusion and partial internal biliary diversion (PIBD). The latter procedure is performed by cholecystocolostomy or using an isolated jejunal loop as a conduit from gall bladder to mid ascending colon[26-28]. At present, only a few reports are available that describe the long-term results of these procedures. One study reported inferior long-term outcome of ileal bypass compared to PEBD with recurrence of symptoms in 50% of the patients. The recurrence in this type of procedure is probably the result of increasing re-absorption of bile acids over time[29]. On the contrary, the first long-term results of PIBD were very promising with outcomes comparable to those of PEBD[30]. A potential complication of PIBD however is choleretic diarrhea resulting from large

amounts of bile salts entering the colon[30]. This problem occurs regularly but can be managed with the use of oral bile salt binders (cholestyramine). Furthermore, the possible side-effects of the direct bile flow on the colonic mucosa, such as the development of colitis or colon carcinoma, need to be investigated. Although long-term results are still limited, PIBD already is frequently used as a stoma-free alternative for PEBD[30-32].

Liver transplantation: Biliary diversion will not be successful if significant fibrosis or cirrhosis is already present. Therefore, liver transplantation is indicated for patients with advanced liver disease or persistent uncontrolled pruritus despite PBD. Living-donor liver transplantation is also a safe and effective option[33,34]. However, since ATP8B1 deficiency is not confined to the liver, extrahepatic manifestations can cause significant morbidity even after transplantation. Features such as pancreatitis, short stature and hearing loss will persist and with liver transplantation diarrhea can be induced or exacerbate[8,35]. ATP8B1 is highly expressed in the small intestine where it might have a role in the regulation of reabsorption of bile salts[36]. After transplantation, normal amounts of bile salts are excreted by the hepatic graft while intestinal ATP8B1 is still impaired. High concentrations of bile salts in the colon may result in diarrhea which however can be diminished by treatment with bile adsorptive resin. Furthermore, significant hepatic steatosis often occurs after transplantation which can progress to cirrhosis and may require re-transplantation[37,38]. The pathogenesis of this steatosis is not totally clear, but it might also be secondary to the continued intestinal malfunction of ATP8B1 since it coincides with diarrhea in practically all patients[37]. Liver transplantation usually gives complete correction of phenotype in patients with ABCB4 and ABCB11 deficiency. However, in some patients with severe ABCB11 deficiency, recurrence has been observed post liver transplantation as a result of the formation of autoantibodies against BSEP[39]. Up to 8% of transplanted PFIC2 patients develop these antibodies. Most of these patients had more severe mutations, resulting in a total absence of BSEP[40]. Treatment options include changes of immunosuppressive therapy, depletion of anti-BSEP antibodies by plasmapheresis or immunoadsorption or treatment with rituximab[41]. A second liver transplantation may be necessary, although almost all re-transplanted patients again developed recurrence of cholestasis.

FUTURE THERAPEUTIC OPTIONS

Symptomatic

Total biliary diversion: In some patients, PBD is not effective in reducing clinical symptoms and improving biochemical parameters. For these patients, total biliary diversion (TBD) might be useful[42]. In four patients with ATP8B1 deficiency, of which three did not benefit from PEBD, total external biliary diversion resulted in a significant reduction or complete disappearance of pruritus and cholestasis. No clinical signs of fat malabsorption were encountered, although fat-soluble vitamin levels turned out to be more difficult to manage than in PBD. If additional long-term results confirm the safety and effectivity of PIBD, this PBD variant can also be converted to a TBD by choledochal duct ligation in patients that do not respond to this procedure. It is likely that at least some residual bile salt export capacity is necessary to respond to TBD and therefore mutation type may be of influence on the effectivity. The four PFIC1 patients that were described all beared the same mutation (c.2932-3C>A), that was shown to predominantly result in aberrant splicing but also in a small percentage of normally spliced product[43]. The exact role of genotype on the outcome of TBD is still unknown. However, in accordance with PBD, TBD might also be beneficial to patients with other mutations in *ATP8B1* or *JAG1*, as well as to patients with mutations in *ABCB11.* Currently, TBD is not recommended as primary therapy since PBD is already effective in the vast majority of the patients and the experience with TBD is limited to only a few patients. Moreover, the long-term effects of total absence of intestinal bile salts are unknown. This is especially important as during the last 15 years it became clear that bile salts are not only necessary for solubilizing lipophilic nutrients in the small intestine but can also act as regulatory molecules that are important in glucose and lipid metabolism. Dysregulation of these pathways might therefore interfere with various physiologic processes, with unknown consequences in the long run[44]. However, given the good results in the described patients and the alternative being a liver transplantation, TBD can be added to the treatment options for patients with severe low-GGT PFIC and persisting symptoms after PBD.

Liver transplantation combined with biliary diversion in PFIC1 patients: Liver transplantation alone is often not a permanent solution in patients with severe ATP8B1 deficiency since the graft is frequently affected by steatohepatitis requiring re-transplantation, and diarrhea is persistent or even worse after transplantation. Diarrhea and malnutrition seem to play an important role in the development of post-transplant steatosis[37]. Recently, biliary diversion has also been used in combination with liver transplantation to improve diarrhea and nutritional status in two patients, which proved to be effective in terms of clinical, histological and biochemical outcomes[45-47]. Again, the long-term safety of total external drainage needs to be established. Yet, liver transplantation combined with variants of biliary diversion seems a promising solution to reduce the frequent complications post transplantation.

Pharmacological diversion of bile acids: Hepatic accumulation and toxicity of bile acids likely plays an

essential role in the etiology of liver damage in PFIC. Therefore, pharmacological inhibition of bile acid uptake transporters could be beneficial clinically as well. The majority of bile acids are absorbed by active transport in the terminal ileum, mediated by the apical sodium-dependent bile acid transporter (ASBT) (Figure 1). Using a mouse model for genetic cholestasis, two studies recently described significantly reduced liver fibrosis and inflammation upon treatment with small molecules that inhibit ASBT[48,49]. Fecal bile acid excretion largely increased, providing a likely explanation for the effect. Although both studies used Abcb4 knockout mice, which is a model for PFIC3 or inflammation of bile ducts and not for low-GGT cholestasis, it is well possible that patients with ATP8B1 or ABCB11 deficiency could be successfuly treated with such ASBT inhibitors. One possible disadvantage of this strategy is the increased hepatic synthesis of bile acids. In this respect, targeting the ileal basolateral bile acid export, mediated by the organic solute transporter (OST) OSTα-OSTβ (OSTab), could be an interesting alternative approach to reduce hepatic bile acid accumulation (Figure 1). OSTab is a heteromeric protein, consisting of 2 subunits, both of which are essential for bile acid transport. OSTa knockout mice show a less cholestatic phenotype upon ligation of the common bile duct, related to increased loss of bile acids in urine[50]. Notably, OSTa depletion leads to enhanced intestinal activation of the farnesoid X receptor (FXR), the main nuclear receptor for bile acids. FXR activation results in increased secretion of FGF19, which reduces bile acid synthesis in the liver[50,51]. Unfortunately, no OSTab inhibitors have been developed to date. Also pharmacological FXR activation leads to increased production of the FGF19 hormone, which reduces hepatic bile acid synthesis (Figure 1). Recently, a nontumorigenic FGF19-like peptide was designed that does not affect proliferation but reduces bile acid production in humans[52,53]. This peptide effectively reversed cholestatic liver injury in Abcb4-deficient mice[54] and could potentially also be beneficial in ATP8B1 and ABCB11 deficiency. Finally, inhibition of hepatic bile acid uptake could be effective to reduce accumulation of toxic bile acids in the liver. The first results of clinical trials with Myrcludex B, a peptidic inhibitor for one of the major uptake proteins for conjugated bile acids, sodium taurocholate cotransporting polypeptide (NTCP), have recently been published[55,56]. Myrcludex B was designed to treat hepatitis B virus (HBV) infections, as NTCP is the docking platform for HBV uptake[57,58]. NTCP inhibition seems well tolerated, and mice and men lacking NTCP are viable[59,60]. However, effects of Myrcludex B on cholestasis are not yet reported.

Hepatocyte transplantation: Given the shortage of donor organs, hepatocyte transplantation might become an alternative to liver transplantation[61,62]. Additional advantages of hepatocyte transplantation are that the procedure is less invasive, can be repeated several times and leaves the native liver *in situ*. The procedure may delay or even eliminate the need for liver transplantation. However, scarcity of donor hepatocytes again is the limiting factor. In addition, immunosuppression with its associated morbidity is still necessary. Therefore, alternative cell sources are being investigated such as induced pluripotent stem cells (iPSCs) derived hepatocyte-like cells or hepatocytes generated by transdifferentiation[63]. Still, other important issues remain: poor engraftment, survival and function of the transplanted cells. Since especially cirrhosis impairs engraftment and survival, extrahepatic transplantation sites need to be investigated. Furthermore, progress has been achieved in the organogenesis from iPSCs. Takebe *et al*[64] succeeded in generating vascularized and functional human liver from iPSCs by transplantation of three-dimensional liver buds created *in vitro*. These liver buds could also be delivered to extrahepatic sites. However, the hepatocyte-like cells were not fully differentiated, as evidenced by lower albumin secretion and lower expression of hepatocyte specific CYP450 enzymes compared to primary human hepatocytes. Furthermore, the absence of cholangiocytes in the organoid might be a problem, since the bile produced by the organoid will be released into the circulation. A promising alternative approach is the clonal expansion of single Lgr 5+ bipotent liver progenitor cells into transplantable liver organoids[65]. Upon transplantation in mice, these ductal organoids differentiate into apparently functional hepatocytes. In contrast to iPSC derived hepatocytes or liver buds, the organoid cells are obtained directly from the liver without the need for genetic modification or introduction of reprogramming factors, diminishing the risk of malignancies[66,67]. Although several issues need to be addressed, these techniques might enable personalized autologous hepatocyte transplantation in the future. However, for patients with ABCB11 deficiency, hepatocyte transplantation is probably less suitable, since premalignant cells are left in place.

Correcting the basic defect

Gene therapy: Despite treatment, the quality of life and life expectancy of many patients with PFIC are still limited. Furthermore, in patients with ATP8B1 deficiency, extra-hepatic features persist, even after liver transplantation. Gene therapy corrects the defective gene responsible for disease development and can be applied to different tissues. However, multiple barriers are still present, including the vector genome and transcriptional persistence as well as the immune response that can limit the viability of transduced cells[68]. In addition, genes like *ATP8B1* and *ABCB11* are large and therefore difficult to insert into conventional viral vectors. Alternatively, autologous transplant with *ex vivo* genetically corrected stem cells or hepatocyte-like cells generated from patient derived iPSCs, may hold promise of success. Because liver

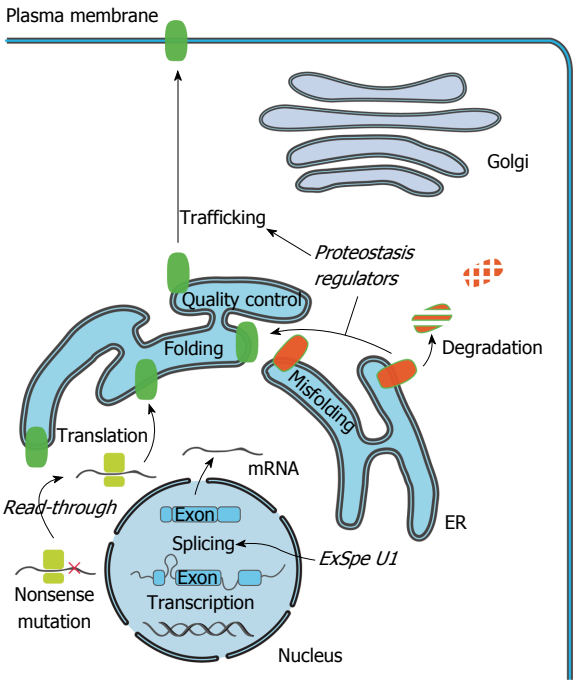

Figure 2 Cellular mechanism of cholestasis-causing mutations and therapeutics. Specific mutations can impair production of full-length ATP8B1/BSEP/MDR3 protein by induction of premature termination codons (PTC). Compounds such as aminoglycosides and Ataluren, could potentially induce read-through of the PTC and allow translation of full-length transporter protein. Mutations that impair pre-mRNA splicing, protein folding or trafficking can lead to a reduced number or total absence of functional transporters at the cell surface. Pre-mRNA splicing could be restored to enhance the levels of correctly spliced transcripts using modified U1snRNA. Exon specific U1 snRNA (ExSpe U1) provide a special class of modified U1 snRNA molecules that could potentially rescue splicing of multiple mutations in a single exon with less potential side-effects. Misfolded transporters are recognized by the endoplasmic reticulum (ER) quality control system and are targeted for degradation *via* the ubiquitin-proteasome system. Compounds that modulate the cellular protein homeostasis machinery (proteostasis regulators) can partially rescue the misprocessing, and restore trafficking to the cell surface, largely *via* unknown mechanisms.

organoids can be based on the *in vitro* expansion of a single progenitor cell, these might also be particular suitable for genetic modifications. Also *in vivo* gene editing using the bacterial CRISPR/Cas system might become an option in the future[69].

Mutation-specific therapy: An alternative approach is molecular rescue of mutation-specific defects. Since these strategies target common disease-causing mechanisms, rare diseases like inherited cholestatic liver diseases can benefit from the experience in other diseases sharing the same underlying mechanisms, such as cystic fibrosis (CF). CF is a multi-organ disease that predominantly affects the lungs, caused by a variety of mutations in the CF transmembrane conductance regulator (CFTR)[70]. CF is particularly suitable to use as a model disease, since it is one of the most common life-threatening monogenic diseases, clinically approved drugs are available and new drugs are being developed.

A considerable number of mutations associated with PFIC have been reported so far[2-4,71-73]. Most of them are rare or limited to a specific population. In line with the classification of CFTR mutations, we can subdivide these mutations in four classes[74,75]. The first class consists of mutations that cause defects in full-length protein synthesis resulting in a non-functional protein. Mutations in this group include nonsense mutations, splice site abnormalities causing a premature termination codon (PTC) or frame shift, and frame shifts due to insertions or deletions. The second class includes mutations resulting in a reduced number of normal transcripts such as some splicing mutants, which allow the synthesis of some residual normal mRNA. This class can also include promotor mutations that reduce transcription. The third class comprises mutations associated with impaired trafficking to the plasma membrane or impaired stability. This can be due to misfolding and subsequent degradation but for ATP8B1 might also be the result of disturbed interaction with CDC50A. The last class comprises mutants that exhibit no or reduced functionality.

To develop targeted therapies, elucidation of the molecular effects, caused by the wide-spectrum of mutations that result in PFIC, is very relevant. Recently, the effect of an increasing number of mutations, resulting in one of the FIC subtypes, has been characterized[43,73,76-82]. Knowing the disease mechanism will allow the classification of mutations according to their functional defect and will help to choose the most adequate therapeutic strategy aimed at correcting this defect (Figure 2).

Restoration of full-length protein synthesis: PTCs cause inappropriate termination of translation leading to a truncated protein, unable to fulfill its functions. If a PTC is caused by a nonsense mutation, a potential pharmacological approach is to selectively promote the translational read-through of the PTC by small molecules, aiming to restore the expression of a full-length protein. Compounds that are known to achieve this goal are the aminoglycoside antibiotics. However, high intravenous or intramuscular concentrations are necessary, leading to serious toxicity. Another compound, PTC124 (Ataluren) was reported to selectively induce ribosomal read-through of premature but not normal termination codons. Unfortunately, clinical trials in patients with CF or Duchenne muscular dystrophy have shown limited efficacy[83-85]. However, in CF patients that did not take chronic inhaled tobramycin, small but significant improvements in pulmonary function were reported[84]. Nevertheless, further optimization or discovery of new compounds is required to achieve clinical efficacy in the future.

Non-functional truncated proteins can also be caused by mutations interfering with pre-messenger RNA splicing. Most of the identified disease-associated *ATP8B1* mutations in exon-intron boundaries were characterized using minigenes, as an alternative to RNA analysis of affected tissue, comparing the

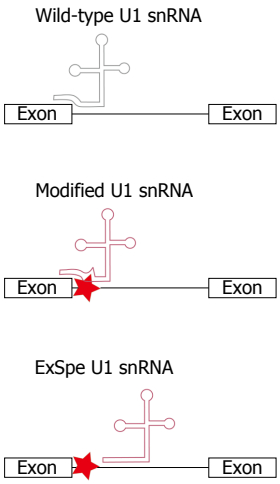

Figure 3 Schematic of the binding of U1 snRNA to exon-intron boundaries to initiate the splicing process. Disease-associated nucleotide changes can affect the recruitment of the cellular splicing machinery, resulting in splice defects. Modification of the U1 snRNA sequence to restore base-pairing between U1 snRNA and the exon-intron boundary can restore splicing. The use of exon-specific U1 constructs, binding at less conserved intronic regions (ExSpe U1 snRNA), may reduce the risk of off-target effects. Simultaneously, ExSpe U1 snRNAs are likely to be effective in a higher number of patients as they can restore splice defects due to several distinct mutations within a certain exon-intron boundary.

splicing patterns of mutant and wild-type exons. All of them resulted to some extent in aberrant splicing resulting in PTCs or large in frame deletions[43]. Also intronic *ABCB11* mutations resulting in abnormal splicing, were identified[81]. The splicing process is normally initiated by binding of the U1 small nuclear RNA (snRNA) to the pre-messenger RNA splice donor site (SDS) by specific base-pairing to the last three nucleotides of an exon and the first six nucleotides of an intron (Figure 3). Mutations interfering with the SDS may reduce complementarity with the U1 snRNA and disturb the splicing process. *In vitro*, modified U1 snRNA constructs, matching the mutated SDS, could rescue splicing very efficiently for a specific group of *ATP8B1* splice-site mutations located at splice donor as well as splice acceptor sites[43]. This same strategy can also be used for the splice site mutations in class 2. Two advantages of the U1 technique compared to conventional gene replacement therapy are that i) the endogenous regulation of transcription is preserved and ii) the small size of the U1 snRNA construct is better suited for insertion in viral vectors. Furthermore, it is becoming increasingly evident that also exonic changes are frequently responsible for missplicing. For instance, a considerable number of missense mutations and single nucleotide polymorphisms in *ABCB11* resulted in aberrant pre-mRNA splicing[78]. Treatment with modified U1 constructs might therefore be applicable to even more patients. However, the safety and efficacy *in vivo* of such treatment needs to be investigated. The use of exon-specific U1 constructs, binding at less conserved intronic regions, may reduce the risk of off-target effects (Figure 3)[43,86,87]. The first *in vivo* data were recently published showing a significant increase of the percentage normal splicing in mice with coagulation factor Ⅶ deficiency or spinal muscular atrophy, without obvious harmful side-effects[87-89]. These promising findings indicate that this strategy may have broad clinical potential for the treatment of a large group of genetic disorders associated with abnormal splicing, including inherited cholestatic liver diseases.

Rescue of impaired protein trafficking: The cellular pathways involved in maintaining the integrity of the proteome are collectively referred to as the proteostasis network. This network coordinates protein synthesis, folding, trafficking and degradation[90]. The inability to restore disturbed proteostasis due to misfolded proteins leads to disease. *In vitro* studies show that some missense mutations, associated with PFIC type 1, 2 and 3, indeed affect protein processing and trafficking, preventing the protein from passing the endoplasmic reticulum (ER) quality control system with subsequent proteasomal breakdown[76-80,82,91,92]. These mutations include the *ATP8B1* mutation p.I661T and *ABCB11* mutations p.E297G and p.D482G, which are the most frequently found mutations in European patients[76,79]. In addition, nine of the 35 characterized *ABCB4* variants also cause retention of ABCB4 in the ER[92]. Cyclosporins were identified as pharmcological chaperones targeting ABCB4 specifically and could improve protein maturation of some of these mutants[80,82]. In contrast to protein-specific compounds like cyclosporins, proteostasis regulators are compounds that can manipulate general proteostasis pathways thereby restoring protein homeostasis and ameliorate disease. These compounds could potentially be beneficial for multiple rare diseases associated with protein misfolding. One of these proteostasis regulator compounds is the histone deacetylase inhibitor 4-phenyl butyric acid (4-PBA). For some of the *ATP8B1*, *ABCB11* and *ABCB4* mutants resulting in retention in the ER, 4-PBA has been shown to result in a marked induction of plasma membrane expression *in vitro*[77,79,92]. Recently, also the first positive *in vivo* results of treatment with 4-PBA were published[93-97]. Five PFIC2 patients were treated with 4-PBA resulting in partial restoration of protein expression at the plasma membrane as well as clinical improvement with disappearance of pruritus and improved liver tests[94-96]. Furthermore, 4-PBA treatment was successful in aborting a cholestatic attack in a BRIC2 patient[97]. Although no beneficial effect on transaminases, GGT and bilirubin was detected, treatment resulted in significant relief of intractable itch in three patients with the progressive form of ATP8B1 deficiency. However, the effect of 4-PBA on the biochemical markers of cholestasis and liver histology might depend on mutation type and the associated degree of residual ATP8B1 activity.

The most common CFTR mutant F508del also

impairs protein folding and various CFTR correctors have been discovered to rescue this misfolding. Some of these CFTR corrector compounds were shown to overcome the trafficking block of ATP8B1 mutant p.I661T at the ER, presumably acting as proteostasis regulators[98]. In addition to 4-PBA, six compounds were able to significantly enhance the plasma membrane expression of p.I661T-ATP8B1 *in vitro*[98]. Two of these compounds were already approved by the Food and Drug Administration for the management of other diseases. This will facilitate the clinical application of these compounds for other indications. Moreover, these data show that p.I661T-ATP8B1, after leaving the ER, is likely to reach its proper location at the canalicular membrane. Evaluation of the functionality of the corresponding p.L622T mutation in P4-ATPase family member ATP8A2, suggested that p.I661T-ATP8B1 maintains functionality. In line with these findings, the transport function of ABCB11 mutants p.E297G and p.D482G, associated with impaired membrane trafficking, remained largely unchanged[76].

The clinical effect of the most promising CFTR corrector, VX-809, was disappointing in CF patients[99]. Despite very promising pre-clinical results of some proteostasis regulator compounds, it needs to be evaluated whether these will indeed be clinically meaningful in PFIC patients. Combination therapy with multiple proteostasis regulator compounds acting at different steps along the folding pathway may however provide a basis for a more effective intervention. Combination of the two most promising corrector compounds (suberoylanilide hydroxamic acid, SAHA and compound C4) *in vitro* did result in an additional improvement of ATP8B1 cell surface abundance[98]. Moreover, it is important to consider that modulating protein misfolding by targeting the proteostasis network is one of the few treatments that potentially can also improve the extrahepatic features in patients with ATP8B1 deficiency. Therefore, these proteostasis regulator compounds hold promise for the future treatment of well-defined patients with inherited cholestatic liver disorders.

TOWARDS PERSONALIZED THERAPY

Molecular characterization

The new therapeutic options described here hold promise for the future treatment of specific subgroups of patients with FIC, paving the way to the development of personalized treatment based on molecular profiles. Below, we describe our view on the challenging route to deliver the benefits of this *in vitro* work to the patients.

The majority of the mutations still cannot be assigned to a specific mutational class with certainty, due to the absence of molecular characterization. Furthermore, some mutations may be included in multiple classes, making rescue therapy even more difficult to achieve. Therefore, the first step, after genotyping, is the characterization of the patient-specific underlying disease-mechanism. Molecular analysis can be used to predict disease phenotype and determine the optimal treatment strategy, most likely to benefit the individual patient. Since residual activity might be one of the main predictors of a positive treatment outcome, a complicating factor in this analysis is that representative functional assays for ATP8B1 remain challenging[100,101]. *ATP8B1* mutations are often in residues that are conserved across the P4-ATPase subfamily. It is therefore also possible to consider measuring the potential impact of these mutations in another P4-ATPase with well characterized transporter activity and specificity such as family member ATP8A2. This ATP8B1 homologue exhibits ATPase activity that is stimulated by phosphatidylserine and to a lesser degree phosphatidylethanolamine and was previously used to evaluate the possible functional consequences of p.I661T-ATP8B1, showing preserved functionality[98]. In addition, a cellular assay for characterizing P4-ATPase-mediated transport in living yeast cells was developed[102].

Pre-clinical assay

The success of personalized medicine depends on having accurate tests that identify patients who can benefit from targeted therapies, avoiding costly treatments with limited benefits and possible side effects. Regular *in vitro* assays using artificial cell lines can be used as pre-clinical assays. However, it is essential that the results of such an assay have a high predictive value for the actual clinical outcomes. Therefore, it is attractive to evaluate the treatment efficacy in a patient's native cellular and genetic background. Now, iPSCs from patient specific cell sources, such as skin fibroblast, hair follicle cells, patient blood samples and even urine containing small amount of epithelial cells, can be generated. These iPSCs and iPSC-derived hepatocytes can serve as disease model. iPSC-derived *in vitro* models can be used to confirm a genetic diagnosis, demonstrate mutation pathogenicity and they can also serve as the basis for drug screening or gene therapy optimization. Patient-specific liver or intestinal organoids could be even better pre-clinical disease models[103]. Although a liver biopsy is necessary, growing an organoid culture from actual patients might show specific defects and will facilitate the determination of specific treatment regimens. Notably, organoids from patients with other liver diseases such as α1-antitrypsin deficiency and Alagille syndrome mirrored the *in vivo* pathology[67]. It might also be possible to use these organoids for indirect functional assays since they can readily be converted into functional hepatocytes *in vitro.* Functional organoid cells secrete albumin as well as bile salts into the medium and exhibit adequate detoxifying qualities. By evaluating these parameters, disease severity and therapy response might be predicted. Given the broad tissue expression of ATP8B1, patient-

derived rectal organoids might be a suitable alternative modelsystem for evaluation of drug-response in patients with ATP8B1 deficiency, circumventing the risk of a liver biopsy.

Clinical efficacy

On the basis of pre-clinical results, patients can be selected that are suitable for a specific therapy. The ultimate way to verify *in vitro* drug responses *in vivo*, is a randomized controlled trial. However, given the low number of patients per mutation, it is practically impossible to conduct such trials. Single patient trials can be considered[104]. Single patient trials, also known as n-of-1 trials, are multi-period crossover experiments comparing two or more treatments within one patient. These trials are only suitable for evaluating long-term treatments for chronic conditions with stable treatment response, quick onset of treatment effect and only modest carryover effects. For most therapies, these data will be unknown and also patients with rapidly progressive disease are not eligible. Furthermore, to evaluate the effect of mutation-specific therapy, we need an objective *in vivo* read-out of response. For patients with PFIC we can use biochemical parameters such as serum liver enzymes and bile acids. However, this is impossible for patients with episodic disease since biochemical parameters normalize in between the cholestatic attacks. In addition, the duration in between episodes is unpredictable. Currently, treatment effect and drug efficacy might therefore be hard to prove, especially in BRIC patients. Still, a lot can be learned from the experience with these therapies in other, more frequent, diseases with a comparable basic defect, like CF.

CONCLUSION

Recently, important steps were taken towards a more personalized treatment strategy for FIC patients focusing on mutation-targeted therapeutic strategies. Promising mutation-specific therapies, like the use of modified U1 snRNA and proteostasis regulators, have much broader potential, and can also be applied to other genetic diseases that are associated with defective pre-mRNA splicing or impaired protein folding. This will facilitate the movement from concept to clinical use enormously. Further research should focus on the establishment of a solid functional assay for ATP8B1, representative patient-specific preclinical assays as well as a good clinical read-out, to make personalized therapy a reality.

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REVIEW

To screen or not to screen? Celiac antibodies in liver diseases

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Abstract

Celiac disease (CD) is a systemic immune-mediated disorder triggered by dietary gluten in genetically predisposed individuals. The typical symptoms are anemia, diarrhea, fatigue, weight loss, and abdominal pain. CD has been reported in patients with primary sclerosing cholangitis, primary biliary cholangitis, autoimmune hepatitis, aminotransferase elevations, nonalcoholic fatty liver disease, hepatitis B, hepatitis C, portal hypertension and liver cirrhosis. We evaluate recommendations for active screening for CD in patients with liver diseases, and the effect of a gluten-free diet in these different settings. Active screening for CD is recommended in patients with liver diseases, particularly in those with autoimmune disorders, steatosis in the absence of metabolic syndrome, noncirrhotic intrahepatic portal hypertension, cryptogenic cirrhosis, and in the context of liver transplantation. In hepatitis C, diagnosis of CD can be important as a relative contraindication to interferon use. Gluten-free diet ameliorates the symptoms associated with CD; however, the associated liver disease may improve, remain the same, or progress.

Key words: Celiac disease; Cholangitis; Sclerosing; Liver cirrhosis; Biliary; Hypertension; Portal; Hepatitis; Autoimmune

Core tip: Liver involvement in celiac disease (CD) has been reported for more than four decades. However, CD antibodies are seldom investigated by clinicians in routine hepatology consultations. In this article, we perform extensive literature review on liver and CD and evaluate if one should screen for celiac antibodies in various liver diseases and clinical settings.

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INTRODUCTION

Celiac disease (CD) is a systemic immune-mediated disorder triggered by dietary gluten in genetically predisposed individuals. Its typical symptoms are anemia, diarrhea, fatigue, weight loss, and abdominal pain[1]. Therefore CD is always considered as a differential diagnosis for malabsorption syndrome and iron-deficiency anemia, but it is often overlooked as a differential diagnosis for liver diseases[2]. Its clinical presentation can comprise varied symptoms, including musculoskeletal, neurological, endocrine, kidney, heart, lung, and liver manifestations, concomitant with other autoimmune diseases and malignancies[1,3-5].

The serological diagnosis of CD is based on the presence of the following antibodies: antigliadin (AGA) immunoglobulin A (IgA) and immunoglobulin G (IgG), antiendomisyal antibody (anti-EmA), antitissue transglutaminase (anti-tTG), and antideamidated gliadin peptide (anti-DGP). AGA has become obsolete and is no longer recommended for diagnosis in the adult population owing to its low levels of sensitivity and specificity[6,7]. Anti-EmA testing is performed using an indirect immunofluorescence assay and detects CD with lower levels of sensitivity than other modern serological assays, particularly in the presence of IgA deficiency[8]. However, EmA antibody is an extremely specific marker of mucosal damage in untreated patients and has been indicated as a useful diagnostic tool[7,9]. Anti-tTG enzyme-linked immunosorbent assay (ELISA) exhibits optimum sensitivity and lack of specificity with positive predictive values that are significantly lower than those obtained for the EmA assay[10]. Therefore, tTG IgA has been recommended as the most efficient single serological test for the detection of CD, whereas EmA IgA can be used as a confirmatory test in case of borderline-positive (low titers) or possibly false-positive results of tests for anti-tTG IgA that may occur in other autoimmune diseases[7,9,11]. Selective IgA deficiency affects approximately 2%-5% of patients diagnosed with CD[12]. Majority of serological tests for CD are IgA based; consequently, these tests do not identify individuals who have both CD and selective IgA deficiency. Therefore, IgA levels must be tested along with other autoantibodies, and individuals with IgA deficiency should go through an IgG-based antibody test[13]. A decade ago, anti-DGP had been introduced as a diagnostic tool for CD[14]. tTG mediates its effects through an ordered and specific deamidation of certain glutamines to glutamates, increasing the antigenicity of peptides; this deamidation creates an epitope that efficiently binds to DQ2 and is recognized by gut-derived T cells[15]. Nowadays, both DGP and tTG antibodies are considered as serological hallmarks of CD[16]. There is new serological biomarker for CD available: the tTg neo-epitope (tTg-neo), with high sensitivity and specificity[17]. tTg-neo IgG has demonstrated better performance when compared to the tTg-IgA, and has been recommended as a novel diagnostic technique for CD[17,18]. Because no diagnostic test is 100% effective in diagnosing CD, a combined search for celiac antibodies is recommended for optimal diagnostic accuracy[19,20]. Hence, combined antibodiy kits have been made commercialy available and have demonstrated excellent diagnostic performance; they may soon be added to the procedures in diagnostic flow charts[21].

Small intestinal biopsy has been central to the confirmation of diagnosis of CD since the late 1950s. Nowadays, distal duodenal biopsies (4-8 fragments) reveal typical histological findings: villous atrophy, crypt hyperplasia, and lymphocytic inflammatory infiltrate[9]. It is important to point out that positive serology with normal histology, formerly termed latent CD[22], are now defined as potential CD[23]. The American College of Gastroenterology recommends human leukocyte antigen (HLA) testing for DQ2 and DQ8 when there is disagreement between serological and histological results[24]; however, certain authors perform HLA determination in patients with positive anti-tTG and negative EmA to identify false-positive tTG results[25].

Liver involvement in CD has been widely described in case reports and case series in the past four decades[26]. In London in 1973, Thatcher *et al*[27] reported a case of Turner's syndrome with CD, thin bones, and abnormal liver function tests. Nowadays, it is well known that hepatic steatosis is the most frequent finding in Turner's syndrome[28], but architectural changes in the liver, including cirrhosis and biliary lesions such as primary biliary cholangitis (PBC), have also been described[29].

CD has been associated not only with autoimmune liver diseases such as primary sclerosing cholangitis (PSC), PBC, and autoimmune hepatitis (AIH) but also with viral hepatitis B and C, and nonalcoholic steatohepatitis, as well as with Wilson's disease, cirrhosis, and portal hypertension[30]. Swedish epidemiological studies have revealed that patients with CD have a 2-6-fold increased risk of future liver disease and an 8-fold increased risk of mortality from liver cirrhosis[31,32]. The development of autoimmune disorders in CD has been related to the age at diagnosis[33]. Early diagnosis and treatment of CD is not associated with an increased prevalence of autoimmune disorders, and autoimmune disorders develop in individuals with unrecognized and untreated CD[33]. Nonetheless, AIH has been reported in patients with treated CD[34].

The aim of the present study was to perform extensive literature review to organize published data on liver and CD and evaluate if one should screen for celiac antibodies in various liver diseases and clinical settings.

LITERATURE SEARCH

We performed a review for liver involvement in CD by conducting a broad search for MeSH terms "celiac

Table 1 Studies regarding the association of primary sclerosing cholangitis and celiac disease

Type of article	No. of patients	Symptoms	Celiac antibodies	Duodenal biopsy	Response to gluten-free diet	Liver biopsy	ERCP	Comorbidities	Country, year	Ref.
Case report	3	Weight loss, steatorrhoea	No	Typical[1]	Yes	Yes	Yes	2 Chronic ulcerative colitis	United States, 1988	Hay *et al*[35]
Abstract	69	Screening	55% AGA (+)	0/26 altered	-	-	-	-	Ireland, 1992	MacMathuna *et al*[46]
Case report	1	Diarrhea, weight loss, growth retardation	No	Typical[1]	Yes	Yes	Yes	Chronic colitis Turner's syndrome	France, 1995	Lacaille *et al*[37]
Case report	2	Anemia	AGA IgA (+)	Villous atrophy	Yes	Yes	Yes	-	Italy, 1996	Fracassetti *et al*[36]
Case report	1	Diarrhea	No	Villous atrophy	Yes	Yes	Yes	Ulcerative colitis	Sweden, 1994	Tysk[41]
Case report	1	Folic acid deficiency	AGA (-) EmA (+)	Typical[1]		Yes	Yes	Ulcerative colitis Hashimoto's thyroiditis	France, 1994	Brazier *et al*[44]
Case report	2	Weight loss	EmA (+)	Typical[1]	Yes	Yes	Yes	-	Italy, 1998	Venturini *et al*[49]
Case report	1	Anemia	No	Not mentioned	Not mentioned	Yes	Yes	Rheumatoid arthritis	United Kingdom, 2001	Gow *et al*[50]
Case series	1	Diarrhea Protruding abdomen Failure to thrive	tTG (+) EmA (-)	Villous atrophy	No adherence	Yes	Not mentioned	Not mentioned	Finland, 2002	Kaukinen *et al*[51]
Case report	2	Active screening for CD	EmA (+) tTG (+)	Typical[1]	Yes	Yes	Yes	Ulcerative colitis	Poland, 2002	Habior *et al*[43]
Prospective cohort	61	Active screening for CD	1.6% EmA (+) 3.3% tTG (+)	100% (1/1) Typical[1]	Yes	Yes	Yes	-	Italy/Spain 2002	Volta *et al*[52]
Case report	2	Weight loss, steatorrhoea	EmA (+)	Villous atrophy	Yes	No	Yes	Ulcerative colitis	United Kingdom, 2003	Wurm *et al*[42]
Case report	1	Routine UDE	AGA (+) EmA (+)	Typical[1]	Yes	Yes	Yes	-	United States, 2004	Al-Osaimi *et al*[53]
Case report	1	Diarrhea	EmA (+)	Typical[1]	Yes	Yes	Yes	-	Spain, 2005	Cadahía *et al*[54]
Prospective cohort	155	Screening	3% EmA (+) 9% tTG (+)	-	-	-	-	-	United States, 2008	Rubio-Tapia *et al*[48]
Case report	1	Short stature and anemia	tTG (+)	Typical[1]	Yes	Yes	No, MRC	-	Saudi Arabia, 2013	Al-Hussaini *et al*[55]

[1]Vilous atrophy, crypt hyperplasia, lymphoplasmocytic infiltrate. ERCP: Endoscopic retrograde cholangio pancreatography; MRCP: Magnetic resonance cholangiopancreatography; AGA: Anti-gliadin antibody; EmA: Endomysial antibody; tTG: Tissue transglutaminase; CD: Celiac disease; UDE: Upper digestive endoscopy.

disease" and "PSC", "primary biliary cirrhosis", "AIH", "alanine transaminase", "nonalcoholic fatty liver disease", "hepatitis B", "hepatitis C", "portal hypertension", "liver cirrhosis", and "liver transplantation", in PubMed, with no data limit. In addition, references of the selected articles were consulted for relevant articles on the subject.

PSC

In 1988, Hay *et al*[35] described the first three cases of a possible association between PSC and CD observed at Mayo Clinic. Patients presented steatorrhea, and CD was assumed based on typical histological findings of small bowel biopsy and clinical or clinical response to a gluten-free diet. Thereafter, a few case reports have been published (Table 1), some of which do not include serological diagnosis of CD. CD was diagnosed "in the old way": on the basis of histological findings and clinical response to a gluten-free diet. In several patients, liver disorder had developed when CD was still undiagnosed. Most cases exhibited clinical improvement in intestinal symptoms and anemia with dietary gluten exclusion, whereas more severe liver lesions showed no response to dietary changes[35-37].

The association between ulcerative colitis and PSC has been well established[38]. CD combined with ulcerative colitis has also been reported[39,40]. Certain cases described associations among ulcerative colitis,

CD, and PSC, which is very rare[35,37,41-43]. Brazier *et al*[44] reported an association between ulcerative colitis, PSC, CD, and Hashimoto's thyroiditis wherein improvements were observed in liver biochemistry and histology with a gluten-free diet.

PSC and CD share some of these predisposing HLA haplotypes. Although it is expected that PSC patients with HLA DR3, DQ2 haplotype would be at risk of CD, such patients have previously been noted to exhibit a more rapid progression of liver disease. Therefore one could imagine whether or not patients with CD who develop PSC suffer from more aggressive liver disease than those without CD[45]. Although this has not been specifically studied, in a previous study, patients with high AGA titers demonstrated advanced portal fibrosis[46].

Based on global reports of less than 20 cases of PSC and CD, it is not wise to establish a true association between both the disorders. This can only be determined if cholangiography is performed in a consecutive series of patients with CD and if celiac antibodies and small bowel biopsies are recorded in all patients with PSC[47]. MacMathuna *et al*[46] studied 69 patients with PSC and observed that 55% of patients presented AGA-positive results, and none of the biopsied patients (0/26) revealed typical findings in duodenal biopsies. Rubio-Tapia *et al*[48] evaluated 155 PSC patients with end-stage autoimmune liver disease; those who expressed HLA-DQ2 or HLA-DQ8 molecules have a high prevalence of CD-associated antibodies: 9% of these were tTG positive and 3% were EmA positive.

Taking into account the aforementioned studies, a diagnosis of CD in patients with PSC requires medical awareness of possible coexistence of the two lesions[47]. In clinical settings, active screening for celiac antibodies in PSC patients cannot be routinely recommended (Table 1[35-37,41-44,46-55]).

PBC

Recently, the governing boards of both the European Association for the Study of the Liver (EASL) and American Association for the Study of Liver Diseases have approved the change of nomenclature for PBC from "primary biliary cirrhosis" to "PBC"[56,57].

The first report of CD in patients with PBC was published in 1978[58]. Four patients presented characteristic symptoms of CD, typical findings on jejunal biopsy, and clinical improvement with a gluten-free diet. Since then, concomitancy of the two diseases has been extensively reported in several cases[59-71] (some of these are enlisted in Table 2), and other disorders have been reported in association with both diseases, including serum IgA deficiency, dermatitis herpetiformis, renal tubular acidosis, Sjögren syndrome, bacterial overgrowth, osteomalacic myopathy, Fanconi syndrome, and *Helicobacter pylori* (*H. pylori*) infection.

In 1998, Kingham and Parker studied the relative prevalence of CD and PBC in the United Kingdom[72]. A 12-year study of a stable population of 250000 individuals revealed a relative prevalence of PBC in 3% of 143 patients with CD and a relative prevalence of CD in 6% of 67 patients with PBC[72]. Accordingly, in Ireland, Dickey *et al*[73] reported that the prevalence of CD in patients with PBC is at least 10 times that in the general population. Sorensen *et al*[74] reported a high risk of PBC in patients with CD. The risk was similar when independently assessed in two separate national hospital databases during two different study periods: an incidence ratio of 27.6 in Denmark and 25.1 in Sweden. Conflicting results were published in Sweden[75], Italy[76,77], and Greece[78], where researchers have failed to demonstrate an increased risk of CD in patients with PBC. Dickey *et al*[79] searched for liver abnormalities in 129 patients with CD and none of the patients were positive for AMA.

According to Bizzaro *et al*[25], 26.7% of PBC patients exhibited tTG positivity on performing at least one of six different ELISA tests (human recombinant, Eurospital; human recombinant, Pharmacia; human placenta, Euroimmun; human red blood cells, Inova; guinea pig liver, Eurospital; and guinea pig liver, Inova); however, a true association between PBC and CD was present in only 2% of patients who exhibited EmA positivity and showed histological patterns indicative of CD. Moreover, Floreani *et al*[80] reported a high prevalence of false-positive results: 27.5% of PBC patients showed serum IgA-tTG above normal limits, only two patients had IgA-tTG > 30 IU, and EmA positivity was detected in only 3.4% of patients. Hence both authors suggest that, in most cases, the false positive results were attributable to the type of substrate used in the tTG assay, suggesting that in PBC patients positive findings in the anti-tTG antibody assay should be confirmed using the EmA test[25,80]. IgA-tTG is characterized by wide heterogeneity in the kit's performance, depending on both the commercial assay variant used and the cut-offs provided by the supplier. The significant range of test accuracies are haphazard[11]. In fact, high-titer IgA-tTG antibodies are specific for detecting CD, whereas in the lower range of titers, there is a broad overlap with other gastrointestinal and liver diseases[81]. Studies on screening CD in patients with PBC are listed in Table 3.

In regions with a low prevalence of CD, in the absence of clinical suspicion, the cost benefit of routinely screening all patients with PBC for CD remains debatable. In addition, gluten-free diets have failed to improve liver biochemistry in patients with coexistent PBC[58,61,72]. The Neuberger report of two patients with PBC who had been referred to their liver unit in the United Kingdom for transplantation because of deteriorating liver tests, lethargy, and diarrhea is noteworthy; however, these patients were diagnosed and treated for CD, with consequent improvements so that transplantation was no longer needed[82]. Abenavoli *et al*[83] reported a case of association among CD, PBC, and *H. pylori* infection, wherein a short period of gluten-

Table 2 Case reports[2] regarding the association of primary biliar cholangitis and celiac disease

Number of patients	Symptoms	Celiac antibodies	Duodenal biopsy	Response to gluten-free diet	Liver biopsy	AMA	Comorbidities	Country, year	Ref.
4	Weight loss, steatorrhea, anemia	No	Typical[1]	Yes	Yes	(+)	serum-IgA deficiency	Scotland, 1978	Logan *et al*[58]
1	Weight loss, diarrhea, anorexia	No	Typical[1]	Yes	Yes	No	-	United Kingdom, 1978	Lee *et al*[59]
1	Malabsorption	No	Typical[1]	Poor adherence	Yes	(+)	-	Canada, 1979	Iliffe *et al*[60]
1	Diarrhea, anemia, short stature		Subtotal villous atrophy	Yes, but PBC was diagnosed afterwards	Yes	(+)	-	Ireland, 1983	Shanahan *et al*[62]
1	Dermatitis herpetiformis	No	Typical[1]	Yes	Yes	(+)	Dermatitis herpetiformis	Norway, 1985	Gabrielsen *et al*[64]
1	Anemia	AGA (-)	Typical[1]	Yes	Yes	(+)	Renal tubular acidosis, Sjögren Syndrome	Ireland, 1987	Whitehead *et al*[68]
1	Weight loss, diarrhea	No	Typical[1]	Yes	Yes	(+)	-	United States, 1992	Ginn *et al*[69]
1	Weight loss, anemia	No	Typical[1]	Yes	Yes	(+)	-	Canada, 1994	Freeman[70]
1	Diarrhea	No	Typical[1]	Yes, but PBC was diagnosed afterwards	Yes	(+)	-	Germany, 1994	Löhr *et al*[196]
1	Diarrhea, weight loss	AGA (+)	Typical[1]	No	Yes	(+)	-	Spain, 1994	Gálvez *et al*[97]
1	Weight loss, steatorrhea	AGA (+) EmA (+)	Typical[1]	Yes	Yes	(+)	Bacterial overgrowth	United States, 1998	DiBaise *et al*[71]
1	Anemia	EmA (+)	Typical	Yes	Yes	No	-	United States, 2002	Sedlack *et al*[84]
1	Diarrhea, weight loss	EmA (+)	Typical[1]	Yes	Yes	(+)	Renal tubular acidosis, Sjögren Syndrome, Graves' disease	Italy, 2004	Fracchia *et al*[98]
1	Inability to walk, anemia	AGA (-) EmA (-)	Typical[1]	Yes	Yes	(+)	Osteomalacic Myopathy	Turkey, 2008	Demirag *et al*[99]
1	Bone pain	AGA (+) EmA (+) tTG (+)	Typical[1]	Yes	Yes	(+)	Fanconi syndrome	Paris, 2008	Terrier *et al*[100]
1	Dispepsia	tTG (+)	Typical[1]	Yes	Yes	(+)	*Helicobacter pylori*	Italy, 2010	Abenavoli *et al*[83]
1	Diarrhea, bloating	EmA (+) tTG (+)	Typical[1]	Yes	Yes	(+)		India, 2013	Lodh *et al*[101]

[1]Vilous atrophy, crypt hyperplasia, lymphoplasmocytic infiltrate; [2]Case reports for which we had access to the full text. ERCP: Endoscopic Retrograde Cholangio Pancreatography; MRCP: Magnetic resonance cholangiopancreatography; AGA: Anti-gliadin antibody; EmA: Anti-endomysial antibody; tTG: Tissue transglutaminase.

free diet associated with eradication therapy of *H. pylori* and ursodeoxycholic acid (UDCA) administration led to marked histological and serological improvements in PBC. In addition, Sedlack *et al*[84] demonstrated clinical and biochemical improvements with a gluten-free diet and UDCA. However, it is important to point out that the patient received the recommended treatment for CBP, which was most likely responsible for the hepatic improvements.

Therefore, it is important to recognize that patients with these two conditions may share several common clinical features. Weight loss, malabsorption, steatorrhea, bone disease, and elevated alkaline phosphatase are frequently observed in both diseases[58,71,85]. Hence, they may not be readily recognized during the early stages.

Numerous theories have been considered to explain the concomitant presence of CD and PBC. A genetic connection has not been determined as CD is strongly linked to HLA-DQ2; HLA associations are less clearer and vary among report centers and different ethnic populations[86]. Intestinal permeability is increased and disrupted intestinal barrier function has been reported[87,88]. Such changes can lead to an augmented absorption of toxins or antigens into portal blood, which can lead to the hepatic injury observed in such patients[89]. It is suggested that immune complexes are formed with molecular mimicry, and this mechanism mediates tissue damage; however, no specific antigen has been identified[90]. It has been proposed that chronic bacterial exposure may initiate the development of antibodies, which then cross react with human antigens in PBC patients[91,92]. Alternatively, diminished function of suppressor T cells in patients with both diseases might allow effector cytotoxic lymphocytes to attack a modifying antigen such as gluten[90]. These effector cells might then recognize an attack on a patient's histocompatibility antigens, which

Table 3 Research on screening celiac disease in patients with primary biliar cholangitis

Study	Screening method	Number of positive patients	Typical duodenal biopsy[1,2]	Response to gluten-free diet	Country, year	Ref.
Prospective	Duodenal biopsy	5/26 (19.2%)	19, 2%	No improvement in liver biochemestry	Sweden, 1982	Olsson *et al*[61]
Retrospective	Previous diagnose	2/18 (11.1%)	Not mentioned	No improvement in liver biochemestry or liver histology	Sweden, 1985	Löfgren *et al*[65]
Prospective	EmA IFI > 1:5	6/57 (11%) EmA (+)	7%	No improvement in liver biochemestry	Ireland, 1997	Dickey *et al*[73]
Prospective cohort	AGA IgG IgA > 1 AU IgA EmA IFI	0/62 (0%) EmA (+) 11/62 (16%) AGA (+)	0/0	-	United States/ Italy	Volta *et al*[76]
Prospective	malabsorption, haematinic deficiency, positive antigliadin antibody, or CD family history	4/67 (6%)	4/67 (6%)	No improvement in liver biochemestry	United Kingdom, 1998	Kingham *et al*[72]
Prospective	AGA IgA > 25 AU/mL IgG > 28 AU/mL EmA IFI > 1:5	4/11 (36, 4%) AGA IgA (+) 1/11 (9%) AGA IgG (+) 1/11 (9%) EmA (+)	18%	-	Argentina, 1998	Niveloni *et al*[102]
Retrospective (stored sera)	EmA IFI > 1:5 tTG IgA ELISA > 140 AU/mL	10/378 (2.6%) EmA (+) + tTG (+) 44/378 (11.6%) EmA (-) + tTG (+)	1.30%	-	United Kingdom, 2000	Gillett *et al*[103]
Prospective	EmA IFI tTG IgA > 10 IU	3/87 (3.4%) EmA (+) 24/87 (27.5%) tTG (+)	0/17	-	Italy, 2002	Floreani *et al*[80]
Prospective	AGA IgA > 50 U/mL AGA IgG > 50 U/mL EmA IgA IFI ⩾ 1:5 IgA tTG > 30 U/mL	13/62 (21%) AGA (+) 0/62 EmA (+) 6/62 (10%) tTG (+)	0/10	-	Greece, 2002	Chatzicostas *et al*[78]
Prospective cohort	EmA IFI > 1:5 tTG IgA > 7 AU AGA IFI	7/173 (4%) EmA (+) 5/173 (2.9%) tTG (+)	7/7	No improvement in liver biochemestry	Italy/Spain 2002.	Volta *et al*[52]
Prospective cohort	IgA tTG > 7 AU IgG anti-Ttg > 30 AU EmA IFI	5/48 (10.4%) tTG (+)	-	-	Italy, 2003	Bizzaro *et al*[104]
	tTG < 1:100 EmA IFI AGA Elisa	7/115 (6.1%) tTG (+) 1/115 (0.9%) EmA (+) 8/115 (7.0%) AGA (+)	1/8	Duodenal histological improvement	Poland, 2003	Habior *et al*[105]
Prospective cohort	Six different ELISA tTG	28/105 (26.7%) tTG IgA (+) 6/105 (5.7%) tTG IgG (+)	100% EmA (+) 0% tTG (+)	-	Italy, 2006	Bizzaro *et al*[25]

[1]Vilous atrophy, crypt hyperplasia, lymphoplasmocytic infiltrate; [2]Only a small number of patients usually undergo intestinal biopsy. EmA: Anti-endomysial antibody; IIF: Indirect immunofluorescence; tTG: Anti-tissue transglutaminase; ELISA: Enzyme-linked immunosorbent assay; CD: Celiac disease; AGA: Anti-gliadin antibody.

are present in high concentrations in biliary as well as intestinal epithelial cells[90]. Moreover, tTG is present in the liver and in other tissues besides the intestinal basal membrane, which suggests a pathological role of humoral immunity (anti-tTG) in the hepatic injury observed in patients with CD[93,94].

Screening for CD in patients with PBC is recommended because a gluten-free diet may remit CD symptoms and prevent the development of other autoimmune diseases and intestinal malignancies[95,96] (Table 3[25,52,61,65,72-73,76,78,80,102-105]).

AIH

AIH has been classified into two or three different subtypes according to the distribution of autoantibodies and clinical presentation[106]. Although not all authors adopt this classification[107], type 1 AIH (the most frequent form) is characterized by the presence of SMA and/or ANA[106,108]. SMA antibodies are directed against microfilaments by the presence of actin, against intermediate filaments by the presence of vimentin, and against microtubules by the presence of tubulin, with a clear predominance of antiactin antibody in type 1 AIH[109,110]. Type 2 AIH is characterized by the detection of specific antiliver/kidney microsomal antibody type 1 (anti-LKM1) or infrequently by that of anti-LKM type 3 (anti-LKM3) and/or antibodies against liver cytosol type 1 antigen (anti-LC1)[106]. The third type was previously known to be seronegative and posteriorly characterized by the presence of antibodies against soluble liver antigen (anti-SLA), which were later found to be identical with previously described antibodies against liver pancreas and consequently termed as anti-SLA/LP antibodies[111]. Thus, initial studies regarding the seroprevalence of CD in AIH considered the presence of such autoantibodies, typical histological lesions, hypergammaglobulinemia, and the absence of viral markers. Since the 1990s, a diagnostic scoring system has been used for this[112].

An interesting peculiarity is that antifilamentous actin antibodies have been described in 90% of pediatric and 60% of adult CD patients and has thus been proposed as a diagnostic tool[110,113]. In the presence of CD and altered liver enzymes, antiactin positivity may reflect villous atrophy and may not be diagnostic of AIH[114].

A genetic link between CD and AIH has been suggested because both disorders express selected combinations of genes coding for class Ⅱ HLA molecules on chromosome 6[115]. Coexistence of the two diseases has been stated in EASL practice guidelines[106]. The prevalence of AIH in adults with CD is 1.6 and in children is 2%[116,117] whereas CD in patients with AIH is ten times more seroprevalent than that in the general population[118]. A similar tendency has been observed in children[117,119,120]. The clinical impact of a gluten-free diet on the outcomes of liver disorders in patients with AIH is still uncertain[114,119,121]. However, probable long-term beneficial effects of a gluten-free diet were suggested because patients with AIH and CD seem less prone to relapse after immunosuppressive withdrawal compared with patients with AIH unrelated to CD[122,123].

Tables 4 and 5[34,76,118,122,124-141] exhibit different studies on the association between the two diseases. Active screening for CD in patients with AIH is strongly recommended[115,142,143].

Asymptomatic persistent elevation of aminotransferases

Asymptomatic persistent elevation of aminotransferases unrelated to the usual causes of liver disease, such as nonalcoholic fatty liver disease (NAFLD), alcohol abuse, viral infection, AIH, or rare genetic and metabolic disorders, is relatively common among patients undergoing outpatient hepatology[144]. Studies suggest that celiac is the cause of liver disease in up to 10% of patients with cryptogenic hepatitis[145,146]. On the other hand, hypertransaminasemia has been reported to be the cause in 9%-40% of individuals with CD[116,147-151]. Abnormal aminotransferases in CD patients habitually normalize with a gluten-free diet. In patients with normal pretreatment liver enzyme levels, a significantly decreased serum levels with a gluten-free diet has been observed[146,148,151,152].

NAFLD

NAFLD is a major cause of chronic liver disease, with an estimated global prevalence of approximately 24%[153]. High prevalence rates of obesity worldwide have influenced the economic and clinical burden of NAFLD[154]. When metabolic syndrome is absent, NAFLD may be related to the concomitant presence of CD. Individuals with CD are at an increased risk of NAFLD compared with the general population[155]. Among patients with hypertransaminasemia and biopsy-proven NAFLD, approximately 3%, in whom liver enzymes normalize after 6 mo of a gluten-free diet, present with CD[156-159]. The association between NASH cirrhosis and refractory CD has been reported[160]. In addition, a pathogenetic link has been proposed between NAFLD and CD involving gut permeability, microbiota, and diet, but the pathogenesis of liver steatosis in CD remains unclear[161,162]. Considering the frequency of subclinical or silent presentations of CD, patients with NAFLD should be screened for celiac antibodies when steatohepatitis is present in the absence of metabolic risk factors and once other causes of liver disease are excluded[161,162].

HEPATITIS C

HCV might be involved in the breaking of tolerance to self-antigens and thus in triggering autoreactivity. HCV has been implicated both in the triggering of autoimmune diseases and in the development of autoantibodies[163].

The association between CD and hepatitis C is controversial and is yet to be elucidated. Although certain authors have reported a higher prevalence of CD among patients with hepatitis C[164,165], this association could not be confirmed in low-prevalence regions[166]. Nonetheless, the primary concern is for hepatitis C patients who will receive interferon-alpha (IFN)-based treatment because studies have reported severe cases of overt CD wherein receiving HCV treatment has led to the discontinuation of IFN[163].

Like CD patients, individuals undergoing IFN-based treatment may present severe diarrhea, refractory anemia, and hypoferritinemia that may persist after treatment discontinuation[167-169]. An early differential diagnosis facilitates the appropriate management of the underlying disease.

The heterogeneity of per capita incomes and health insurance systems across the world has determined the necessity to continue the use of IFN-based regimens in certain nations; however, newer drugs have become the first choice in most developed countries[170]. Considering the aforementioned exposed possibilities, patients should be screened for CD antibodies before treatment, and those with positive serology should be selected for IFN-free treatment regimens. If newer drugs are unavailable, a gluten-free diet must be preemptively initiated, and patients should be carefully monitored during the IFN treatment period[163,164,171]. It is important to emphasize that CD behavior with newer treatments is unknown, but it seems to be a safer alternative considering its mechanism of action.

HEPATITIS B

Studies that evaluated the coexistence of hepatitis B and CD have provided no evidence of an association between the two diseases. When serological screening for CD is performed in patients with chronic hepatitis B, EmA and tTG positivity vary in the ranges of 0%-8% and 0%-10%, respectively, and only 6% exhibit

Table 4 Case reports regarding the association of autoimmune hepatitis and celiac disease

Number of patients	Symptoms	AIH antibodies	Celiac antibodies	Duodenal biopsy	Response to gluten-free diet	Liver biopsy	Comorbidities	Country, year	Authors
1	Anemia, infection	ASM 1:500 anti-vimentin 1:500	AGA IgA and IgG (+) EmA (+)	Typical[1]	Yes[2]	Active chronic hepatitis	Erythroblastopenia	France, 2001	Bridoux-Henno *et al*[124]
1	Weight loss, fatigue, abdominal pain, and diarrhea	ANA 1:1280, p-ANCA 1:2560, SMA 1:1200, LKM1 1:50	Reticulin antibodies to 1:2000 AGA IgA (+)	Typical[1]	No, developed AIH despite of a gluten-free diet	Chronic inflammation In the portal area and proliferation of the small hepatic Ductules. Patchy degeneration of the liver cells.	Thyrotoxicosis	Finland, 2002	Arvola *et al*[34]
2	Diarrhea, abdominal enlargement and failure to thrive.	ANA (+) SMA (+) antiactine (+)	?	?	case 1: poor response to a gluten-free diet for the treatment of hepatitis; case 2: developed AIH despite the diet	Acute hepatitis With portal bridging necrosis and fibrosis and a Peri-portal inflammatory infiltrate of lymphocytes, Plasma cells and neutrophils	-	Italy, 2003	Leonardi *et al*[125]
1	Elevated liver enzymes detected, hypesthesia of the left foot, purpura and skin ulcers of both legs.	ANA (+)	AGA (+) EmA (+)	Typical[1]	Poor adherence to diet	Moderately active, chronic hepatitis with Interface lesions and fibrosis of the portal tract, Bile duct lesions and ductular Proliferations.	Cryoglobulinaemia	Switzerland, 2003	Biecker *et al*[126]
1	Jaundice and pale stools.	All negative. Score probable	AGA IgA (+) AGA IgG (+) Ema (+) tTG IgA (+)	Typical[1]	Liver disease progressed despite the diet	Moderate to severe lobular inflammatory activity, mononuclear portal inflammation, interface hepatitis, and portal and periportal fibrosis with septae; rosetting of liver cells and some giant cells.	.-	Italy, 2004	Iorio *et al*[127]
1	Ferropenia and elevation of aminotransferases.	-	tTG (+)	Villous atrophy	Elevation of aminotranferases despite the diet.	severe ymphocytic inflammatory infiltrate with slight increase of collagen in portal tracts, foci of lobular necrosis and presence acidophilus bodies		Peru, 2006	Tagle *et al*[128]
1	Anorexia, severe diarrhea, rapid loss of weight, amenorrhea and anemia.	ANA (+) SMA (+)	EmA (+) tTG (+)	Villous atrophy	Developed cirrhosis despite the diet	Cirrhosis	Holmes-Adie syndrome	Hungary, 2006	Csak *et al*[129]
1	Jaundice	SMA (+)	AGA (+) EmA (+) tTG IgA (+) IgG (+)	Typical[1]	Poor adherence to diet	Confirmed the diagnosis of acute AIH	Multiple sclerosis	Italy, 2008	Ferrò *et al*[130]

1	Weight loss, anorexia, fatigue, and diarrhea.	ANA+++	AGA IgA (+) AGA IgG (+) EmA (+) tTG (+)	Typical[1]	Liver disease was diagnosed on a gluten-free diet	Moderately active, chronic hepatitis with interface lesions And fibrosis of the portal tracts, ductular injury and ductopenia.	Autoimmune cholangitis overlap, Autoimmune thyroiditis	Turkey, 2009	Ozaslan *et al*[131]
1	Malaise, intermittent pyrexia and vomiting, an urticarial-vasculitic rash and joint pains.	ANA, SMA, LKM-1, mitochondrial, anti-LC1, anti-SLA/LP, parietal cell antibodies, all negative	EmA (+) tTG (+)	Typical[1]	No, developed AIH despite of a gluten-free diet	Lymphoplasmacytic hepatitis (portal interface and lobular) With moderate to marked activity and minimal Chronicity (fibrosis stage 1/6).	-	United Kingdom, 2009	Quail *et al*[132]
1	Two miscarriages, iron deficiency anemia, osteopenia and alternating bowel habit, elevated aminotransferases	ANA +++, homogeneous; SMA ++, anti-dsDNA 0.1527778	EmA 1:160	Severe villous atrophy	Yes[2]	Chronic Active hepatitis with piecemeal necrosis and lympho-plasmacellular periportal infiltrate	Lupus	Italy, 2010	Tovoli *et al*[133]
1	anemia, weakness and high aminotransferase levels	ANA 1:640, SMA 1:320, pANCA 1:160	EmA (+) tTG (+)	Flat mucosa	No, developed acute liver failure	Severe fibrosis	None	Italy, 2013	Volta *et al*[134]
1	Miscontrol of diabetes Altered liver enzymes	ANA 1:160	IgA tTG (+) EmA (-)	Typical[1]	Yes[2]	Moderate interface hepatitis and chronic inflammatory infiltrate, and foci of necrosis	Autoimmune thyroiditis and type 1 diabetes	Spain, 2016	Dieli-crimi *et al*[135]

[1]Vilous atrophy, crypt hyperplasia, lymphoplasmocytic infiltrate; [2]Patient under corticosteroids and azathioprine. AGA: Anti-gliadin antibody; EmA: Anti-endomysial antibody; tTG: Anti-tissue transglutaminase antibody; ANA: Anti-nuclear antibody; ASM: Anti-smooth muscle antibodies; anti-LP: Antibodies against liver pancreas; anti-SLA: Antibodies against soluble liver antigen.

compatible histological changes[172-175].

Several studies have reported lower efficacy of anti-HBV vaccines in individuals with CD[176,177] , which has been confirmed by a recent meta-analysis[178]. Therefore, novel immunization strategies have been proposed to ensure complete protection in such cases; these strategies include higher doses of vaccine and/or additional injection and intramuscular or preferably intradermal administration of booster doses of HBV vaccine because direct administration into the skin can activate an immune response mediated by dendritic cells through lower doses of antigen as opposed to intramuscular route of administration, which acts on cellular immune response. Moreover, administration of an additional booster dose of vaccine every 10 years is recommended for all patients with CD, including those who had developed anti-HBs with vaccine, because it has been shown that CD patients are predisposed to losing their memory antibodies[179,180].

NONCIRRHOTIC PORTAL HYPERTENSION

CD has been repetitively reported in association with idiopathic noncirrhotic intrahepatic portal hypertension (NCIHPH)[181-184], including a case of variceal hemorrage[185]. It has been suggested that in CD, repetitive stimulation by antigens along the portal vein - as well as immune responses to these result in the development of idiopathic portal hypertension[183]. In India, 10% of NCIHPH patients present with biopsy-proven CD[186]. Moreover, the presence of CD predicts reduced transplant-free survival in such patients[187]. Current data advises that all patients with unexplained portal hypertension should be screened for CD[186,188], although there is no evidence that

Table 5 Research screening celiac disease in patients with autoimmune hepatitis

Study	Screening method	Number of positive patients	Typical duodenal biopsy[1,2]	Response to gluten-free diet	Country, year	Authors
Prospective cohort	AGA IgG IgA > 1 AU IgA EmA IFI	8/181 (4.4%) EmA (+) 7/181 (3.9%) AGA (+)	5/5	-	United States/ Italy, 1998	Volta *et al*[76]
Retrospective	EmA tTG IgA IgG	3/47 (6.4%)	3/3	-	Italy, 2005	Villalta *et al*[136]
Retrospective	EmA tTG IgA IgG	19/140 (14%)	?	No	Italy, 2008	Caprai *et al*[120]
Retrospective	AGA IgA, IgG EmA IgA tTG IgA	5/40 (13%)	5/5	Mild decrease of transaminases, but never a complete normalization	Italy, 2008	Diamanti *et al*[137]
Retrospective	AGA IgA, IgG EmA IgA tTG IgA	7/15 (47%)	7/7	-	Turkey, 2009	Tosun *et al*[138]
Retrospective	?	3/278 (1.1%)	?	-	Germany, 2010	Teufel *et al*[139]
Prospective	IgA EmA IFI tTG ELISA	4/26 (15%)	3/4	-	Egypt, 2011	El-Shabrawi *et al*[140]
Retrospective	EmA IgA, IgG tTG IgA, IgG	15/79 (19%)	?	All of the 15 patients achieved sustained remission when treated with prednisone and azathioprine or cyclosporine	Italy, 2013	Nastasio *et al*[122]
Prospective	tTG IgA ELISA	3/64 (4.7%) tTG (+)	3/3	-	Iran, 2014	Najafi *et al*[141]
Prospective	IgA EmA IIF tTG ELISA HLA DQ2 DQ8	6 previous diagnoses 10/460 tTG + EmA + HLA -3.50%	-	-	Netherlands, 2014	van Gerven *et al*[118]

[1]Vilous atrophy, crypt hyperplasia, lymphoplasmocytic infiltrate; [2]Only a small number of patients usually undergo intestinal biopsy. EmA: Anti-endomysial antibody; IIF: Indirect immunofluorescence; tTG: Anti-tissue transglutaminase; ELISA: Enzyme-linked immunosorbent assay; HLA: Human leukocyte antigen; AGA: Anti-gliadin antibody.

a gluten-free diet can change the evolution of the disease or improve survival.

LIVER FAILURE AND CIRRHOSIS

CD is at least twice more common in cirrhotic patients than in the general population[189]. An association between cryptogenic cirrhosis and CD has been suggested[190,191]. The absence of a common histological pattern of liver injury in patients with CD does not favor the assumption that this disease directly damages the liver[192]. There have been case reports of patients with decompensated cirrhosis that reversed after the introduction of a gluten-free diet[55,192,193]. These data suggest that all cirrhotic patients, particularly those with hypoalbuminemia and ascites[189,192], should be screened for CD, because independent of the etiology of liver cirrhosis, patients with advanced liver disease and CD may benefit from a gluten-free diet.

LIVER TRANSPLANTATION

Prevalence of celiac antibodies was evaluated before and after transplantation; it was observed that patients with end-stage autoimmune liver disease, particularly those who are HLA-DQ2 or -DQ8 positive, had a high prevalence of celiac antibodies. Liver transplantation and/or immunosuppressive drugs used to prevent allograft rejection produced a significant decrease in serum levels of tTG and EmA antibody titers, but the clinical impact on CD outcomes, particularly on the risk of malignancy, remains unclear[48]. The reason(s) behind the significant and sustained decrease/normalization of CD related antibody serology after liver transplantation, particularly in the presence of gluten challenge, is obscure[194]. Regardless, pretransplant monitoring of CD-related autoantibodies could be helpful, particularly in HLA-DQ2- or HLA-DQ8-positive patients with end-stage autoimmune liver disease; moreover, the diagnosis of CD in this patient group, either before or after transplant, must be based on duodenal biopsies and response to gluten-free diet[194].

Diarrhea following orthotropic liver transplantation in patients receiving mycophenolic acid therapy is a noteworthy entity because it causes significant morbidity and mortality. The significance of duodenal histopathological findings and prevalence of tTG has been evaluated in this setting. Celiac-like changes and an increase in apoptotic counts are common in duodenal biopsies. Increased awareness of the clinical difference between CD and mycophenolate mofetil-induced villous atrophy is imperative because in the latter case, patients do not require a gluten-free diet and may instead need discontinuation of mycophenolic acid therapy[195].

Active screening for CD is recommended in pa-

tients with liver diseases, particularly in those with autoimmune disorders, steatosis in the absence of metabolic syndrome, NCIHPH, cryptogenic cirrhosis, and in the context of liver transplantation. In HCV, diagnosis of CD can be important as a relative contraindication to interferon use. Gluten-free diet ameliorates the symptoms associated with CD and may prevent the emergence of other autoimmune diseases and bowel cancer; however, the associated liver disease may improve, remain the same, or progress.

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ORIGINAL ARTICLE

Basic Study

Sinusoidal endotheliitis as a histological parameter for diagnosing acute liver allograft rejection

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Abstract

AIM

To investigated the feasibility of using sinusoidal endotheliitis (SE) as a histological marker for liver allograft rejection.

METHODS

We compared the histological features of 88 liver allograft biopsies with acute cellular rejection (ACR) and 59 cases with no evidence of ACR. SE was scored as: (1) focal linear lifting up of the endothelial cells by lymphocytes with no obvious damage to adjacent hepatocytes; (2) focal disruption of the endothelial lining by a cluster of subendothelial lymphocytes (a group of > 3 lymphocytes); and (3) severe confluent endotheliitis with hemorrhage and adjacent hepatocyte loss.

RESULTS

The sensitivity and specificity of SE was 81% and 85%, respectively. Using SE as the only parameter, the positive predictive value for ACR (PPV) was 0.89, whereas the negative predictive value for ACR (NPV) was 0.75. The correlation between RAI and SE was moderate (R = 0.44, $P < 0.001$) (Figure 3A), whereas it became strong (R = 0.65, $P < 0.001$) when correlating SE with the venous endotheliitis activity index only.

CONCLUSION

Our data suggest that SE scoring could be a reliable and reproducible supplemental parameter to the existing Banff schema for diagnosing acute liver allograft rejection.

Key words: Liver transplantation; Acute cellular rejection; Sinusoidal endotheliitis

Core tip: In this clinico-pathological study, we have found that scoring of the sinusoidal endotheliitis could be a reliable and practically reproducible supplemental parameter to the existing Banff schema for diagnosing early acute cellular rejection in liver allograft as well as predicting the occurrence of acute cellular rejection in appropriate clinical setting.

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INTRODUCTION

Liver transplantation (LT) has become a viable option for the treatment of end stage liver diseases due to the establishment of the concept of brain death in donors, and the availability of effective immunsuppressive agents, including calcineurin inhibitors for allograft recipients. The liver allograft is unique in that Kupffer cells are capable of sequestering cytotoxic antibodies formed against the graft, and the venous vascular endothelium could be gradually replaced by host hematopoietic cells over time[1]. Therefore, although HLA cross matching is not routinely performed in LT, antibody-mediated hyperacute rejection rarely occurs in liver allografts[2]. Acute cellular rejection (ACR) in the liver allograft often occurs between 5 and 30 d after LT[3]. The overall frequency of ACR varies with the baseline immunosuppression regimen used, ranging from 30% to 70%[4,5]. The diagnosis of ACR is usually suspected based on clinical manifestations and abnormal liver function tests, and confirmed by the examination of a core needle biopsy. The Banff schema is currently the standard system for diagnosing and grading the severity of ACR[3,6]. In the Banff schema, the severity of three morphological parameters, *i.e.,* portal inflammation, bile duct inflammation, and venous endotheliitis, are assigned individual scores from 0 to 3, and the sum of these scores is called the rejection activity index (RAI). However, experienced transplant pathologists often pay more attention to the presence of, and the degree of, vascular endothelial damage in portal veins and in central veins.

The sinusoidal endothelium, with its relatively low hydrostatic pressure and large surface area, forms a unique interface between the graft and the recipient's immune system. Sinusoidal endothelial damage has been recognized as a histological parameter for diagnosing graft-*vs*-host disease in the setting of hematopoietic stem cell transplantation[7,8]. Sinusoidal inflammation was recently incorporated as part of antibody-mediated liver allograft rejection in the updated Banff criteria[9]. However, the association between sinusoidal endothelial damage and ACR has not been examined systematically. In our assessment of liver allograft biopsies from patients with concurrent or subsequent ACR, we frequently observed sinusoidal subendothelial lymphocytic infiltration, with a range of severity that increases from lifting of the endothelium by a linear arrangement or clustering of lymphocytes, to disruption of the intact endothelial lining, to hemorrhage and damage to adjacent hepatocytes. In the present study, we investigated whether sinusoidal endotheliitis (SE) could be a reliable and reproducible supplemental parameter to the existing Banff schema for diagnosing ACR in liver allograft biopsies.

MATERIALS AND METHODS

Patients

After obtaining the approval of the institutional review board (IRB), all biopsies from 2010 to 2015 at the McGill University Health Center (MUHC) were studied. Most liver transplant cases were performed for hepatitis C-related cirrhosis, the leading indication for liver transplantation in Canada[10]. Cases with detailed clinical history and a definite histopathologic diagnosis of ACR were recruited into our study. Liver allograft biopsies were divided into two groups: cases with or without histological evidence of ACR according to the Banff schema. Biopsies were performed between 6 and 180 d post-transplant either for clinical indication or protocol biopsy. There is no skewed distribution in terms of biopsy time or clinical indication/protocol biopsy between the two groups. All patients received baseline immunosuppressive therapy or antiviral treatment if the primary liver disease was hepatitis C. A total of 88 cases with a definitive histological diagnosis of ACR were obtained and were designated

as the ACR-positive group. The primary liver diseases of this group included 82 cases of hepatitis C, two cases of postpartum liver failure, one case of primary sclerosing cholangitis, one case of non-alcoholic steatohepatitis (NASH), one case of hepatitis B, and one case of cholestasis of uncertain etiology. Cases with recurrent hepatitis C were excluded from the ACR-positive group if serum HCV RNA levels were high (> 8.00 log IU/mL) or if there was histologic evidence of hepatitis C infection. The ACR-negative group was comprised of 59 cases, including 45 cases of recurrent hepatitis C, seven cases of NASH, six cases of cholestasis of uncertain etiology, and one case of primary biliary cholangitis.

Biopsy preparation for light microscopy

Tissue was fixed in 10% neutral buffered formalin, embedded in paraffin, processed and cut into 3 μm-thick sections. The slides were stained with hematoxylin-eosin, Masson trichrome, reticulin, PAS, PAS + diastase, and Prussian blue iron stains.

Grading of acute liver allograft rejection

The liver biopsies were evaluated by three pathologists to reach a consensus on the diagnosis of ACR. Following the guidelines of the 1997 Banff schema for grading liver allograft rejection, the rejection activity index (RAI) was determined by summing the individual scores of the parameters (on a 0 to 3 scale), *i.e.*, portal inflammation, bile duct inflammation and, and venous endotheliitis.

Definition and grading of sinusoidal endotheliitis

SE was defined as subendothelial lymphocytic infiltration with lifting and/or damage to the sinusoidal endothelial cells. Sinusoidal lymphocytes were counted on HE slides in five high-power fields (HPF). Greater than 100 lymphocytes/HPF was considered an increase. Increase of intrasinusoidal lymphocytes and adhesion of lymphocytes to the endothelium were not considered to be SE. Grading of SE was as follows: (1) focal linear lifting up of the endothelial cells by lymphocytes with no obvious damage to adjacent hepatocytes; (2) focal disruption of the endothelial lining with a cluster of subendothelial lymphocytes (a group of > 3 lymphocytes); and (3) severe confluent endotheliitis with hemorrhage, adjacent hepatocyte loss, with or without fibrosis.

Statistical analysis

The linear correlation coefficient (r) was calculated to evaluate the correlation between SE and RAI and between SE and the score of portal venous endotheliitis using Excel software (Pearson correlation coefficient test). The sensitivity, specificity, positive, and negative predictive value of SE for ACR were calculated using the total Banff RAI scores.

RESULTS

Histologic findings of acute cellular rejection

Among the 88 cases with ACR, 82 cases had ACR with average RAI scores of 5. Six cases had a RAI below 3, but with definitive evidence of endotheliitis in the portal or central veins. The 59 cases without evidence of ACR had no or very minimal inflammation in biopsies or showed histologic features and clinical presentation (*e.g.,* elevated hepatitis C viral load, or morbid obesity, *etc*.) pointing to another etiology.

When using the Banff criteria to diagnose ACR, the participating pathologists felt that portal vein or central vein endotheliitis was more reliable and reproducible than portal inflammation or ductulitis. The spectrum of portal and central venous pathology is illustrated in Figure 1. The non-inflamed portal tract has little or no inflammatory cells (Figure 1A). Mild portal inflammation without venular endothelial damage was considered negative (Figure 1B). Portal vein endotheliitis was unequivocal when the endothelium was lifted up by subendothelial lymphocytic infiltration (Figure 1C). A severe case of endotheliitis is characterized by perivenular liver cell necrosis in addition to endothelial damage (Figure 1D).

Increase of intrasinusoidal lymphocytic infiltration without sinusoidal damage is a universal finding (100%) in ACR-positive cases (Figure 2A), and is also frequently seen in ACR-negative cases (specificity 57%). These lymphocytes may appear to attach to the sinusoid wall, or float in the lumen at different levels of the section. Therefore, an increase in lymphocytes in the sinusoids was not considered as reliable evidence of ACR due to poor reproducibility and lack of specificity. Sometimes, diffuse lymphocytic infiltration, Kupffer cell hypertrophy and hyperplasia can occur with unknown significance. True SE is characterized by lymphocytic infiltration underneath the sinusoidal endothelium, typically lifting up and detaching the overlying endothelium from the basement membrane (Figure 2B and C). There were total 80 cases with SE, including 35 cases of grade 1, 32 cases of grade 2, and 13 cases of grade 3. In grade 1, we observed focal linear lymphocytic infiltration with the formation of a "pearl band" along the sinusoidal subendothelial space (Figure 2B). Focal clustering (> 3 lymphocytes per cluster) of lymphocytic infiltration between the endothelium and the basement membrane that interrupts the integrity of the endothelium represents grade 2 SE (Figure 2C). In the grade 1 and grade 2 SE case scenarios, SE was associated with sinusoidal dilatation. Grade 3 endotheliitis is characterized by further damage causing hemorrhage and adjacent hepatocyte loss with mixed lymphohistiocytic infiltration (Figure 2D). The findings of a collapsed reticulin framework (Figure 2E) and deposition of collagen bands (Figure 2F) are consistent with the loss of hepatocytes. The presence of hemorrhage and adjacent SE can help to distinguish

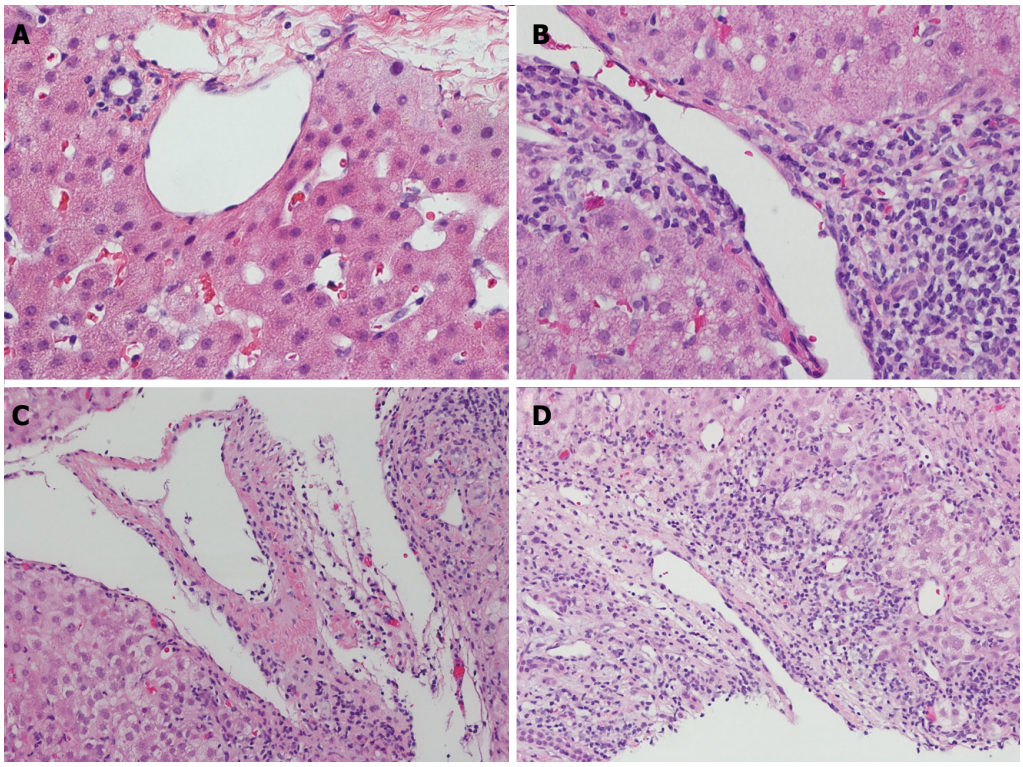

Figure 1 Spectrum of portal and central venous pathologic findings. A: Intact portal triad; B: Portal inflammation with intact venule; C: Portal vein with subendothelial lymphocytic infiltration (endotheliitis); D: Severe endotheliitis with perivenular hepatocyte necrosis (hematoxylin-eosin staining, magnification × 400).

Table 1 Association between sinusoidal endotheliitis and acute cellular rejection

	ACR positive	ACR negative	Total
SE positive	71	9	80
SE negative	17	50	67
Total	88	59	147

ACR: Acute cellular rejection; SE: Sinusoidal endotheliitis.

it from lobular hepatitis-related hepatocyte loss.

Evaluation of sinusoidal endotheliitis as a new parameter for diagnosing acute cellular rejection

As shown in Table 1, in the 88 ACR-positive cases and 59 ACR-negative cases, as diagnosed by the Banff schema, the sensitivity of SE was 81% and the specificity was 85%.

Using SE as the only parameter, the positive predictive value for ACR (PPV) was 0.89, whereas the negative predictive value for ACR (NPV) was 0.75. The correlation between RAI and SE was moderate (R = 0.44, P < 0.001) (Figure 3A), whereas it became strong (R = 0.65, P < 0.001) when correlating SE with the venous endotheliitis activity index only (Figure 3B).

DISCUSSION

The Banff schema is currently the gold standard for the diagnosis of ACR and for the assessment of its severity. However, this seemingly uncomplicated practice is not without challenges. For instance, the number of portal tracts in each liver biopsy varies and portal changes are often patchy, resulting in false negative biopsies. Additionally, portal inflammation and bile duct injury are features which are often shared by other liver diseases, in particular recurrent hepatitis C[11] or drug toxicity[3]. In this study, we demonstrate that SE is a useful supplemental parameter for diagnosing ACR in the liver with high sensitivity, specificity, and positive and negative predictive value. The SE score showed a strong correlation with the portal venous endotheliitis index score of the Banff criteria. In 6 cases, SE was an early sign that predicted the development of subsequent ACR.

Sinusoids are low-pressure vascular channels lined by a specialized endothelium with slit-like spaces, which lie between plates of hepatocytes, providing these cells with a large interface for the exchange of various substances with the circulating blood[12]. In the normal human liver, a small number of functional T lymphocytes can be seen in the portal tracts and scattered throughout the liver parenchyma. Lymphocytic infiltration of the liver could be the result of an immune response to many insults. Lymphocyte recruitment to the human liver is mediated by distinct combinations of molecules depending on whether recruitment occurs *via* the portal vascular endothelium or the hepatic sinusoids[13]. Intravital microscopy has revealed that leucocyte recruitment to the hepatic parenchyma can occur through the sinusoids in a process that involves direct adhesive interactions with

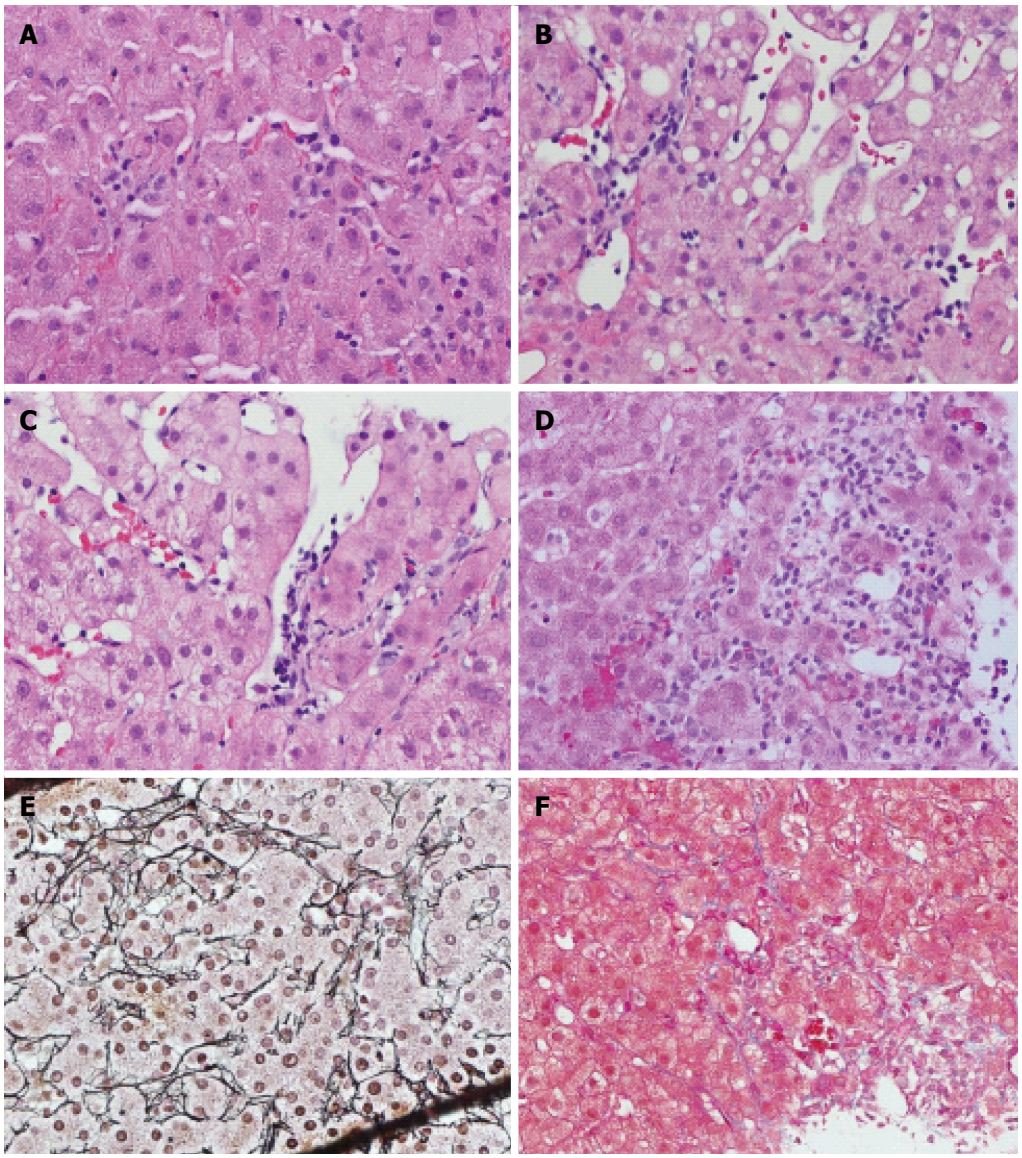

Figure 2 Spectrum of sinusoidal pathology. A: lymphocytes in sinusoidal spaces, with adhesion to endothelium but without lifting of endothelial cells; B: Grade 1 sinusoidal endotheliitis with subendothelial linear lymphocytic infiltration; C: Grade 2 sinusoidal endotheliitis with subendothelial lymphocyte clusters and partially disrupted endothelium; D: Grade 3 sinusoidal endotheliitis with endothelial damage, fresh hemorrhage, lymphohistiocytic infiltration and adjacent liver cell necrosis; E: Grade 3 sinusoidal endotheliitis with collapsed liver cell plates on reticulin staining; F: Grade 3 sinusoidal endotheliitis with collagen deposition on Masson trichrome staining (hematoxylin-eosin staining, magnification × 400).

the sinusoidal endothelium[14]. An animal model of liver injury in rat has demonstrated that most lymphocytes are recruited to the liver *via* the hepatic sinusoids with subsequent redistribution to the hepatic parenchyma in lobular hepatitis or to the portal tracts in portal and interface hepatitis[15]. Given the much larger surface area, the low pressure and relatively slow blood flow, sinusoidal endothelium should, theoretically, bear more immunological damage than either portal or central venous endothelium in patients with ACR. Sinusoidal lymphocyte infiltration has been recognized as a common histological finding in the liver in experimental and clinical graft-*vs*-host disease[7,8]. Previous studies have demonstrated that infiltration of lymphocytes in the sinusoidal space, and particularly adherence of lymphocytes to the endothelium are associated with various liver diseases[16-18]. In reality, it is often difficult to determine whether sinusoidal lymphocytes are attached to the endothelium or simply the result of tangential cuts. Furthermore there is no consensus regarding the upper limit of the number of lymphocytes in the sinusoids[19]. In the present study, presence of > 100 lymphocytes/HPF was considered an increase in intrasinusoidal lymphocytes. However, we found that the increase in sinusoidal lymphocytes was not specific to ACR because it was observed not only in all ACR-positive cases but also in some ACR-negative cases. Therefore, to ensure reproducibility, the definition of SE in the current study is identical to endotheliitis that occurs in the portal or central vein, as characterized by linear or clustered subendothelial lymphocytic infiltration that lifts the endothelial cells

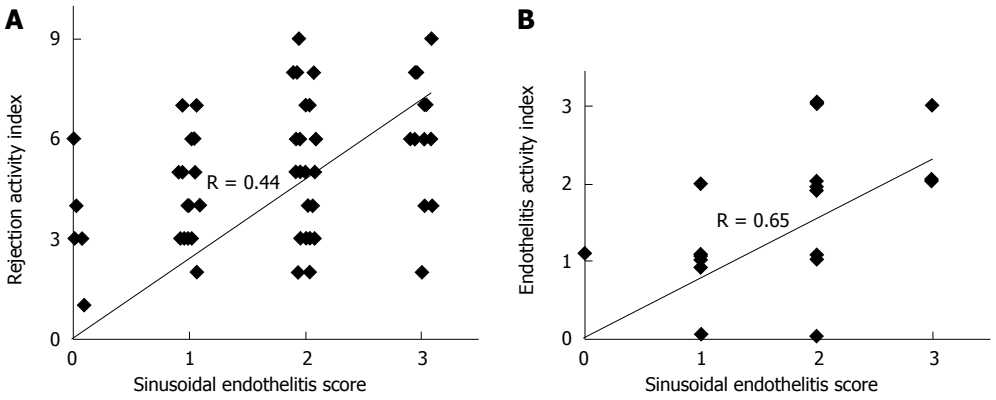

Figure 3 Correlation between scores of sinusoidal endotheliitis and the rejection activity index of the Banff schema. A: The sinusoidal endotheliitis score (SES) moderately correlates with the overall rejection activity index; B: The SES strongly correlates with the portal and central vein endotheliitis activity score (hematoxylin-eosin staining, magnification × 400).

or disrupts the integrity of the sinusoidal endothelium with or without peripheral hepatocyte necrosis.

Recurrent hepatitis C in post-transplant biopsy is problematic, because portal inflammation, bile duct damage, and lobular hepatitis can mimic ACR. The combination of clinical presentation, the pattern of elevated liver enzymes, the viral titer, and careful examination of the histological pattern are often required to distinguish it from bona fide ACR[11]. To complicate the matter even further, cases of mixed ACR and recurrent HCV do exist. In this study, cases with elevated HCV RNA levels or histologic evidence of hepatitis C infection were excluded from the ACR-positive group so that we could focus exclusively on the latter.

The 3-tier grading of SE is a measure of the severity of the rejection process, but also somewhat reflects the evolving process of the disease: starting from subendothelial linear lymphocytes that lift up the intact sinusoidal endothelium, to the formation of clusters of lymphocytes that interrupt the intact endothelial lining, to causing hemorrhage and adjacent hepatocyte necrosis and subsequent collagen deposition. Not surprisingly, the scores of SE were more strongly correlated with the portal vein or central vein endotheliitis activity index of the Banff schema than they were with the overall RAI. Both SE and portal or central vein endotheliitis are more specific for ACR than other parameters, and they should be given more weight in scoring the severity of ACR.

In the six cases that were negative for ACR by the Banff criteria, but positive for SE, a follow up repeat biopsy revealed the subsequent development of ACR. This could be due to patchiness of portal vein endotheliitis with limited available portal numbers in a biopsy. However, it is more likely that SE was a precursor, presenting earlier than portal or central vein endotheliitis because of the larger surface area of sinusoids and easier access to lymphocytes.

The limitations of our study include the study population and the sample size. Our study population was composed predominantly of patients with hepatitis C as primary disease, so that these findings may not necessarily hold true for patients with other primary disease leading to liver transplantation. Hepatitis C virus (HCV)-caused cirrhosis is the most common indication for liver transplantation (LT) in Canada.[10] Despite advances in antiviral therapy, reinfection of HCV in liver allografts is almost universal[20,21]. The recurrence of HCV as defined by elevation of HCV RNA in serum, and histologic evidence of HCV can be demonstrated in 70%-90% of recipients after 1 year and in 90%-95% after 5 years[22,23]. Most post-transplant liver biopsies in our institute were cases with or without serum HCV RNA to rule out acute cellular rejection (ACR). Since recurrent HCV shares some histology features with ACR, cases of ACR with high HCV RNA were excluded from ACR group to simplify the comparison. In ACR negative group, cases with high HCV RNA were included because there weren't enough cases of HCV RNA negative patients in this group. Post-liver transplant patients with neither ACR nor HCV RNA were rarely indicated for biopsy. Exceptions are the cases with other etiology liver diseases, such as non-alcoholic steatohepatitis (NASH), or cholestatic disease. Therefore, there were more non-HCV cases in ACR negative group than those in ACR group. Secondly, despite our study including a significant number of cases, confirmation in larger numbers of biopsies and with follow up repeat biopsies should be carried out to provide further support of SE as an early and reliable histological marker of ACR.

In summary, we demonstrated that SE scoring could be a reliable, practical supplemental parameter to the existing Banff schema for diagnosing ACR of liver allograft as well as for predicting the occurrence of ACR in an appropriate clinical setting.

COMMENTS

Background

In the last two decades, Banff schema has been the standard system for diagnosing and grading the severity of acute cellular rejection in liver allografts.

The Banff schema evaluates portal inflammation; bile duct damage; and venous endotheliitis. Each component is scored on a scale of 0-3 and added together to report a final rejection activity index (RAI). In practice, experienced transplant pathologists often pay more attention to the presence of, and the degree of, vascular endothelial damage in portal veins and in central veins. One of the limitations of this system is the variation in portal tract number in each liver biopsy and the patchiness of portal changes, causing false negative biopsies. Additionally, portal inflammation and bile duct injury are features which are often shared by other liver diseases.

Research frontiers

The sinusoidal endothelium, with its relatively low hydrostatic pressure and large surface area, form a unique interface between the graft and the recipient' s immune system. Sinusoidal endothelial damage has been recognized as a histological parameter for diagnosing graft-*vs*-host disease in the setting of hematopoietic stem cell transplantation. Sinusoidal inflammation was recently incorporated as part of antibody-mediated liver allograft rejection in the updated Banff criteria. However, the association between sinusoidal endothelial damage and ACR has not been examined systematically.

Innovations and breakthroughs

In this study, the authors investigated the feasibility of using sinusoidal endotheliitis (SE) as an early diagnostic marker for liver allograft rejection by comparing the histological features of 82 liver transplant (LT) biopsies with acute rejection (AR) and 65 cases with no evidence of AR. The sensitivity and specificity of SE was 81% and 85%, respectively. Using SE as the only parameter, the positive predictive value for ACR (PPV) was 0.89, whereas the negative predictive value for ACR (NPV) was 0.75. The correlation between RAI and SE was moderate (R = 0.44, $P < 0.001$) (Figure 3A), whereas it became strong (R = 0.65, $P < 0.001$) when correlating SE with the venous endotheliitis activity index only. This is the first study to propose the concept of SE and its diagnostic value in ACR. It represents a significant contribution to understanding ACR in routine pathology practice and potential improvement in diagnosing ACR.

Applications

The authors' data suggest that SE scoring was a sensitive and specific parameter for diagnosing ACR. These results could be useful to pathologist in daily practice, especially when liver biopsy with limited portal tract number or showing the patchiness of portal changes.

Terminology

SE was defined as subendothelial lymphocytic infiltration with lifting and/or damage to the sinusoidal endothelial cells. Sinusoidal lymphocytes were counted on HE slides in five high-power fields (HPF). Greater than 100 lymphocytes/HPF was considered an increase. Increase of intrasinusoidal lymphocytes and adhesion of lymphocytes to the endothelium were not considered to be SE. Grading of SE was as follows: (1) focal linear lifting up of the endothelial cells by lymphocytes with no obvious damage to adjacent hepatocytes; (2) focal disruption of the endothelial lining with a cluster of subendothelial lymphocytes (a group of > 3 lymphocytes); and (3) severe confluent endotheliitis with hemorrhage, adjacent hepatocyte loss, with or without fibrosis.

Peer-review

The work of Shi and co-workers investigates the impact of sinusoidal endotheliitis for qualification of liver graft rejection. This parameter is an additional parameter to the qualification categories of the RAI Score currently used in clinical routine to express the degree of rejection activity after liver transplantation. Since quantification of sinusoidal endotheliitis reached a sensitivity of 81% and a specificity of 85% it may reflect a more sensitive parameter than the currently used categories (lymphocyte infiltration around portal veins, centrilobular veins and bile ducts). Alternatively, this new category might reflect an additional parameter, which would improve the accurateness of the RAI score.

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ORIGINAL ARTICLE

Basic Study

Genomic variability of *Helicobacter pylori* isolates of gastric regions from two Colombian populations

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Author contributions: All the authors that were involved in the acquisition and interpretation of the results, read and approved the final manuscript; Matta AJ, Pazos AJ and Bustamante-Rengifo JA conducted the microbiological and molecular tests; Matta AJ and Bravo LE analyzed the data; Matta AJ, Pazos AJ, Bustamante-Rengifo JA and Bravo LE wrote, edited, and revised the manuscript.

Institutional review board statement: All procedures involving human participants were reviewed and approved by the Ethics Committee at Universidad del Valle, Cali, Colombia.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: The authors declare that there is no conflict of interest related to this study.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at andres.matta@correounivalle.edu.co Participants gave informed consent for data sharing.

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Abstract

AIM

To compare the genomic variability and the multiple colonization of *Helicobacter pylori* (*H. pylori*) in patients with chronic gastritis from two Colombian populations with contrast in the risk of developing gastric cancer (GC): Túquerres-Nariño (High risk) and Tumaco-Nariño (Low risk).

METHODS

Four hundred and nine patients from both genders with dyspeptic symptoms were studied. Seventy-two patients were included in whom *H. pylori* was isolated from three anatomic regions of the gastric mucosa, (31/206) of the high risk population of GC (Túquerres) and (41/203) of the low risk population of GC (Tumaco). The isolates were genotyped by PCR-RAPD. Genetic diversity between the isolates was evaluated by conglomerates analysis and multiple correspondence analyses.

RESULTS

The proportion of virulent genotypes of *H. pylori* was 99% in Túquerres and 94% in Tumaco. The coefficient of similarity of Nei-Li showed greater genetic diversity

among isolates of Túquerres (0.13) than those of Tumaco (0.07). After adjusting by age, gender and type of gastritis, the multiple colonization was 1.7 times more frequent in Túquerres than in Tumaco (P = 0.05).

CONCLUSION

In Túquerres, high risk of GC there was a greater probability of multiple colonization by *H. pylori*. From the analysis of the results of the PCR-RAPD, it was found higher genetic variability in the isolates of *H. pylori* in the population of high risk for the development of GC.

Key words: *Helicobacter pylori*; Pathogenicity islet *cag*; *cagA*; *vacA*; Multiple colonization; PCR-RAPD

Core tip: Multiple colonization of *Helicobacter pylori (H. pylori)* occurred more frequently in individuals living in the Colombian population with higher risk of gastric cancer (GC) (Túquerres). In the two populations contrasted in relation to the risk of developing GC. (Túquerres high risk and Tumaco low risk) *H. pylori* was identified with specific genetic characteristics for each region and with varying stages of genomic variability. The diversity of *H. pylori* dependent of the anatomic regions of the gastric mucosa, obstructs the eradication of the microorganism. Identifying the multiple colonization and evaluating the genetic diversity of *H. pylori* individuals may be sifted that require particular schemes of early treatment and prevention of the precursor lesions of GC.

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INTRODUCTION

Helicobacter pylori (H. pylori) is recognized as the principal etiologic agent of chronic gastritis; it is related to the development of peptic ulcer, and is the most important factor in the pathogenesis of gastric adenocarcinoma[1].

The high genetic variability between strains of *H. pylori* is associated to the population or geographic origin of the individuals of the population[2]. Variations in the genomic DNA of *H. pylori* allows the identification of individuals infected by multiple strains that colonize different anatomic regions of the gastric mucosa, phenomenon described as multiple colonization[3]. In Colombia, there are regions of high and low risk for the development of gastric cancer (GC), although the prevalence of the infection by *H. pylori* is similar (> 80%)[4,5]. In Tumaco (low risk of GC) and Túquerres (high risk of GC), strains of *H. pylori* were identified with different stages of genetic variability for each region. The genetic variability of *H. pylori* that colonizes the different anatomical regions from the gastric mucosa has not been studied in Colombia and it makes the eradication of the microorganism difficult. Therefore it is important to study the multiple colonization of *H. pylori* as a strategy to prevent the precursor lesions of GC. The high genetic diversity between strains of *H. pylori* has been evaluated by several methods of characterization, including polymorphisms in the length of the restriction fragments (RFLP), Restriction fragment polymorphism amplified by PCR (AFLP-PCR), ribotyping and random amplification of polymorphic DNA (RAPD)[6,7].

The technique most used to discriminate isolates of *H. pylori*, in the genotyping studies, is the RAPD with a high level of discrimination (0.99-1.0), able to reveal a marked genetic diversity between isolated strains of different patients and it is discriminatory in the determination of the similarity of the isolated strains from the same patient in the biopsies taken from different anatomical sites from the gastric mucosa[8,9].

Previous investigations describe diversity of *H. pylori* strains with differences in their virulence factors such as the gene associated with *cagA*[10] cytotoxicity and the gene related with the production of vacuolizing toxin *vacA*[11].

A standard treatment to eradicate the *H. pylori* doesn't exist, possibly because the gastric mucosa of the same host may be colonized by multiple strains of the microorganism with differences in the virulence factors and differences in the genomic DNA and eventually antimicrobial susceptible and resistant isolates coexist[12]. This phenomena, not studied in Colombia, makes the eradication of *H. pylori* difficult and doesn't allow the establishment of therapeutic efficacy as a strategy for the prevention of precursor lesions of GC.

It is important to genotype the isolated *H. pylori* from the different locations of the gastric mucosa (antrum and body) to evaluate genetic diversity and determine the multiple colonization, in the two Colombian populations with contrast in the risk of developing GC.

MATERIALS AND METHODS

Subjects, samples and histopathology

Adult men and women with symptoms of dyspepsia were included (n = 203) from Tumaco and (n = 206) from Túquerres. Four biopsies from the gastric mucosa were obtained from each patient, two from the antrum and two from the gastric body for its histopathologic evaluation. Histological sections cut into microtome at 6 μm, were colored with hematoxylin-eosin and were interpreted according to the Sydney[13] classification system. The presence of *H. pylori* was determined with Giemsa's staining.

For cultivation and genotyping of *H. pylori* three biopsies of gastric mucosa were used, two from the antrum and one from the gastric body, that were preserved in thioglycolate and glycerol at (25%). The biopsies were immediately frizzed in liquid nitrogen and subsequently transported on dry ice at -70 ℃ until its analysis in the Microbiology and Histopathological Laboratory of the Pathology Department of the Universidad del Valle-Colombia. This research counts with the approval of the Human Ethics Committee (CIREH) of the Universidad del Valle. All the participants signed the informed consent.

H. pylori isolation and cultivation

Fragments of gastric mucosa were homogenized in 200 μL of sterile saline solution 0.89%. The homogenate was seeded in columbia agar plates (Oxoid, Basingstoke, Hampshire, England) with defibrillated lamb blood to 7% plus selective supplement for *H. pylori* (Dent) and incubated in microaerophilic conditions (6% O_2, 6% CO_2, 88% N_2 using CampyPak Plus Envelop, BBL, Nashville, TN United States) at 37 ℃ during 4 to 8 d[4]. The compatible colonies with *H. pylori* were transferred to columbia agar defibrillated lamb blood to 10%, to purify and identify them by the tests of urease, catalase, oxidase, Gram staining. A PCR of the *ureA* gene was used to confirm the species[14].

DNA extraction from *H. pylori* isolates

From a pure cultivation in petri dish colonies were transferred to 1.0 mL of PBS 1 × pH = 7.2, and it was centrifuge at 13000 RPM × 2 min. The cell pellet was re-suspended in 300 μL of extraction buffer [Proteinase K 100 μg/mL, sodium dodecyl sulfate (SDS) 0.5%, ethylenediaminetetraacetic acid (EDTA) 5 mmol/L, Tris-HCl 10 mmol/L, pH = 8.0 and 276 μL of distilled water], the cell button was homogenized and taken to the dry block (Labnet®) at 56 ℃ for 18 h, subsequently the proteinase K was inactivated at 76 ℃ for 10 min and NaCl 5M was added. It was shaken in vortex for 15 seg, and then it was centrifuge at 13000 RPM for 5 min. To the supernatant only, 2 volumes of absolute ethanol were added. It was centrifuge at 13000 RPM for 20 min at 4 ℃. The decanted was precipitated by addition of 2 volumes of absolute ethanol at 70%, it was mixed and centrifuge for 5 min at 13000 RPM and the supernatant was discharged. The DNA pellet dried by tube inversion for 10 min. The precipitate DNA was re-suspended in 100 μL of TE buffer (Tris 10 mmol, EDTA 1 mmol) and it was stored at -20 ℃. The yield and purity of the DNA were determined by optical density at 260/280 nm in Gene Quant II® spectrophotometer (Pharmacia Biotech, Piscataway, NJ, United States) according to the manufacturer's instructions[15].

ureA gene amplification

The molecular identification of *H. pylori* was performed by PCR amplification of the *ureA* gene. The following reagents were added to a 0.2 mL tube: 1 × PCR buffer (Buffer green 5 ×, Promega®); $MgCl_2$ 1 μmol/L (Promega®); 0.25 mmol/L of dNTPs (deoxyribonucleoside 5'-triphosphates - dATP, dCTP, dGTP and dTTP Promega®); 50 pmol/L of each primer (sense 3'-AAGACATCACTATCAACG-5'/anti-sense 5'-CCCGCTCGCAATGTCTAA-3'); 0.5 U of GoTaq DNA polymerase (Promega®) and 25 ng of genomic DNA from *H. pylori* in a final volume of 25 μL. Amplification was performed at 95 ℃/2 min followed by 35 cycles (95 ℃/1 min, 54 ℃/1 min and 72 ℃/1 min) and a final extension at 72 ℃/15 min.

Detection of *cagA* and *vacA* genes

The virulence factors were analyzed by PCR amplification of *cagA* gene. An amplicon of 183 bp was obtained with specific primers (CagAF and CagAR). The negative isolates for the amplification of *cagA* gene were confirmed by PCR assay *empty-site* with primers [ES-F (+) - ES-R (-)]. The *vacA* gene alleles (*s1*/*s2*, *m1*/*m2*) were analyzed with the specific primers (VA1F and VA1R) to obtain the sizes of the amplicons; 176 bp and 203 bp for *vacA s1* and *s2*, respectively. PCR was performed for *vacA* gene alleles *m1*/*m2* with primers (HPMGF- HPMGR) to obtain the sizes of 401 bp amplicons for *vacA m1* and 476 bp for *vacA m2*. PCR was performed under the following conditions: 2 min of pre-incubation at 95 ℃, followed by 40 cycles of 1 min at 95 ℃, 1 min at 52 ℃, a min at 72 ℃, and subsequently, a final extension of 72 ℃ for 5 min. In each experiment, *H. pylori* reference strain Tx30a (ATCC 51932) was included as a negative control. A positive control, *H. pylori* reference strain 700392 (ATCC 26695), along with inhibition controls for each sample, were used to rule out false negative results[16].

RAPD-PCR

PCR amplification was performed in separate reactions with the primers, 1281 (5'-AACGCGCAAC-3') and 1254 (5'-CCGCAGCCAA-3')[17], under the following amplification conditions: two cycles of denaturation at 94 ℃ for 5 min; amplification at 36 ℃ for 1.30 min and extension at 72 ℃ for 5 min; 40 cycles of denaturation at 94 ℃ for 1 min; amplification at 36 ℃ for 1.30 min; extension at 72 ℃ for 2 min and a final cycle of extension at 72 ℃ for 10 min. The components of the PCR were the buffer (10 mmol/L Tris pH = 9.0, 50 mmol/L KCl, Triton 100 × 0.1%); $MgCl_2$ 3 mmol/L 1 × (Promega®); dNTPs 0.2 mmol/L (5'-deoxyribonucleoside triphosphates - dATP, dCTP, dGTP and dTTP - Promega®); 25 pmol of the primer 1281 and 25 pmol of the primer 1254; 1 μL of genomic bacterial DNA; 0.2 U of enzyme Go*Taq* DNA polymerase (Promega®) and milliQ water until completing at 12.5 mL. A reaction without DNA was used as a negative control. The amplification was performed in a thermocycler (Swift MiniPro™, Esco).

Electrophoresis of amplicons

All the amplicons obtained in each of the previously described PCR reactions were run on agarose gel (SeaKim, FMC Bioloabs) at 2%, stained with ethidium bromide (Invitrogen, Carlsbad, CA, United States) at 0.5 μg/mL, in an electrophoresis chamber (Fotodyne Inc., Hartland, WI, United States). This process was performed by an EC-105 Compact Power Supply (Thermo Fisher Scientific Inc., Asheville, NC, United States) at 75 V for 40 min.

Statistical analysis

The images obtained through the amplification by RAPD-PCR were analyzed by Gel-Pro Analyzer 4.5 for Windows (Media Cybernetics Inc.). A Binary data matrix was constructed based on the presence (1) and absence (0) of bands observed on electrophoresis gel. Relations between isolates were established by cluster analysis and multiple correspondence analysis (MCA), whereby similarities between individuals were evaluated in terms of their molecular profiles, in which the distance between each pair of individuals is proportional to its molecular differences. The estimation of distances between each pair or group of isolates of *H. pylori* was calculated with the similarity of Neid, 1973. *H. pylori* isolates were classified as virulent when *cagA* (+) gene amplification was detected. Low virulence was established when there was an absence of *cag* marker by the amplification of *empty site cagA*. To identify multiple colonization in relation to virulence of *H. pylori* isolates, patients were sub-grouped according to the following characteristics: gender, age, type of gastritis and cancer risk. Univariate and bivariate analyses were performed using the χ^2 test. Subsequently, the risk of presenting multiple colonization was then evaluated according to the characteristics of the populations through Odds Ratio with a 95%CI. Statistical analysis was performed using the SAS statistical package, version 9.0. The statistical significance was accepted with a P value $\leqslant 0.05$.

RESULTS

General results

The prevalence of the infection by *H. pylori* diagnosed by histopathology was higher in the population of low risk of GC (Tumaco, 88.7%) than in the population of high risk of GC (Túquerres, 86.4%). However, the bacteria was isolated from three anatomical places of the stomach (antrum greater curvature, antrum lesser curvature and body greater curvature) in 41 (19.7%) of the infected patients in Tumaco. Through histological tests it was possible to determine that from 206 patients in the population of Túquerres, 178 (86.4%) were positive for *H. pylori*; of which 165 (80.1%) were positive for isolation of *H. pylori* and 31 (15.04%) with positive isolation in the three anatomical places of the gastric mucosa described previously. From the 41 positive patients for isolation of *H. pylori* of Tumaco in the three anatomical regions of the gastric mucosa, (70.8%) were women, with ages between 19 and 68 years old; (90.24%) had non atrophic gastritis and (9.75%) atrophic gastritis. From the 31 positive patients for isolation of *H. pylori* in the three anatomical regions of the gastric mucosa of the population of Túquerres, (54.8%) were women, with ages between 19 and 68 years old; (93.5%) had non atrophic gastritis and (6.5%) had atrophic gastritis.

cagA gene amplification

In the population of Tumaco it was found that the prevalence of the virulence marker *cagA* (+) of *H. pylori*, in the lesser curvature of the gastric antrum was (85.4%); (100%) in the greater gastric antrum curvature and (100%) in the greater curvature of body, prevalence significantly higher than the marker *cagA* (-) (P = 0.002) (Table 1). In the population of Túquerres the prevalence of the virulence gene *cagA* (+) of *H. pylori* on the lesser curvature of the gastric antrum was 96.4%; 100% in the greater curvature of the antrum and 100% in the greater curvature of body, prevalence significantly higher than the marker *cagA* (-) (P = 0.036) (Table 1).

vacA gene amplification

In the population of Tumaco the prevalence of the allele *vacA m1* of *H. pylori,* in the lesser curvature of the gastric antrum was 73.2%; 78.1% in the greater curvature of the antrum and 70.7% in the greater curvature of body, prevalence higher than the marker *vacA m2,* without being significant (P = 0.36) (Table 1). The allele *s1* from the gene *vacA* of *H. pylori* was more frequent than the allele *s2*. 80.5% in the lesser curvature of the gastric antrum, 78.5% in the greater curvature of the antrum and 80.5% in the greater curvature of body, without the differences being significant (P = 0.73) (Table 1).

In the population of Túquerres the prevalence of the allele m1 of *H. pylori,* in the lesser curvature of the gastric antrum was 71%; 61.3% in the greater curvature of the antrum and 58.1% in the greater curvature of body, prevalence higher than the marker *vacA m2,* without being significant (P = 0.257). The allele *s1* from the gene *vacA* was more frequent than the allele *s2,* 74.2% in the lesser curvature of the gastric antrum, 80.7% in the greater curvature of the antrum and 64.5% in the greater curvature of body, without being significant (P = 0.44) (Table 1).

Colonization of the gastric mucosa by multiple H. pylori isolates according to virulence genotypes

The study included DNA of *H. pylori* from 72 patients, 41 from Tumaco and 31 from Túquerres. Through bivariate analysis it was found that the infection with multiple strains wasn't significant with respect to the age (P = 0.063), being more frequent in patients from

Table 1 Prevalence of *cagA* and *vacA* alleles of *Helicobacter pylori* according to site of the stomach

Alleles	Tumaco n = 41			P value	Túquerres n = 31			P value
	Antrum		Body		Antrum		Body	
	Lesser curvature	Greater curvature	Greater curvature		Lesser curvature	Greater curvature	Greater curvature	
cagA								
cagA(+)	85.4%	100%	100%	0.002	96.4%	100%	100%	0.36
cagA (-)	14.6%	0%	0%		0%	0%	0%	
vacA								
m1	73.2%	78.1%	70.7%	0.360	71.0%	61.3%	58.1%	0.26
m2	26.8%	21.9%	24.3%		12.9%	22.6%	35.5%	
vacA								
s1	80.5%	78.5%	80.5%	0.730	74.2%	80.7%	64.5%	0.44
s2	19.5%	19.5%	19.5%		19.4%	19.4%	25.8%	

Table 2 Type of colonization of *Helicobacter pylori* according to gender, age and cancer risk

Features	Type of colonization		Total n = 72	P value
	Single n = 34	Multiple n = 38		
Gender				0.067
Male	26.5%	47.4%	37.5%	
Female	73.5%	52.6%	62.5%	
Age (yr)				0.633
18-35	41.2%	36.8%	38.9%	
36-47	26.5%	36.8%	31.9%	
48-63	32.4%	26.3%	29.2%	
Cancer risk				0.027
Low (Tumaco)	70.6%	44.7%	56.9%	
High (Túquerres)	29.4%	55.3%	43.1%	
Type of gastritis				0.203
Non atrophic	79.4%	89.5%	84.7%	
MAG	20.6%	7.9%	13.9%	

Table 3 Risk of multiple colonization of *Helicobacter pylori* in the stomach

Characteristics	Odds ratio	95%CI		$P > z$
Gender				
Female	1.000			
Male	2.477	0.858	7.154	0.094
Age (yr)				
18-35	1.000			
36-47	1.401	0.412	4.769	0.589
48-63	1.160	0.330	4.076	0.816
Cancer risk				
Low (Tumaco)	1.000			
High (Túquerres)	2.721	0.973	7.609	0.056
Type of gastritis				
Non atrophic	1.000			
MAG	0.325	0.066	1.594	0.166

18 to 35 years and 36 to 47 years (36.8%), than in patients between 48 and 68 years. It was observed that the infection with strains of multiple genotype was higher in women than in men (52.6% and 47.4%) respectively, without being this difference statistically significant (P = 0.067). Significant differences were observed of multiple colonization according the risk population of GC, being higher in the population of Túquerres (55.3%) than in the Tumaco (44.7%) (P = 0.027). It was found that the infection with multiple strains wasn't significant with respect to the type of gastric lesion (P = 0.203) (Table 2).

The multivariate analysis allowed to determine there aren't significant differences in the risk of presenting colonization with multiple strains according the population characteristics, OR 0.13. It was observed that the risk of colonization by multiple strains of *H. pylori* is (2477) higher in men than in women and (1.4) times higher in patients in the age range between 36 and 47 years, than in patients between the two intervals studied. Depending the place of origin the risk of multiple colonization also increases, being (2721) times higher in the population of Túquerres (high risk) than in Tumaco (P = 0.056), while the risk of multiple colonization decreases with respect to the type of gastritis, being lower in atrophic gastritis (Table 3).

Analysis of RAPD profiles

In the 123 isolates from Tumaco and 93 isolates from Túquerres the amplification by PCR-RAPD gave as result well defined DNA bands with each of the primers, 1281, 1254. Isolates with slight variation were found in the banding patterns, which varied between 100 pb and 1000 pb (Figure 1).

The analysis of the dendogram executed with the UPGMA classification method and the coefficient of similarity of Neid, showed that there is no relation between the groups formed and the characteristics of the population as gender, age and type of gastritis at a level of similarity of 0.07. In the population of Tumaco the dendogram was formed with ten groups (Figure 2). Group one was formed by 35 isolates, from these 33 were from patients with chronic non atrophic gastritis and two patients with chronic atrophic gastritis. Group two was structured with 47 isolates among which, four were from patients with chronic atrophic gastritis and 43 from patients with chronic non atrophic gastritis; group four was formed by 16 isolates, among which one was from a patient with chronic atrophic gastritis and the rest were from patients with non-atrophic gastritis, group five was formed by seven isolates of patients

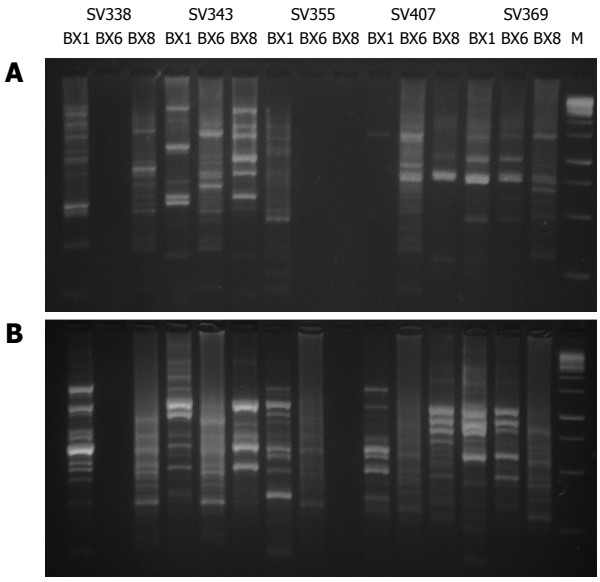

Figure 1 DNA profiles of *Helicobacter pylori* isolates, generated with RAPD-PCR. A: Profiles with primer 1254; B: Profiles with primer 1281. PCR products from DNA of the *Helicobacter pylori* (*H. pylori*) isolates obtained from three anatomical sites of the gastric mucosa by each patient (SV338, SV343, SV355, SV407 and SV369). Line M: 100 bp DNA ladder. Note that the size of bands for each of the three isolates are not coincident, indicating that they are different isolates of patients with multiple strain colonization by *H. pylori*.

with non-atrophic gastritis; group six was formed by three isolates of patients with atrophic gastritis, group seven was also formed with three isolates from which two were from patients with non-atrophic gastritis and one from a patient with atrophic gastritis, the groups 8, 9, 10 were formed by one isolate of a patient with non-atrophic gastritis (Figure 2).

Through multiple correspondence multivariate analysis (MCA), the three dimensional representation of the group of the isolates of the population of Tumaco, showed that there is no relation between the groups and the characteristics of the population, but a low variation is shown between the isolates of Tumaco by the formation of a relatively homogeneous group.

In the population of Túquerres the dendogram was formed by seven groups. In these there was no relation between the segregation of the isolates in the groups and the characteristics of the population at a similarity level of 0.13.

Group one was formed by 30 isolates, from which 27 were from patients with chronic non-atrophic gastritis and three with chronic atrophic gastritis. Group two joined 16 isolates, among which was found one with chronic atrophic gastritis and 15 with chronic non atrophic gastritis; group three was formed by 13 isolates of patients with chronic non atrophic gastritis and two of patients with chronic atrophic gastritis; group four, with three isolates of patients with chronic non atrophic gastritis; in group five with five isolates, from which one was from a patient with chronic atrophic gastritis; groups six and seven were formed by three and two isolates of patients with chronic non-atrophic gastritis respectively (Figure 2). Through the MCA, it is confirmed that there is no relation between the groups and the characteristics of the population, but a higher variation is showed between the isolates of Túquerres by the formation of a less homogeneous group (Figure 2).

DISCUSSION

The prevalence of the infection of *H. pylori* was high and similar in both populations: (88.7%) in Tumaco and (86.4%) in Túquerres[5]. However, there are strong differences in the incidence rates of gastric cancer among these populations, separated by only 200 km, being up to 25 times higher in the population of Túquerres in the Colombian Andes with predominant ancestry Amerindian and European, than in the population of Tumaco, with African ancestors and coming from the pacific coast in Colombia[18].

It was found that the multiple colonization evaluated with respect to genes of virulence of *H. pylori,* was 52.8% of all the analyzed cases (Table 2), and significantly higher in the population of Túquerres (55.3%), than in Tumaco (44.7%) ($P = 0.027$) (Table 2). Similar findings, of multiple colonization are described in different geographical regions of the world, including: Korea (60%), México (65%), Chile (32%), Portugal (30%) and Brazil (15%)[18,19].

The genetic similarity was higher between the isolates of *H. pylori* from the patients of Tumaco than those from Túquerres. Additional to this finding, the MCA showed in the population of Túquerres higher diversity with the formation of a less homogeneous group (Figure 2). People living on the Nariño Pacific Coast (Tumaco), have African ancestors and are infected with *H. pylori* strains, from African origin that, presumably they acquired when their ancestors brought to the new world during the slave trade and that they have coevolved with his host towards commensalism and in the time adapted, and in consequence are less virulent[18]. In contrast, in Túquerres (Colombian Andes), the phylogenetic origin of *H. pylori* that colonize the people of the region is predominantly European, and possibly by selective Amerindian strains tend to disappear gradually over time[5]. The disruption of host-bacteria coevolution may favor multiple colonization and determine less favorable biological relations for the host[5].

The *H. pylori* strains are genetically heterogeneous, with deep variations between patients and in the same patient[20]. An individual may be colonized with one strain or with genetically predominant strains with a same genomic DNA, or by different strains that show high genomic diversity, evident facts in the DNA sequences found in different anatomical places of patients[21]. This genomic diversity reflects a process of persistent accumulation of mutations in the strains as a result of horizontal transfer and DNA incorporation by genetic transformation or spontaneous mutations.

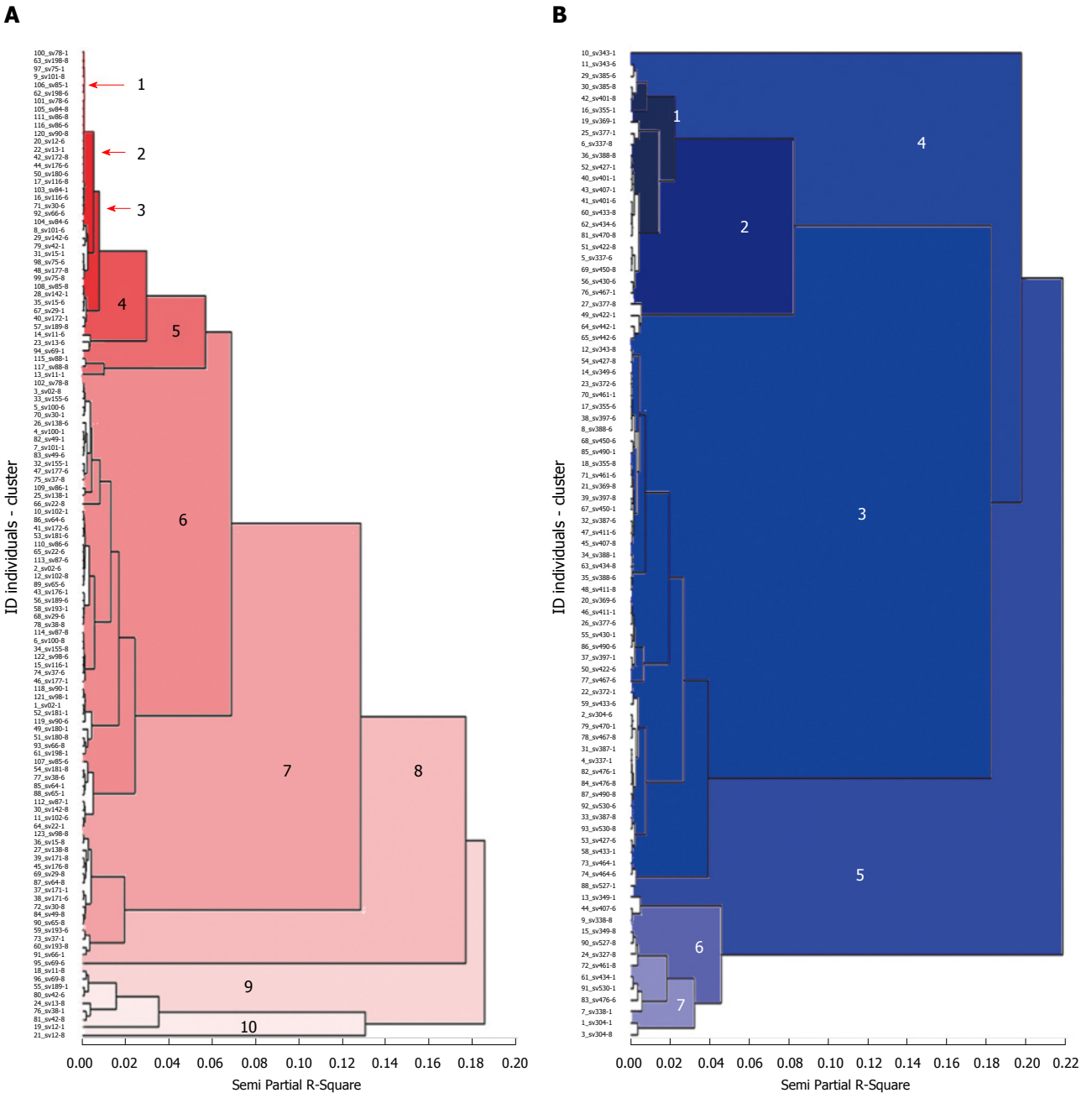

Figure 2 Dendrogram generated by the mixture of 1281 and 1254 DNA fingerprints in *Helicobacter pylori* isolates of patients from two Colombian regions with contrast risk of gastric cancer. A: Low risk population of gastric cancer (GC) was formed by 10 groups (red) with a similarity coefficient of 0.07 less than the similarity (0.13) between isolates of the high risk population of gastric cancer, dendrogram B; B: In the high-risk population of gastric cancer 7 groups (blue) were formed, with greater genetic distance between population groups at risk of gastric cancer and with greater genetic variability of isolates of *Helicobacter pylori* (*H. pylori*). Cluster analyses were designed by following UPGMA clustering method and estimate of the distances between each pair of *H. pylori* isolated was calculated with the similarity of Neid. The fusions produced near to the origin of the scale (left) indicate that the cluster formed is quite homogeneous. Conversely, fusions produced on the final zone of the scale (right) indicate the cluster formed is quite heterogeneous.

The persistent accumulation of mutations, principally in the virulence genes, provides a set of variants to *H. pylori* that can be selected to colonize particular gastric niches[11]. When all the individuals in a microbial population are identical, the concept of establishment in the host is relatively simple. However, the genetic variability of the microorganism that colonizes different regions in the gastric mucosa of a same host carries a more complex relationship[22].

The host responses to genomic variations of the bacteria leads immune local mechanisms for the anatomical place of the gastric mucosa where the microorganism colonizes. These variations in the local response towards the microorganisms represents an environmental pressure for the strains of *H. pylori,* living in the gastric mucosa where the microorganism colonizes. These variations in the local response towards the microorganisms represent an environmental

pressure for the strains of *H. pylori*, living in the gastric mucosa. Such selective pressures will affect the relative proportions of the bacterial strains in the population or may involve changes in the alleles of important genes and cause the displacement of the balance point, that favors the percentage of new genetic variants of the bacteria. The continuous rate of mutation assures that the dynamic equilibrium of the strains of *H. pylori* is maintained present in the population[23].

A more complex condition occurs when two or more strains of *H. pylori* colonizes one same gastric mucosa, since there is a space to promote genetic recombination which gives rise to new more resistant genotypes to the environmental conditions and they compete with their precursor strains. Each bacterial cell may create independent signals in the host and trigger immune specific answers. However, a strong localized response of the host may affect all the bacterial cells from the environment. The selective pressure results as negative influence in the development of most of the autochthonous bacterial cells, but the new recombinants survive because they may selectively adapt to hostile environments, they can form population structures more organized and optimize the use of resources from the host[24].

The results from the genotype comparison by RAPD-PCR between isolates collected from different patients and the same patient in different anatomical places of the gastric mucosa, is a secure method for the study of the genomic variability of *H. pylori*.

In this investigation the stage of genomic diversity between the clinical isolates obtained with the primers 1281 and 1254, based in a clear distinction of the patterns with multiple differences in the bands, reveal that 4 of 41 patients shelter one unique strain of *H. pylori,* while 37 showed heterogeneous traces of DNA that suggests infection by multiple strains in different anatomical places of the gastric mucosa in the same patient in the population of Tumaco (Figure 2). In the population of Túquerres 30 patients shelter different strains between the regions of the gastric mucosa of the same patient (Figure 2). Cellini *et al*[25], reported similar results in a population with different strains of *H. pylori* that showed genotypic variation, in the same patient. The genetic diversity of the *H. pylori* may also be due to multiple genotypes worldwide. A great number of variants *H. pylori* coexist as product of natural transformation and re genetic arrangements or alterations during the adaptation process and colonization in the host[26]. Nevertheless, the evidence of the high variety of strains of *H. pylori* may be the result of a continuous evolution, which occurs when the stomach of a person gets infected, since nucleotide mutation may occur, excisions in the isle *cag* PAI, transposition and insertion of elements *cag* PAI, recombination with DNA of surrounding strains that aren't involved in the disease and horizontal transmission of new genes[26]. It is proposed that the high diversity is due to the limited direct competition between most of the strains, even if they are residents in different people in the same community. The diversity emphasizes by the geographical breakup of many towns of the world and in consequence the phyllogeographic origin of *H. pylori* plays a preponderant role in the high diversity and in the pathogenicity of the bacteria[27,28]. The divergence between strains of *H. pylori* increases even more, by differences between the characteristics of the people and the individual features of the strains[28]. Some features include types of Lewis antigens for the adhesion of *H. pylori,* the specificity and intensity of the inflammatory response and the complex regulation of gastric acid secretion.

In conclusion, the population of Túquerres located in the Colombian Andes (high risk of gastric cancer), are more likely to have a multiple colonization of *H. pylori,* with greater genetic diversity than the infectious strains from the patients of Tumaco (low risk of GC), which should be taken into account when formulating eradication programs of the microorganisms as a strategy for the primary prevention of gastric cancer in these populations of Colombia.

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COMMENTS

Background

Infection by virulent strains of *Helicobacter pylori* (*H. pylori*) is one of the most important risk factors for the development of precursor lesions of gastric cancer. Ideal treatment is not yet possible because the gastric mucosa of a host can be colonized by multiple strains of *H. pylori* (virulent and avirulent). The strains of *H. pylori* can coexist with susceptible bacterial isolates and resist antibiotics. This phenomenon makes eradication of the *H. pylori* difficult as a strategy in treatment regimens and prevention of gastric cancer precursor lesions.

Research frontiers

The eradication of *H. pylori* from the gastric mucosa is the cornerstone in the treatment of diseases such as chronic gastritis, peptic ulcer, metaplasia and dysplasia; it is necessary to have genotyping isolates of *H. pylori* obtained from different gastric locations (antrum and body) to evaluate multiple strain colonization of *H. pylori* by analysis of genetic diversity in patients with gastritis.

Innovations and breakthroughs

In this research, the degree of variability of genomic DNA from clinical isolates, obtained with primers 1281 and 1254, based on a clear distinction of patterns with multiple differences in bands, shows that 4 out of 41 patients harbor a single strain of *H. pylori*, while 37 showed heterogeneous DNA traces that indicate infection by multiple strains in different anatomical sites in the same patient in the population of Tumaco. In the population of Túquerres, 30 patients harbor different strains within their gastric mucosa. While the difference in risk is associated with the virulence of the infecting strain, it is considered that the multiple colonization of gastric mucosa and its interrelation with environmental agents is a condition that could boost the development of precancerous lesions and eventually lead to the development of gastric carcinoma. The eradication of *H. pylori* is a valid strategy for the prevention of gastric carcinoma. However, treatment failure is inherent to multiple colonization by multiple strains of *H. pylori* (virulent and avirulent) and the coexistence of susceptible bacteria with

bacteria that are resistant to antibiotics.

Applications

These *in vitro* findings are especially important because of their possible implications for the treatment and evolution of gastro-duodenal diseases caused by *H. pylori*, suggesting that in most of the cases, polycolonization by virulent strains of *H. pylori* is one of the most important risk factors for the development of precursor lesions of GC and gastric carcinoma. Our results suggest differences in genetic characteristics of the circulating strains, with different degrees of greater genetic diversity between anatomical regions in the same patient, showing multiple colonization. Eradication of *H. pylori* from the gastric mucosa is the only valid strategy for GC prevention. However, therapeutic failure of polymicrobial treatment is inherent and can be attributed to the colonization of the gastric mucosa by multiple strains of *H. pylori* (virulent and avirulent).

Terminology

The phenomenon known as multiple colonization refers to the colonization of the gastric mucosa by multiple strains of *H. pylori* with different characteristics at the genetic level with varying degrees of pathogenicity (virulent and avirulent) between anatomical regions in a single patient. In Colombia, the risk of gastric cancer varies according to geographical areas with high and low risk regions for developing GC. This contrast of risk between nearby towns with a prevalence of similar infection by *H. pylori* is explained by the high genetic diversity of bacteria, with varying levels of pathogenicity.

Peer-review

Personalized medicine has important role in treatment. Authors should list comparison of the genomic variability of *H. pylori* isolates of gastric regions from Columbian population.

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ORIGINAL ARTICLE

Basic Study

Gut microbial diversity analysis using Illumina sequencing for functional dyspepsia with liver depression-spleen deficiency syndrome and the interventional Xiaoyaosan in a rat model

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Author contributions: Qiu JJ and Liu Z contributed equally to this work; Guo HS and Cai ZX are the joint corresponding authors of this article; Guo HS and Cai ZX designed the research; Qiu JJ, Liu Z, Zhao P, Wang XJ, Li YC and Sui H performed the research; Qiu JJ, Li YC and Sui H analyzed the data; Owusu L, Guo HS and Cai ZX revised the manuscript; Qiu JJ wrote the paper.

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Abstract

AIM

To investigate gut microbial diversity and the interventional effect of Xiaoyaosan (XYS) in a rat model of functional dyspepsia (FD) with liver depression-spleen deficiency syndrome.

METHODS

The FD with liver depression-spleen deficiency syndrome rat model was established through classic chronic mild unpredictable stimulation every day. XYS group rats received XYS 1 h before the stimulation. The models were assessed by parameters including state of

the rat, weight, sucrose test result and open-field test result. After 3 wk, the stools of rats were collected and genomic DNA was extracted. PCR products of the V4 region of 16S rDNA were sequenced using a barcoded Illumina paired-end sequencing technique. The primary composition of the microbiome in the stool samples was determined and analyzed by cluster analysis.

RESULTS

Rat models were successfully established, per data from rat state, weight and open-field test. The microbiomes contained 20 phyla from all samples. *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Cyanobacteria* and *Tenericutes* were the most abundant taxonomic groups. The relative abundance of *Firmicutes*, *Proteobacteria* and *Cyanobacteria* in the model group was higher than that in the normal group. On the contrary, the relative abundance of *Bacteroidetes* in the model group was lower than that in the normal group. Upon XYS treatment, the relative abundance of all dysregulated phyla was restored to levels similar to those observed in the normal group. Abundance clustering heat map of phyla corroborated the taxonomic distribution.

CONCLUSION

The microbiome relative abundance of FD rats with liver depression-spleen deficiency syndrome was significantly different from the normal cohort. XYS intervention may effectively adjust the gut dysbacteriosis in FD.

Key words: Functional dyspepsia with liver depression-spleen deficiency syndrome; Illumina sequencing; Gut microbial diversity; Xiaoyaosan

Core tip: Gut microflora has been found to associate with promotion of health and occurrence and development of different gastrointestinal/non-gastrointestinal disease. Some traditional Chinese drugs exert effects on diseases by regulating the equilibrium of intestinal microflora. In the present study, we described gut microbial diversity in functional dyspepsia with liver depression-spleen deficiency syndrome in rat models and the effect of Xiaoyaosan intervention.

Qiu JJ, Liu Z, Zhao P, Wang XJ, Li YC, Sui H, Owusu L, Guo HS, Cai ZX. Gut microbial diversity analysis using Illumina sequencing for functional dyspepsia with liver depression-spleen deficiency syndrome and the interventional Xiaoyaosan in a rat model. *World J Gastroenterol* 2017; 23(5): 810-816 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i5/810.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i5.810

INTRODUCTION

Functional dyspepsia (FD) is a common gastrointestinal disorder[1]. FD is not life-threatening but adversely impacts the patient's quality of life. The pathogenesis of FD is uncertain because of complicated multiple factors. Clinical observation has shown that patients with FD suffer from obvious gastrointestinal motility obstacles, visceral hypersensitivity, *Helicobacter pylori* infection, neuropsychological factors and so on[2]. The occurrence and development of FD is closely related with mood disorders, such as anxiety and depression. Traditional Chinese Medicine (TCM) holds that gastrointestinal function abatement is usually due to spleen deficiency (pixu) and mood disorders with associated liver depression (ganyu). Thus, liver depression-spleen deficiency is regarded as one of the main pathogenesis of FD. In all syndrome types, FD with liver depression-spleen deficiency syndrome is the most common FD in traditional Chinese medical syndrome typing[3].

Xiaoyaosan (XYS) is a well-known Chinese herbal formula and is prescribed to sooth liver, tonify spleen, and nourish blood[4,5]. The Song Dynasty (960-1127 AD) book of "Taiping Huimin Heji Jufang" recorded that XYS comprises the following eight Chinese herbs: Bupleurum root, Chinese angelica root, white peony root, Perenniporia, bighead Atractylodes rhizome, roasted ginger, prepared licorice root, menthol and peppermint. It has long been used for the treatment of FD associated with the syndrome of "liver depression" and "spleen deficiency" in China.

Gastrointestinal microflora plays an important role in the host's life activities[6,7]. When the host's balance of microflora in the gastrointestinal tract is disrupted, the host may become ill. In recent years, the gastrointestinal microflora has been shown to be associated with promotion of health and occurrence and development of different gastrointestinal/non-gastrointestinal diseases[8,9]. Therefore, more experimental research has focused on the relationship between disease and gastrointestinal microflora. Besides, research has shown that some traditional Chinese drugs exert their effects on disease by regulating the equilibrium of intestinal microflora[10,11]. Many molecular biology techniques have been used to illustrate gut microbial diversity, including denaturing/temperature gradient gel electrophoresis, terminal restriction fragment length polymorphism, and high-throughput sequencing[12]. High-throughput sequencing has the advantage of providing more and detailed information on microbial diversity with high accuracy, and thus has been widely used in the study of gut microbial diversity. Here, we used the Illumina sequencing to analyze gut microbial diversity of FD. The goal of the current study was to describe gut microbial diversity on FD with liver depression-spleen deficiency syndrome in a rat model and to assess the effect of XYS on microflora.

MATERIALS AND METHODS

Establishment and validation of rat models

Male Sprague-Dawley rats of clean grade were su-

pplied by the Experimental Animal Center of Dalian Medical University, Dalian, China. Rats were kept at a temperature of 23 ± 2 ℃ and a relative humidity of 55% ± 2% on a 12 h light-dark cycle for 7 d before experimentations. Rats were randomly divided into three groups: normal group (FD1), FD with liver depression-spleen deficiency syndrome group (FD2) and XYS-treated group (FD3). The FD with liver depression-spleen deficiency syndrome rat model was established by including classic chronic mild unpredictable stimulation (bondage, swim-induced fatigue, electrical stimulation, fasting and concussion) every day[13]. The normal and FD model groups were given normal saline intragastrically before initiating stimulation. The XYS group was given XYS intragastrically 1 h before initiating stimulation. The dose of XYS was 15.3 g/kg per day (gavage volume 2 mL).

The models were assessed by state of the rat, weight, sucrose test result and open-field test result. All the procedures of animal experiments were approved by the local Animal Care Committee and in accordance with the Guide for the Care and Use of Laboratory Animals published by the Science and Technology Commission of P.R.C. (STCC Publication No. 2, revised 1988).

Sample collection and DNA extraction

Stool samples were collected from rats of FD1, FD2 and FD3 groups. The stools were suspended, respectively, in TE buffer with lysozyme. Total bacterial DNAs were extracted using the Stool DNA Isolation Kit (FORE GENE, China) according to the manufacturer's instruction. The total DNAs were determined using electrophoresis on 1% agarose gel containing ethidium bromide. The concentration of DNAs was measured using NanoVue plus (GE, United States).

V4 region of the 16S rDNA PCR amplification

The variable V4 region of 16S rRNA was amplified by PCR using the following barcode primers: upstream primer 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and downstream primer 806R (5'-GGACTACHVGGGTWTCT AAT-3'). The PCR reaction mixture contained 12.5 μL of Phusion Master Mix (Phusion® High-Fidelity PCR Master Mix with GC Buffer; New England Biolabs, United States), 1 μL of template DNA, 1.25 μL of 10 μmol primer 515F, 1.25 μL of 10 μmol primer 806R and 10 μL of double-distilled H_2O. The amplification of PCR was performed as follows: 98 ℃ for 30 s; 30 cycles of 98 ℃ for 10 s, 54 ℃ for 30 s, and 72 ℃ for 30 s; and 72 ℃ for 7 min. The amplified products were identified by electrophoresis on a 2% agarose gel containing ethidium bromide. The PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen, Germany).

Sequencing PCR amplicons of 16S rDNA

Library construction was performed using the TruSeq® PCR-Free DNA Sample Preparation Kit and quantified by Qubit and Q-PCR. After library accreditation, purified PCR products of 16S rDNA were sequenced using HiSeq2500 sequencer PE250. Sequencing work was performed in collaboration with Novogene Bioinformatics Technology Co., Ltd (Beijing, China).

Animal care and use statement

The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions (23 ± 2 ℃, 12 h/12 h light/dark, 55% ± 2% humidity, *ad libitum* access to food and water) for 1 wk prior to experimentation. All animals were euthanized by ethyl ether for tissue collection.

Statistical analysis

After amputation of the barcode and primer sequences, Raw Tags were obtained by joining reads of each sample using FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/)[14]. Clean Tags were obtained through the strict filtering process[15]. Effective Tags of all samples were clustered using Uparse software (Uparse v7.0.1001, http://drive5.com/uparse/)[16]. Operational Taxonomic Units (OTUs) were defined by clustering 97% identity sequences. Based on the principles of the algorithm, the highest frequency OTUs were screened as the representative OTUs. The microbiomes species at phylum level were annotated by analyzing the representative OTUs with RDP Classifier (version 2.2, http://sourceforge.net/projects/rdp-classifier/) method[17] and GreenGene database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi)[18]. The phylogenetic relationships of representative OTUs were identified by multiple sequence alignment using PyNAST software (version 1.2)[19] and "Core Set" of GreenGene database.

RESULTS

Model validation

The FD with liver depression-spleen deficiency syndrome rat model was established successfully using chronic mild unpredictable stimulation. Compared with the good mental state of the normal group rats, the model group rats were restless and exhibited high alertness. The fur of the model group rats was slightly rough. It was difficult for the model group rats to grasp. The weight of the model group rats significantly decreased, compared to that of the normal group rats (Table 1). The saccharine preference index of the model group rats was significantly lower than that of the normal group rats (Figure 1). At the end of the modeling process, the numbers of crossed-grids, standing and grooming times were calculated. There were significant differences between the model group and the normal group in all these parameters (Table 2).

Identification of PCR products of the V4 region of 16S rDNA

Amplified PCR products were examined by agarose gel

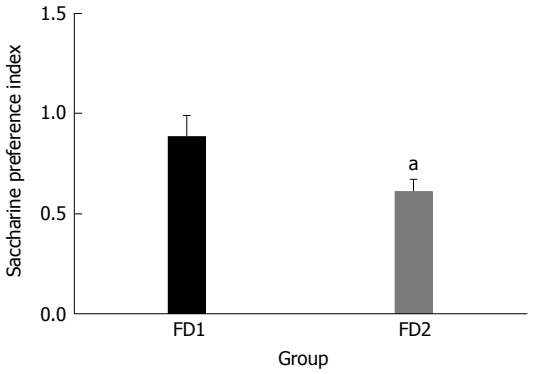

Figure 1 Saccharine preference index of the normal group (FD1) and the model group (FD2). The data are presented as mean ± SE. $^{a}P < 0.01$ *vs* the normal group.

Table 1 Rat weight (g, mean ± SE)

	Model before	Model after
FD1	220.33 ± 1.20	264.17 ± 1.17
FD2	220.50 ± 1.06	236.00 ± 1.17[a]

Normal group: FD1; Model group: FD2. $^{a}P < 0.01$ *vs* the normal group.

Table 2 Open-field test (mean ± SE)

	Model before	Model after
Stand-up times		
FD1	21.83 ± 0.95	18 ± 1.00
FD2	22.00 ± 0.89	3.00 ± 0.78[a]
Number of crossings		
FD1	55.33 ± 1.31	50.67 ± 0.80
FD2	54.83 ± 2.23	8.33 ± 0.92[a]
Number of cleanings		
FD1	9.83 ± 0.60	8.83 ± 0.31
FD2	9.83 ± 0.48	2.17 ± 0.40[a]

Normal group: FD1; Model group: FD2. $^{a}P < 0.01$ *vs* the normal group.

electrophoresis (Figure 2). The V4 region PCR products of 16S rDNA were obtained.

Sequencing and quality control

The number of Effective Tags and OTUs observed after sequencing are shown in Figure 3. The Effective Tags of all samples were enough to cluster to OTUs. The representative OTUs were used for annotating.

Phylum level taxonomic distribution of microbiomes

The microbiomes contained 20 phyla and 156 genera in all rat stool samples (Figure 4). The composition microbiome of all samples was similar at the phyla level. *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Cyanobacteria* and *Tenericutes* were the most abundant taxonomic groups. The relative abundance of *Firmicutes, Proteobacteria* and *Cyanobacteria* in the FD model group was individually higher than in the normal group. On the contrary, the relative abundance of *Bacteroidetes* in the FD model group was lower than observed in the normal group. After XYS treatment, the relative abundance of all altered phyla readjusted to levels comparable to those observed in the normal rats.

Species abundance clustering heat map at the phylum level

The abundance clustering heat map of phyla was consistent with results of phylum level taxonomic distribution (Figure 5).

DISCUSSION

In this study, we modeled FD with liver depression-spleen deficiency syndrome in rats and observed changes in gut microbial diversity with or without XYS intervention.

FD is a common clinical digestive disease with epigastric discomfort, postprandial full bilge, abdominal bloating, belching, anorexia, nausea, vomiting, heartburn and other symptoms that occur persistently or recurrently[20]. FD with liver depression-spleen deficiency syndrome is one of the most common FD subtypes in TCM. Modern medicinal knowledge has indicated psychiatric factors as the most important etiologic factors. We used unpredictable stimulations to mimic psychiatric stress to induce FD in rats[21]. These stimulations led to successful FD with liver depression-spleen deficiency syndrome through making rats nervous and depressive for a long time.

Some studies have indicated the influence that gut microbial diversity may have on different diseases, including gastrointestinal disease, incretion disease and mental disease[22]. Therefore, understanding the interaction between the microbiota and the host's physiology may facilitate the exploration of new therapeutic targets for certain diseases. FD with liver depression-spleen deficiency syndrome belongs to a kind of gastrointestinal disease caused by psychiatric factors. While XYS was the TCM prescription used for the treatment of FD with liver depression-spleen deficiency syndrome[23], it has become widely accepted in microbial diversity analysis by employing the sequencing technique in this postmetagenomic era.

In our study, the microbiomes of the normal group, the FD model group and the XYS-treated FD group rats were classified at the phylum level. *Firmicutes*, *Bacteroidetes* and *Proteobacteria* were the three most abundant taxonomic groups in all group samples. These groups of microorganisms have, in fact, been indicated as the three predominant phyla in the gut of animals and humans[24]. The experimental groups had similar microbiome distributions but with varied phyla abundances within each microbiome. *Firmicutes* was the most favored microflora and the highest in proportion in the gut of mammals. Many bacteria of

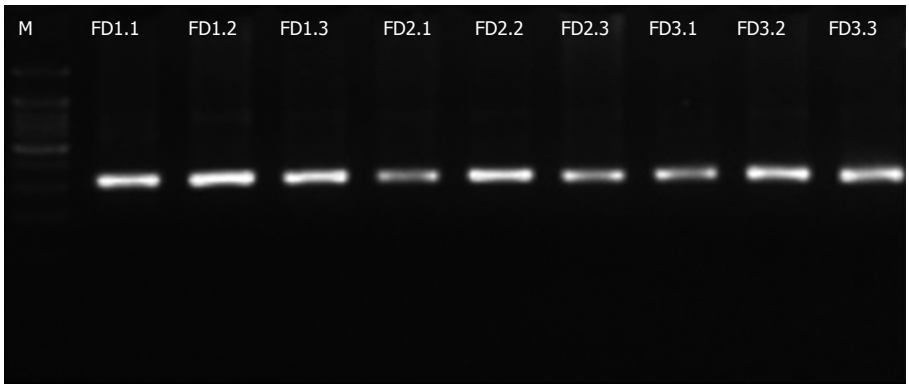

Figure 2 Agarose gel electrophoresis of PCR products of the V4 region of 16S rDNA. Normal group: FD (1.1-1.3); Model group: FD (2.1-2.3); Xiaoyaosan group: FD (3.1-3.3). FD: Functional dyspepsia.

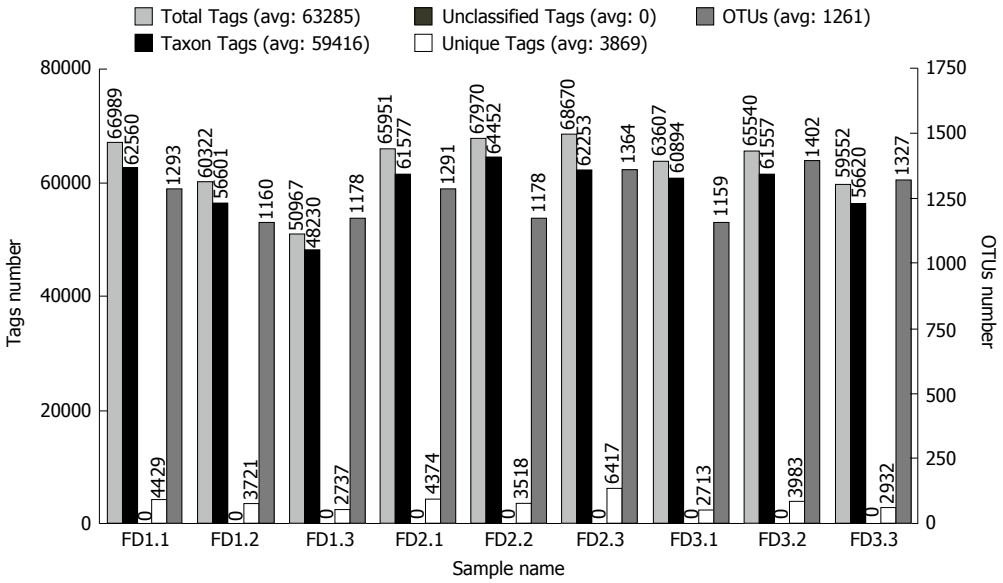

Figure 3 Statistical graph of Tags and Operational Taxonomic Unit clustering. Normal group: FD (1.1-1.3); Model group: FD (2.1-2.3); Xiaoyaosan group: FD (3.1-3.3). Total Tags: Effective Tags; Unique Tags: Uneffective Tags; Taxon Tags: Annotated Tags for OTUs; Unclassified Tags: Unannotated Tags. OTUs: Operational Taxonomic Units; FD: Functional dyspepsia.

the phyla *Firmicutes* can produce butyrate. Butyrate is an important source of energy for colonic epithelial cells[25]. *Bacteroidetes* and *Proteobacteria* were the second and third favored microflora, respectively. Many *Bacteroidetes* are involved in the metabolic processing of polysaccharides, steroids and bile acids, whereas *Proteobacteria* are pathogenic[26,27]. The increased relative abundance of *Firmicutes* and *Proteobacteria* coupled with the decreased relative abundance of *Bacteroidetes* in the FD microbiome as compared with the microbiome of normal rats suggests an association between increased *Proteobacteria* with decreased *Bacteroidetes* and the occurrence of FD with liver depression-spleen deficiency syndrome. Although the relative abundance of *Fusobacteria* was generally low, it indicated a plausible positive association with *Proteobacteria*. *Fusobacteria* is a common resident pathogen in gut mucosa and has been linked to gastrointestinal disease[28]. Upon treatment of FD rats with XYS, the relative abundances of *Firmicutes, Proteobacteria* and *Bacteroidetes* were adjusted to similar levels, as identified in the microbiome of normal rats, with enhanced decrease of *Fusobacteria.* We infer that the therapeutic function of XYS on FD may, at least partly, be due to its ability to restore gut microbial homeostasis. Notwithstanding, further studies are needed to define the molecular factors and downstream mechanisms that are chiefly influenced during the dysbiosis-microbiome restoration activities in FD.

In summary, this study investigated the gut microbial diversity of FD in a rat model and indicated that the microbiome composition of FD with liver depression-spleen deficiency syndrome was significantly different from the normal cohort. Intervention with XYS restored the gut dysbiosis in FD to normalcy. The data thus shed light on a plausible means by which XYS may achieve its therapeutic function in FD.

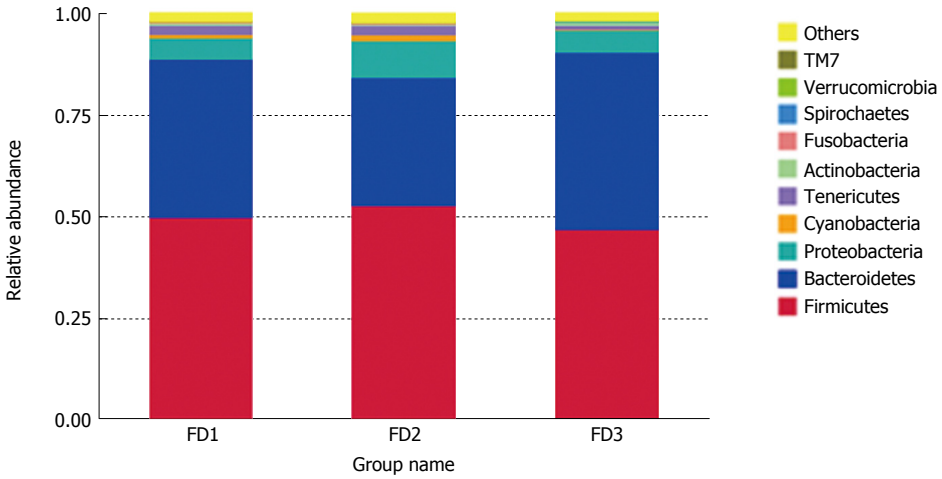

Figure 4 Relative abundance of the microbiomes at the phylum level. Normal group: FD1; Model group: FD2; Xiaoyaosan group: FD3. FD: Functional dyspepsia.

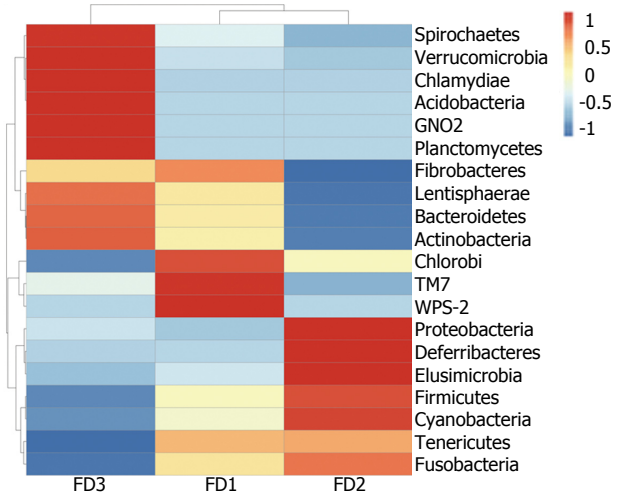

Figure 5 Heat map of species abundance clustering at the phylum level. Normal group: FD1; Model group: FD2; Xiaoyaosan group: FD3. FD: Functional dyspepsia.

COMMENTS

Background

Functional dyspepsia (FD) with liver depression-spleen deficiency syndrome is not life-threatening but adversely impacts the patient's quality of life. Xiaoyaosan (XYS) is a common Chinese herbal formula and has long been used for the treatment of FD associated with the syndrome of "liver depression" and "spleen deficiency" in China. Gastrointestinal microflora plays an important role in the host's life activities. Descriptions of the gut microbial diversity of FD rat models and those intervened with XYS will facilitate the understanding of FD pathogenesis and clarify the mechanism of XYS.

Research frontiers

Gastrointestinal microflora has been found to associate with promotion of health and occurrence and development of different gastrointestinal/non-gastrointestinal diseases in recent years. Besides, research has shown that some traditional Chinese drugs exert their effects on disease by regulating the equilibrium of intestinal microflora.

Innovations and breakthroughs

Gut microbial diversity is related to different diseases, such as gastrointestinal disease, incretion disease and mental disease. Some traditional Chinese drugs exert their effects on disease by regulating the equilibrium of intestinal microflora. There is no report to date on the relationship between gut microbial diversity, FD with liver depression-spleen deficiency syndrome and XYS.

Applications

These data will facilitate the exploration of new therapeutic targets of disease by increasing the understanding of the interaction between the microbiota and the host's physiology. Clarification of the XYS intervention will provide a theoretical basis for promotion and application of Chinese traditional medicine.

Terminology

Illumina sequencing, also known as next-generation sequencing, describes sequencing of millions of gene fragments by synthesis with reversible terminators.

Peer-review

The manuscript is interesting and worthy of publication.

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ORIGINAL ARTICLE

Basic Study

Dendritic cells engineered to secrete anti-DcR3 antibody augment cytotoxic T lymphocyte response against pancreatic cancer *in vitro*

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Author contributions: Chen J performed the majority of experiments and analyzed the data; Li HY and Zhao JJ performed the molecular investigations; Guo XZ designed and coordinated the research; Chen J and Xu WD wrote the paper.

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Abstract

AIM

To investigate the enhanced cytotoxic T lymphocyte responses against pancreatic cancer (PC) *in vitro* induced by dendritic cells (DCs) engineered to secrete anti-DcR3 monoclonal antibody (mAb).

METHODS

DCs, T lymphocytes and primary PC cells were obtained from PC patients. DCs were transfected with a designed humanized anti-DcR3 monoclonal antibody heavy and light chain mRNA and/or total tumor RNA (DC-tumor-anti-DcR3 RNA or DC-total tumor RNA) by using electroporation technology. The identification, concentration and function of anti-DcR3 mAb secreted by DC-tumor-anti-DcR3 RNA were determined by western blotting and enzyme-linked immunosorbent assay. After co-culturing of autologous isolated PC cells with target DCs, the effects of secreting anti-DcR3 mAb on RNA-DCs' viability and apoptosis were assessed by MTT assay and flow cytometry. Analysis of enhanced antigen-specific immune response against PC induced by anti-DcR3 mAb secreting DCs was performed using a ^{51}Cr releasing test. T cell responses induced by RNA-loaded DCs were analyzed by measuring cytokine levels, including IFN-γ, IL-10, IL4, TNF-α and IL-12.

RESULTS

The anti-DcR3 mAb secreted by DCs reacted with

recombinant human DcR3 protein and generated a band with 35 kDa molecular weight. The secreting mAb was transient, peaking at 24 h and becoming undetectable after 72 h. After co-incubation with DC-tumor-anti-DcR3 RNA for designated times, the DcR3 level in the supernatant of autologous PC cells was significantly down-regulated ($P < 0.05$). DCs secreting anti-DcR3 mAb could improve cell viability and slow down the apoptosis of RNA-loaded DCs, compared with DC-total tumor RNA ($P < 0.01$). The anti-DcR3 mAb secreted by DC-tumor-anti-DcR3 RNA could enhance the induction of cytotoxic T lymphocytes (CTLs) activity toward RNA-transfected DCs, primary tumor cells, and PC cell lines, compared with CTLs stimulated by DC-total tumor RNA or control group ($P < 0.05$). Meanwhile, the antigen-specific CTL responses were MHC class I-restricted. The CD4+ T cells and CD8+ T cells incubated with anti-DcR3 mAb secreting DCs could produce extremely higher level IFN-γ and lower level IL4 than those incubated with DC-total tumor RNA or controls ($P < 0.01$).

CONCLUSION

DCs engineered to secrete anti-DcR3 antibody can augment CTL responses against PC *in vitro*, and the immune-enhancing effects may be partly due to their capability of down-regulating DC apoptosis and adjusting the Th1/Th2 cytokine network.

Key words: Dendritic cell; Antibody-encoding RNA; DcR3; Cytotoxic T lymphocyte response; Pancreatic Cancer

Core tip: Dendritic cells co-transfection with tumor-associated antigens RNA and humanized anti-DcR3 monoclonal antibody mRNA may augment cytotoxic T lymphocyte responses against pancreatic cancer *in vitro*. This finding lays a good foundation for further investigation of tumor dendritic cells' vaccine targeting DcR3 protein against pancreatic cancer.

Chen J, Guo XZ, Li HY, Zhao JJ, Xu WD. Dendritic cells engineered to secrete anti-DcR3 antibody augment cytotoxic T lymphocyte response against pancreatic cancer *in vitro*. *World J Gastroenterol* 2017; 23(5): 817-829 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i5/817.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i5.817

INTRODUCTION

Pancreatic cancer (PC) is the fourth leading cause of cancer-related deaths in the US, with 40560 deaths in 2015 alone[1,2]. It has an extremely poor prognosis with a total 5-year survival rate of < 5%[3]. The poor prognosis and high mortality rate in PC patients may be attributed in part to lack of effective treatments[2]. Existing therapies for PC are limited to systemic chemotherapy and surgical resection. However, neither of these two strategies can cure PC completely[4]. Thus, more effective therapeutic methods are urgently needed.

Cellular immunotherapy is a promising alternative that is currently considered the fourth line of cancer treatment[5]. In this approach, different kinds of immune cells, such as cytokine-induced killer cells, lymphokine-activated killer cells, natural killer (NK) cells and dendritic cells (DCs) are adopted for immunotherapy. Of these, DC is the most commonly used immune effector cell because of its potent antigen-presenting function in the initiation of antitumor immune responses and its pivotal function in cancer immunosurveillance. Cytotoxic T lymphocytes (CTLs) are capable of eliminating cancer cells directly *in vivo*, but their activities are primarily managed by DCs. The use of DC-based tumor vaccines has therefore become a promising alternative treatment method for cancer[2,6].

In clinical practice, antigen choice is essential in the design of an effective vaccine. Because of lacking the expression of MHC class II molecules and co-stimulatory molecules, PCs, with low level of expression of tumor-associated antigens (TAA), display weak antigenicity and high heterogeneity[7]. Therefore, loading whole antigens from PC cells may be an alternative method that can both generate a broad T cell immune response to TAA and reduce the possibility of PC escape from immune recognition. We have previously reported that DCs transfected with total tumor RNA can effectively induce anti-PC tumor-specific CTL responses[2]. However, although this method has been demonstrated to generate a vaccine-induced rise in tumor-specific cells, the immune and tumor reactions stay modest, suggesting the need for novel strategies to improve antitumor immunity[8]. Evidently, one cause of this insufficiency is that tumor cells can produce certain immunosuppressive molecules to induce an immunosuppressive microenvironment and inhibit the function of tumor-associated cells, such as T lymphocytes and DCs[9]. Decoy receptor (DcR) 3 is possibly one of these cells in the tumor microenvironment (TME)[10].

DcR3 is a decoy receptor for Fas ligand (FasL) and is a member of the tumor necrosis factor receptor (TNFR) superfamily[11]. DcR3 displays inducible expression, interacts with the herpes virus entry mediator (HVEM) and is presented by TNF-like molecule 1A (TL1A) and T lymphocytes (LIGHT)[12,13]. DcR3 lacks a transmembrane domain and works as a secreted protein instead of a membrane-bound one. DcR3 can bind to LIGHT and FasL, thereby blocking the interaction between LIGHT and LT-receptor (LTβR) or HVEM to inhibit the apoptosis induced by Fas-FasL interaction or LIGHT-mediated biological effects. DcR3 is frequently overexpressed in various

tumors, including lung cancers[14], gastrointestinal tract tumors[15], virus-associated lymphomas[16], and PCs[17]. It has been reported that overexpression of DcR3 is correlated with shortened total survival time of cancer patients[18]. DcR3 has been postulated to promote tumor growth by escaping FasL- and LIGHT-mediated immunosurveillance. Specifically, DcR3 is able to suppress the activation and differentiation of DCs and macrophages[19], enhance osteoclast differentiation and angiogenesis[20], and sensitize T lymphocytes to TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in cancer patients[21]. In addition, DcR3 is also regarded as an important immunosuppressive factor in defects associated with immune effector cell function. Therefore, we sought to determine whether neutralizing DcR3 expression in the TME can augment the CTL responses against PC *in vitro* induced by DCs loaded with total tumor RNA.

In the current study, we evaluated the novel approach of co-transfecting DCs with total tumor RNA and mRNA encoding humanized heavy (H) and light (L) chains of an anti-human DcR3 mAb together to achieve anti-DcR3 protein stimulation. Through co-culturing of autologous isolated PC cells with DCs, we found that DCs transfected with these RNAs secrete operational immune modulating proteins that can reduce DcR3 expression in TME of cultured PC cells. Then we demonstrated that CTLs induced by DCs co-transfected with total tumor RNA and anti-DcR3 monoclonal antibody (mAb) mRNA show more effective cytotoxic activities against PC cells *in vitro* compared with DCs loaded only with total tumor RNA alone. Furthermore, the immune-enhancing effect of DCs engineered to secrete anti-DcR3 mAb is partly due to their capability of down-regulating apoptosis of DCs and adjusting the T helper (Th)1/Th2 cytokine network. These findings are crucial for the development of tumor DC vaccines targeting DcR3 protein against PC.

MATERIALS AND METHODS

Patient eligibility and tumor cells preparation

Fifteen HLA-A2+ PC patients (9 males and 6 females; median age of 53.5 years, ranging from 35 years to 72 years) were included in this study. According to the TNM classification of AJCC[22], there were 10 stage II patients and 5 stage Ⅲ patients. The location of tumor was divided into head (7 cases) and body/tail (8 cases). All patients underwent surgical resection and were pathologically diagnosed with invasive ducal adenocarcinoma.

Peripheral blood monocyte cells (PBMCs), isolated by Ficoll-Hypaque (Sigma, St Louis, MO, United States) density gradient separation, and was used as the nonmalignant control tissues. Pancreatic cancer specimens were obtained at the time of surgery and were stored in RNAlate (Ambion, Austin, TX, United States) at 4 ℃ until processing.

Autologous tumor cells were obtained as described by Wang *et al*[23]. Approximately 10 g of each tumor specimen was harvested in the operating room for primary cell culture. The tumor tissue was mechanically disrupted to generate approximately 1 mm^3 sections. The tissue was digested in 10 mL of RPMI-1640 medium supplemented with 0.05% collagenase (Hyclone, South Logan, UT, United States) with gentle agitation at room temperature for 4-6 h. After culturing for 7 d, the immunohistochemistry technique was used to detect the expression of DcR3 protein (anti-DcR3 mAb obtained from Sigma).

The human PC cell lines Capan-2 (HLA-A2+) and AsPC-1 (HLA-A2-), as well as the leukemia cell line K562, were obtained from the American Type Culture Collection (Manassas, VA, United States). The cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine (Hyclone), 50 U/mL penicillin, and 50 mg/mL streptomycin (Hyclone). All cells were cultured for 7 d and maintained in the logarithmic phase growth at 37 ℃ in a humidified atmosphere supplemented with 5% CO_2.

Preparation of RNA

Total cellular RNA was extracted from autologous PC cells and PBMCs by using TRIzol Reagent (Sigma) according to the manufacturer's instructions. Only RNA exhibiting a ratio of 28S:18S > 1 was subjected to further analysis.

Total RNA of anti-human DcR3 hybridoma clone 1B1 (a kindly gift from Dr. CF Wu of Jilin University, China)[24], was isolated with the RNeasy mini kit (Qiagen, Valencia, CA, United States). Five micrograms of RNA were used in a reverse transcription reaction with the RT primer 5′- ATT CTA GAG GCC GAG GCG GCC GAC ATG (T-30) VN-3′ and PowerScript RT (Clontech, Mountain View, CA, United States) and the primer 5′-AAG CAG TGG TAT CAA CGC AGA GTG GCC ATA TTG GCCr GrGrG 3AmMC7/-3′. The heavy (H) chain was amplified from 10% of the RT reaction by using Advantage 2 HF PCR mix (Clontech) and the primers 5′- AAA GAA TTC GGC CTT GTT GGC CTC ATT TAC CCA GAG ACC GGG AGA TG -3′ and 5′-GAA AAG CTT GGC CAT TGG GGC GGT ATC AAC GCA GAG TGG CCA TAT TG-3′. The L chain was amplified with the primers 5′-AAG AAT TCG GCC TTG TTG GCC TAA CAC TCA TTC CTG TTG AAG CTC TTG-3′ and 5′-GAA AAG CTT GGC CAT TGG GGC GGT ATC AAC GCA GAG TGG CCA TAT TG-3′. The resulting PCR fragments were digested with *Hind*Ⅲ and *Eco*RI and cloned into the *Hind*Ⅲ and *Eco*RI sites of the plasmid pSP73-Sph/A64, which possesses a T7 promoter and 64T nucleotides that allow for the production of *in vitro* transcribed RNA with a *polyA* tail of 64 residues. The gene encoding the full-length enhanced actin (as controls) was inserted into the pSP73-Sph/A64 plasmid, as well.

In vitro transcription of mRNA

All plasmids were digested with *Spe*I for use as a template for *in vitro* transcription reactions using the

mMESSAGE mMACHINE T7 kit (Ambion) according to the manufacturer's protocol. mRNA was purified with the RNeasy mini kit.

Generation and electroporation of DCs

DCs' generation was performed as previously described by Zhu *et al*[25]. A concentrated leukocyte fraction was isolated from PBMCs that processed 200 mL of blood during each collection. Leukapheresis products were separated by density-gradient centrifugation over polysucrose sodium diatrizoate (Sigma), and cells were resuspended in serum-free AIM-V medium (Gibco, Burlington, Canada). Cells were incubated in a humidified incubator for 2 h at 37 ℃ to allow plastic adherence. The non-adherent fraction was removed, and the adherent cells were cultured for 7 d in serum-free AIM-V medium supplemented with human rIL-4 (500 U/mL) and recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF) (800 U/mL) (R&D Systems, Minneapolis, MN, United States) at 37 ℃ under 5% CO_2.

The DCs were transfected with RNA using a Gene Pulser II (Bio-Rad, Hercules, CA, United States). After 7 d, the immature DCs were transferred into a low-conductance medium (Cytofusion Medium Formula C; CytoPulse Sciences, Columbia, MO, United States) after centrifugation at 170 × *g* at 4 ℃ for three times, each time for 7 min. Viable cells were resuspended to a final concentration of (10-40) × 10^6 cells/mL in the low-conductance medium. Subsequently, 0.5 mL cell suspension was mixed with 3 μg per 10^6 DCs and electroporated in a 0.4 cm cuvette at an optimum condition[26]. The cells were recovered for 5 min, and then the same protocols were repeated with 10 μg of H chain antibody RNA and 5 μg of L chain antibody RNA per 10^6 DCs. Cells were recovered for 15 min, and then the transfected DCs were matured by adding 10 ng/mL of TNF-α (Roche Molecular Biochemicals, Mannheim, Germany) for 24 h. The electroporation of actin mRNA and PBMC RNA was used as controls. Inverted phase contrast microscopy (I × 70; Olympus, Tokyo, Japan) and electron microscopy (scanning electron microscope JSM-7300EX; Hitachi, Tokyo, Japan) were used for the morphological characterization of DCs transfected with different RNA. The DcR3 protein expression in RNA-DCs was low to negative (mature DC ⩽ 11%-14% and immature DC ⩽ 9%-11%, data not shown).

Western blot analysis

The recombinant human DcR3 protein (Sigma) was dissolved in 0.02 mol/L phosphate-buffered saline (PBS, pH 7.4). The concentration was determined by the BCA Protein Assay Reagent method (Pierce Chemical Company, Rockford, IL, United States). Then, the proteins were resolved on sodium dodecyl sulfate (SDS)-polyacrylamide denaturing gels, and transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) overnight at 4 ℃. The membranes were blocked in Tris-buffered saline containing 2% non-fat dry milk (Bio-Rad) and 0.05% Tween 20 (Sigma) for 1 h. Using 1:5000 dilution of the supernatant from DCs co-transfected with autologous PC cell total RNA and anti-DcR3 mAb-encoding mRNA for 24 h, the supernatant from DCs was transfected with actin mRNA for 24 h (negative control) and the commercial anti-DcR3 mAb (positive control; Qiagen) as primary antibodies, followed by incubation for 1 h in a horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ, United States).

Enzyme-linked immunosorbent assay

As described by our previous study[26], autologous tumor cells (1 × 10^6 cells) were co-incubated with DCs (1 × 10^6 cells) encoding anti-DcR3 mAb mRNA or actin mRNA (negative control) in 96-well plates in an overall volume of 200 μL at 37 ℃ for 0-72 h. A 1:5000 dilution of the commercial anti-DcR3 mAb was applied as positive control. Triplicate supernatant samples from these co-cultures were examined for specific DcR3 secretion using DcR3 enzyme-linked immunosorbent assay (ELISA) kit (Genzyme, Waltham, MA, United States). Each well was measured at 450 nm, and the optical density values were used to calculate the concentration of the samples.

As previously described by Pruitt[8], an indirect ELISA was simultaneously used to measure the DcR3 mAb concentration in the supernatants of DCs co-transfected with total tumor RNA and anti-DcR3 mAb-encoding mRNA for 0-72 h. DCs transfected with actin mRNA were used as positive control.

Assay for DCs viability

As described by Chen[27], the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) cell proliferation assay was used to defined the cell viability. In brief, RNA loaded DCs were inoculated into 96-well tissue culture plates (BD PharMingen, San Diego, CA, United States) with a density of 3000 cells per well and co-incubation with or without 3000 autologous PC cells. The cells were handled according to the instructions of MTT at the time points of 0-96 h. The formazan crystals that we acquired were dissolved in dimethylsulfoxide (BD Biosciences, Franklin Lakes, NJ, United States). Absorbance was monitored at 490 nm and the percentage of exposed cells to controls was used to show the cell viability. Cells that did not receive RNA transfections were regarded as the control cultures.

Phenotypic analysis of DCs by flow cytometry

As described by our previous study[2], DCs were prepared using 2% paraformaldehyde after washing for three times with frigid PBS containing 0.5% of bovine serum albumin. Four fluorescein isothiocyanate

(FITC)-conjugated mAbs, including anti-HLA-DR, anti-CD80, anti-CD86 and anti-CD83, were used and which came from BD PharMingen. Flow cytometry was used to analyze those stained cells.

Flow cytometric analysis of DC apoptosis

Following the manner of Lin *et al*[28], apoptotic DCs were quantified by using annexin V-FITC and propidium iodide (PI) double staining. Briefly, autologous tumor cells were co-incubated with DC-total tumor RNA and DC-tumor-anti-DcR3 mAb mRNA for the specific times, and then 1×10^6 cells were resuspended in 100 μL binding buffer, after washing with frigid PBS. Two microliters of PI and FITC-annexin V were added into the cells that were resuspended and cultured for 15 min protecting from light. The cells were then added into 0.5 mL binding buffer and analyzed with flow cytometry.

Induction of antigen-specific CTL and in vitro cytotoxicity assay

Antigen-specific CTLs were produced utilizing a protocol described by Chen *et al*[2,26,27]. The PBMCs without adherence were cultured in serum-free medium containing 10 ng/mL IL-7 and 20 U/mL IL-2 (R&D Systems). The cells were encouraged weekly for a minimum of two times with RNA-DCs at a stimulator-to-effector ratio of 1:10. As determined by flow cytometric analysis, a minimum of 45% of purified effector cells were CD8+ T cells after 16 d of culture.

Target cells, including autologous DCs transfected with tumor antigen-encoding RNA and tumor cells, were resuspended in 1 mL RPMI-1640 medium at 37 ℃ in 5% CO_2 for 1 h, and which contained 100 μCi $NaCrO_4$ solution (Isotope Products, Beijing, China). The serial dilutions of effector CTLs at various E:T ratios and the 5×10^3 ^{51}Cr-labeled target cells were incubated in 200 μL RPMI-1640 in 96-well plates for 6 h. Fifty microliters of supernatant were then taken away, and ^{51}Cr secretion was measured by a gamma counter (Beckmann, Heidelberg, Germany). In all the tests, the spontaneous discharge was less than 15% of the total release of the detergent. Specific lysis percentage was computed as [(experimental cpm-spontaneous cpm)/(maximum cpm-spontaneous cpm)] × 100.

Analysis of cytokines released by T cells

As described by our previous study[2], after subjecting to different treatments, 5×10^3 DCs (DC-total tumor RNA and DC-tumor-anti-DcR3 RNA) were cultured in 96-well round bottom plates. T cells were isolated from proliferating peripheral blood lymphocytes (PBLCs) and 5×10^4 T cells were stimulated with RNA-DCs in a whole volume of 200 μL in 96-well plates for 24 h. The cytokines interleukin (IL)-12p70, interferon-γ (IFN-γ), IL-10, and TNF-α released by T cells were measured by ELISA kits (Endogen, Woburn, MA, United States). The results were obtained from triplicate wells and the examination of supernatant from cultured T cells alone for the four cytokines were used as control groups.

RNA-loaded DC-induced CD4+ and CD8+ T cell responses

The measurement of multi-antigen specific CD4+ and CD8+ T cell responses were conducted by cytokine release assay as described by Chen *et al*[2] and Miyazawa *et al*[29]. Through using the instrument of autoMACS™ (Miltenyi Biotec, Bergisch Gladbach, Germany), CD4+ and CD8+ T cells were separated from proliferating PBLCs and that were civilized after three cycles of re-stimulation *ex vivo*. The cells were then incubated with CD4 or CD8 microbeads (Miltenyi Biotec) for 15 min at 4 ℃ and washed before separation. Separation was executed adopting an autoMACS column (Miltenyi Biotec). The pillar was set in the magnetic field, and magnetically-labeled cells were preserved in the pillar and then flushed out as positively chosen cells while the magnetic field was turned off. The sorted populations' purity was determined by flow cytometry. The selected CD4+ and CD8+ T cells (5×10^4) were stimulated with RNA-DCs (DC-actin mRNA, DC-PBMC RNA, DC-total tumor RNA, and DC-tumor-anti-DcR3 mAb RNA, 5×10^3) in an overall volume of 200 μL of the entire medium in 96-well plates for 24 h. The supernatants were collected, and the IL-4 and IFN-γ levels were measured using IL-4 and human IFN-γ ELISA kits (Endogen). Each assay was carried out on duplicate samples.

Statistical analysis

Quantitative data are presented as mean ± SD. The ANOVA and post hoc test (S-N-K method) analyses were performed using Excel software (Microsoft, Redmond, WA, United States). $P < 0.05$ was considered for statistical significance.

RESULTS

Generation of primary tumor cells and DCs

Primary tumor cells and DCs were cultured from HLA-A2+ PC patients. Most cultured primary tumor cells (> 90%) were found to be positive for DcR3 in the cytoplasm (Figure 1A). For DCs, cells cultured from PBMCs with stimulation of GM-CSF, TNF-α and IL-4 showed a series of typical morphologies of mature DCs. Most DCs without transfection with RNAs assembled non-cohesive colonies. Cells that were ablated from these colonies demonstrated typical villiform processes as shown by inverted phase contrast microscopy (Figure 1B). Both transfection with total tumor RNA (Figure 1C) and simultaneously loading with the total tumor RNA and anti-DcR3 mAb mRNA (Figure 1D) showed typical morphological characteristics of DCs.

DC-tumor-anti-DcR3 RNA secrete functional anti-DcR3 mAb

To determine if we could produce specific mAb by

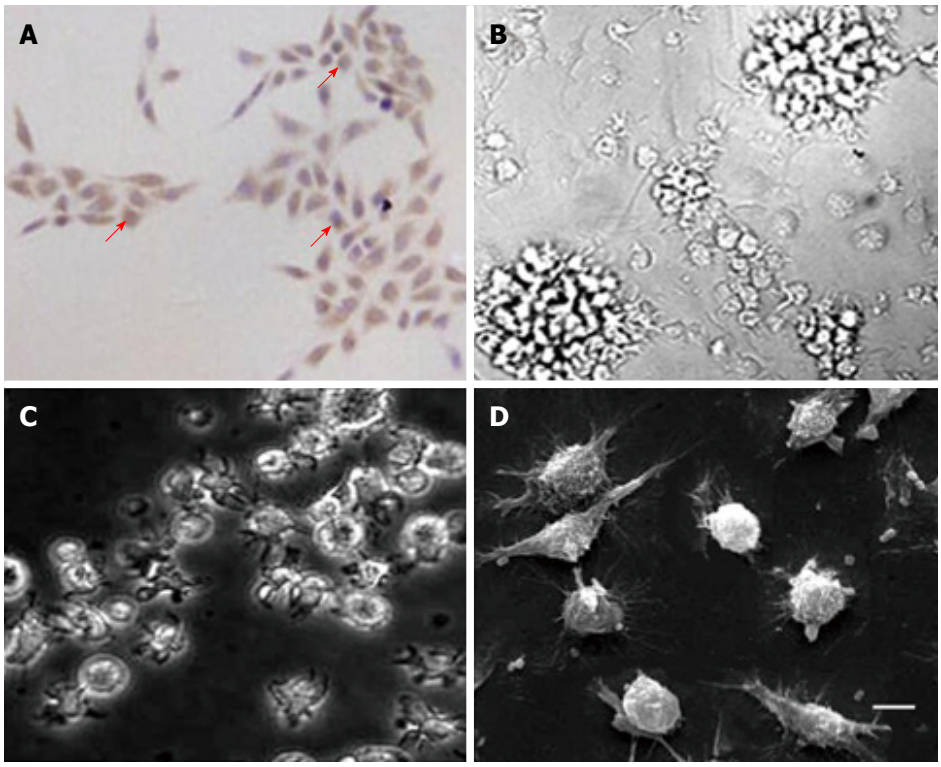

Figure 1 Cells obtained represented typical morphological characteristic of primary tumor cells and mature dendritic cells. Cells were derived from five pancreatic cancer (PC) patients and with similar shape of target cells. A: Most of the cultured primary tumor cells (over 90%) showed a DcR3-positive expression (magnification × 200); B: After cultured for 7 d, matured dendritic cells (DCs) were transfected without RNA and assembled into non-cohesive colonies, and the ablated cell showed a distinctive villiform process (magnification × 200). DCs transfected with total tumor RNA alone (C) or together with anti-DcR3 mAb mRNA (D) showed similar cell morphology to DCs transfected without RNA (C: magnification × 400; D: scanning electron microscope, bar represents 10 μm).

pulsing DCs with anti-DcR3 mAb-encoding RNA, we electroporated tumor-antigens-loaded DCs with IVT RNA encoding H and L mAb chains (anti-DcR3 H+L mRNA). The supernatants of DCs were obtained at the designated time points to identify the target mAb and measure their concentration. The supernatants of co-cultured autologous PC tumor cells and DC-tumor-anti-DcR3 RNA were harvested at specific time points to determine the effects of the mAb (Figure 2).

First, we used Western blotting to identify the anti-DcR3 mAb produced by DCs co-transfected with total tumor RNA and anti-DcR3 mAb mRNA. As shown in Figure 2A, the supernatant of DC-tumor-anti-DcR3 RNA could specifically neutralize the recombinant human DcR3 protein and generate a band with slightly higher molecular weight than 30 kDa, which was in line with the theoretical molecular weight of DcR3 protein. A commercially available anti-DcR3 mAb was used as positive control, while DC-actin was used as a negative control.

The amounts of anti-DcR3 mAb secreted by RNA-pulsed DCs were analyzed using an indirect ELISA assay. As shown in Figure 2B, mAb production by DC-tumor-anti-DcR3 RNA was transient and peaked at 24 h (containing 13.15 ± 1.9 ng of anti-DcR3 mAb per 1×10^6 cells) and then could not be detected after 72 h. However, no anti-DcR3 mAb was found in the supernatant of DC-actin RNA at any point.

The specific antigen-binding effect of anti-DcR3 mAb secreted by DCs co-transfected with total tumor RNA and humanized anti-DcR3 mAb mRNA was confirmed by ELISA. As shown in Figure 2C, the soluble DcR3 protein in the supernatant of autologous PC cells (1×10^6 cells) co-cultured with DC-tumor-anti-DcR3 RNA (1×10^6 cells) was significantly lower than that of tumor cells and the DC-actin RNA co-incubation group from 12 h to 72 h ($^aP < 0.05$).

Enhanced tumor-specific immune response induced by anti-DcR3 mAb secreting DCs

We next sought to determine whether anti-tumor responses could be enhanced by immunizing DCs co-transfected with total tumor RNA and anti-DcR3 mAb mRNA. Using cells from HLA-A2+ PC patients, we evaluated the ability of DC-tumor-anti-DcR3 RNA to augment the induction of anti-PC CTLs in response to DCs. As shown in the left panel of Figure 3A, DC-tumor-anti-DcR3 RNA was used as not only stimulator cells but also target cells, while DCs transfected with total tumor RNA alone or other autologous RNA-DCs were used as targets. DC-tumor-anti-DcR3 RNA demonstrated further enhancement of antigen-specific CTL induction compared with DCs only loaded with total tumor RNA without any increase in CTL activity above non-specific background.

CTL activity against PC cells was also assessed.

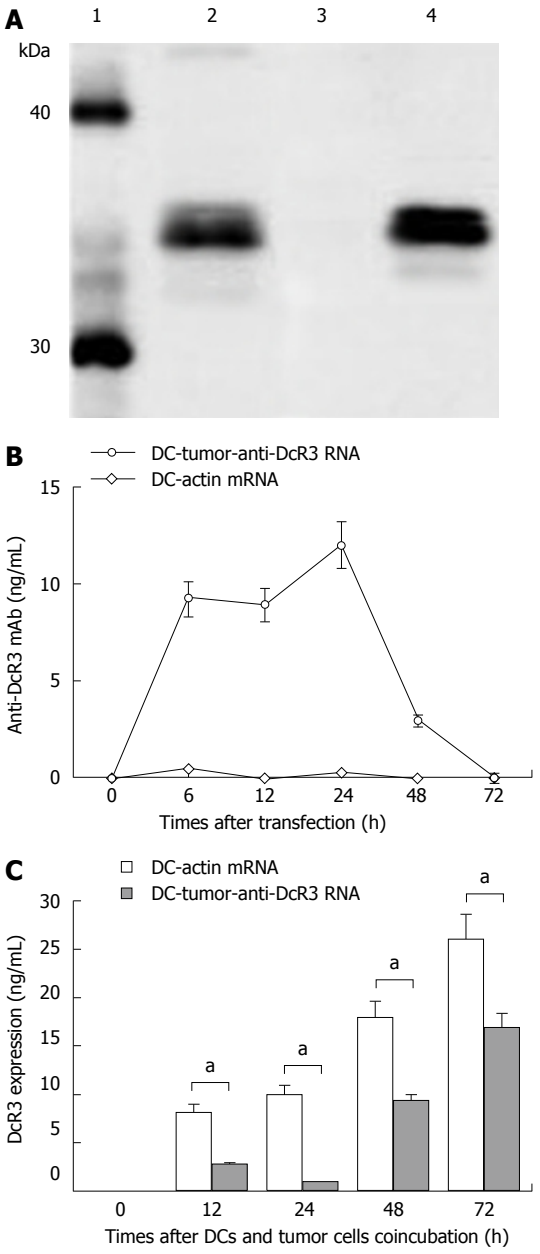

Figure 2 Identification, concentration and function of anti-DcR3 monoclonal antibody secreted by dendritic cells co-transfected with total tumor RNA and anti-DcR3 monoclonal antibody mRNA. A: Western blotting analysis showed that, similar to commercial anti-DcR3 mAb (lane 2), mAb secreted by dendritic cells (DCs) co-transfected with total tumor RNA and humanized anti-DcR3 H+L mRNA (lane 4) could also react with the recombinant human DcR3 protein (molecular weight of 35 kDa) and generate a band with molecular weight slightly greater than 30 kDa, whereas the supernatant harvested from DC-actin RNA could not bind the DcR3 protein (lane 3); B: The amounts of anti-DcR3 mAb produced by RNA transfected DCs were analyzed using indirect ELISA assay. The mAb secreted by DC-tumor-anti-DcR3 RNA was transient, peaked at 24 h, and then could not be detected after 72 h. However, no anti-DcR3 mAb was found in the supernatant of DC-actin RNA continuously; C: The specific antigen binding effect of anti-DcR3 mAb secreted by RNA transfected DCs was determined by measuring the levels of DcR3 protein in the supernatant of autologous tumor cells (1×10^6 cells) co-cultured with defined DCs (1×10^6 cells). After co-incubation with DC-tumor-anti-DcR3 RNA for 12-72 h, the soluble DcR3 protein level in the supernatant of autologous PC cells was significantly lower than those of tumor cells and the DC-actin RNA co-cultured group ($^aP < 0.05$). Except for those of western blotting and histogram, data represent the means of three experiments, and the histograms are representative of three experiments. Error bars represent SD. mAb: Monoclonal antibody.

Both CTLs induced by DC-tumor-anti-DcR3 RNA and DC-total tumor RNA were able to lyse their own cancer cells effectively, while CTLs induced by DC-PBMC RNA or DC-actin RNA were not, as shown in Figure 3A (right panel). Furthermore, DC-tumor-anti-DcR3 RNA showed greater effectiveness and superior ability to recognize and lyse HLA-A2+ autologous PC cells ($P < 0.05$). Meanwhile, with increase in the E:T ratio (from 10:1 to 40:1), the killing intensity increased concomitantly ($P < 0.05$).

No lysis of normal PBMCs or NK-sensitive K562 cells was observed. However, evident lysis against the cultured PC cell line occurred (Figure 3B). Effector T cells (HLA-A2+) stimulated by DCs transfected with total tumor RNA alone or together with anti-DcR3 mAb mRNA could lyse the Capan-2 cells, which expressed the HLA-A2+ antigen endogenously. On the other hand, HLA-A2- AsPC-1 cells were not identified and lysed. DC-tumor-anti-DcR3 RNA was more potent at inducing cytotoxicity in CTLs against Capan-2 cells with HLA-A2 adaptation in comparison with the CTLs induced by DC-total tumor RNA alone ($P < 0.05$). The expression of HLA alleles other than A2 was not evaluated in these experiments.

Improvement in cell viability by anti-DcR3 mAb secreting DCs

The effect of anti-DcR3 mAb secreting DCs on viability of RNA-loaded DCs was determined by MTT assay. As shown in Figure 4A, when RNA-DCs were co-incubated without autologous tumor cells, cell viability did not vary significantly at designed times, with around 85% survival throughout ($P > 0.05$). By contrast, the viability of DC-total tumor RNA cultured with tumor cells was evidently lower than that of DC-tumor-anti-DcR3 RNA ($P < 0.01$). The results of DC-PBMC RNA and DC-actin RNA were similar to those of DC-total tumor RNA. Meanwhile, both the DC-tumor-anti-DcR3 RNA and DC-tumor RNA demonstrated positive expression of CD80, CD83, CD86 (co-stimulatory molecules) and HLA-DR (MHC II molecules) after co-incubation with PC cells for 24 h, but there was no significant difference between the two methodologies (data not shown).

Annexin V and PI double staining was performed to demonstrate the inhibition of apoptosis of DCs co-transfected with total tumor RNA and anti-DcR3 mAb mRNA. After incubation with autologous PC cells for 0-96 h, annexin V-positive apoptotic cells were found to increase sharply in a time-dependent manner in DC-total tumor RNA, whereas apoptosis of DC-tumor-anti-DcR3 RNA were mitigated by anti-DcR3 mAb (Figure 4B).

T cell cytokine production pulsed by RNA-DCs

There were no significant differences in the amount of IL-10, TNF-α, IL-12p70 and IFN-γ cytokines released in the culture supernatants of T cells when measured by ELISA (data not shown). However, high levels of cytokines could be secreted by T cells after pulsing

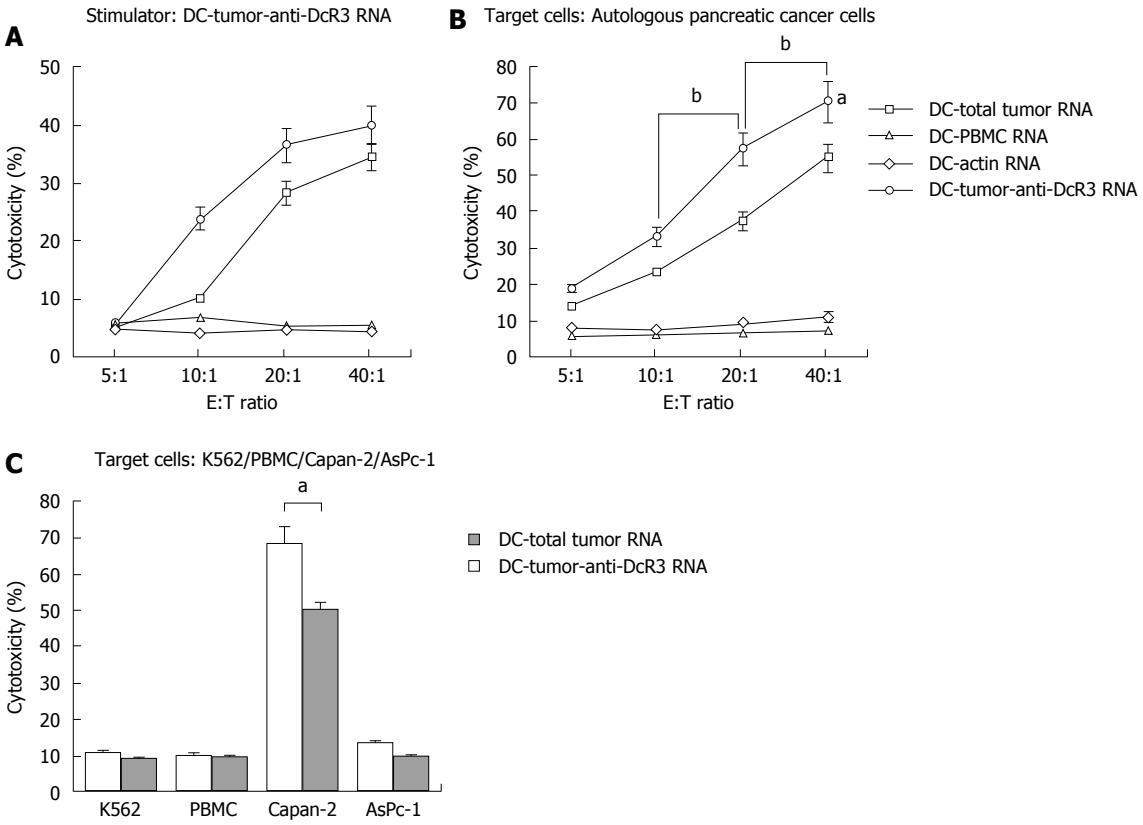

Figure 3 Antigen-specific immune response against pancreatic cancer is enhanced by anti-DcR3 monoclonal antibody secreting dendritic cell. Dendritic cells (DCs)-total tumor RNA, DC-PBMC RNA, DC-actin RNA and DC-tumor-anti-DcR3 RNA were used to stimulate autologous T cells weekly for two times followed by a cytotoxic T lymphocyte (CTL) assay. Induction of tumor antigen-specific CTLs was measured by using RNA-transfected DCs and tumor targets (primary tumor cells, K562, Capan-2 and AsPC-1 cell line cells). A: Left panel: DC-tumor-anti-DcR3 RNA was used as not only stimulator cells but also target cells, and CTLs stimulated by DC-tumor-anti-DcR3 RNA could recognize and lyse tumor antigen-specific cancer targets (DC-total tumor RNA and DC-tumor-anti-DcR3 RNA). No cross-reactivity was apparent against DCs loaded with normal tissue surrounding PC or actin (DC-PBMC RNA and DC-actin RNA). Compared with CTLs stimulated by DC-total tumor RNA, DC-tumor-anti-DcR3 RNA further enhanced the induction of CTL activity. Right panel: Both DCs co-transfected with total tumor RNA and anti-DcR3 mAb mRNA and DCs transfected with total tumor RNA alone showed an effective and superior ability in recognizing and lysing HLA-A2+ autologous PC cells, whereas T cells activated by DC-PBMC RNA or DC-actin RNA could not ($^{a}P < 0.05$). Moreover, CTLs induced by DC-tumor-anti-DcR3 RNA could produce a more powerful killing activity toward the tumor cells compared with the DC-total tumor RNA with the E:T ratio increasing from 10:1 to 40:1 ($^{b}P < 0.05$); B: At the E:T ratio of 40:1, the effector T cells (HLA-A2+) stimulated by DCs transfected with total tumor RNA alone or together with anti-DcR3 mAb mRNA could lyse the Capan-2 cell line cells, which endogenously expressed the HLA-A2 antigen effectively. By contrast, the cells of the AsPC-1 line (HLA-A2-) were not recognized and lysed. DC-tumor-anti-DcR3 RNA showed a more powerful capability in inducing the cytotoxicity of CTLs against HLA-A2-matched tumor cell line (Capan-2) compared with that induced by DC-total tumor RNA alone ($^{a}P < 0.05$). The experiment was repeated thrice representatively, and the data are shown as mean ± SD. mAb: Monoclonal antibody; DC: Dendritic cell; CTLs: Cytotoxic T lymphocytes.

with RNA-DC (Figure 5A). The IFN-γ and IL-12p70 produced by DC-tumor-anti-DcR3 RNA pulsed T cells were higher than those secreted by T cell pulsed by DC-total tumor RNA ($P < 0.01$). At the same time, compared with the DC-total tumor RNA group, the TNF-α levels detected in the DC-tumor-anti-DcR3 RNA group was not changed significantly ($P > 0.05$). In addition, the IL-10 level was lower when it was detected in DCs co-transfected with both total tumor RNA and anti-DcR3 mAb RNA ($P < 0.05$).

CD4+ and CD8+ T cell responses induced by anti-DcR3 mAb secreting DCs

As shown in Figure 5B, the CD4+ and CD8+ T cells incubated with DCs transfected with total tumor RNA alone or together with anti-DcR3 mAb mRNA produced significantly higher level of IFN-γ than those incubated with control DC (DC-actin RNA) or DCs exposed to normal tissues (DC-PBMC RNA) ($P < 0.01$). In addition, the CD4+ and CD8+ T cells that were cultured with DCs co-transfected with both total tumor RNA and anti-DcR3 mAb RNA produced a higher level of IFN-γ than those cultured with DC-total tumor RNA ($P < 0.01$). Meanwhile, CD4+ and CD8+ T cells incubated with DCs transfected with total tumor RNA alone or together with anti-DcR3 mAb mRNA were able to produce IL-4, but negative or weak secretion of IL-4 was observed in control groups (DC-actin RNA and DC-PBMC RNA) ($P < 0.01$). Furthermore, decreased levels of IL-4 production were detected in CD4+ T cells stimulated by DC-tumor-anti-DcR3 RNA compared with DC-total tumor RNA ($P < 0.01$). No significant difference in IL-4 secretion was detected between CD8+ T cells stimulated by DC-tumor RNA and those stimulated by DC-tumor-anti-DcR3 RNA.

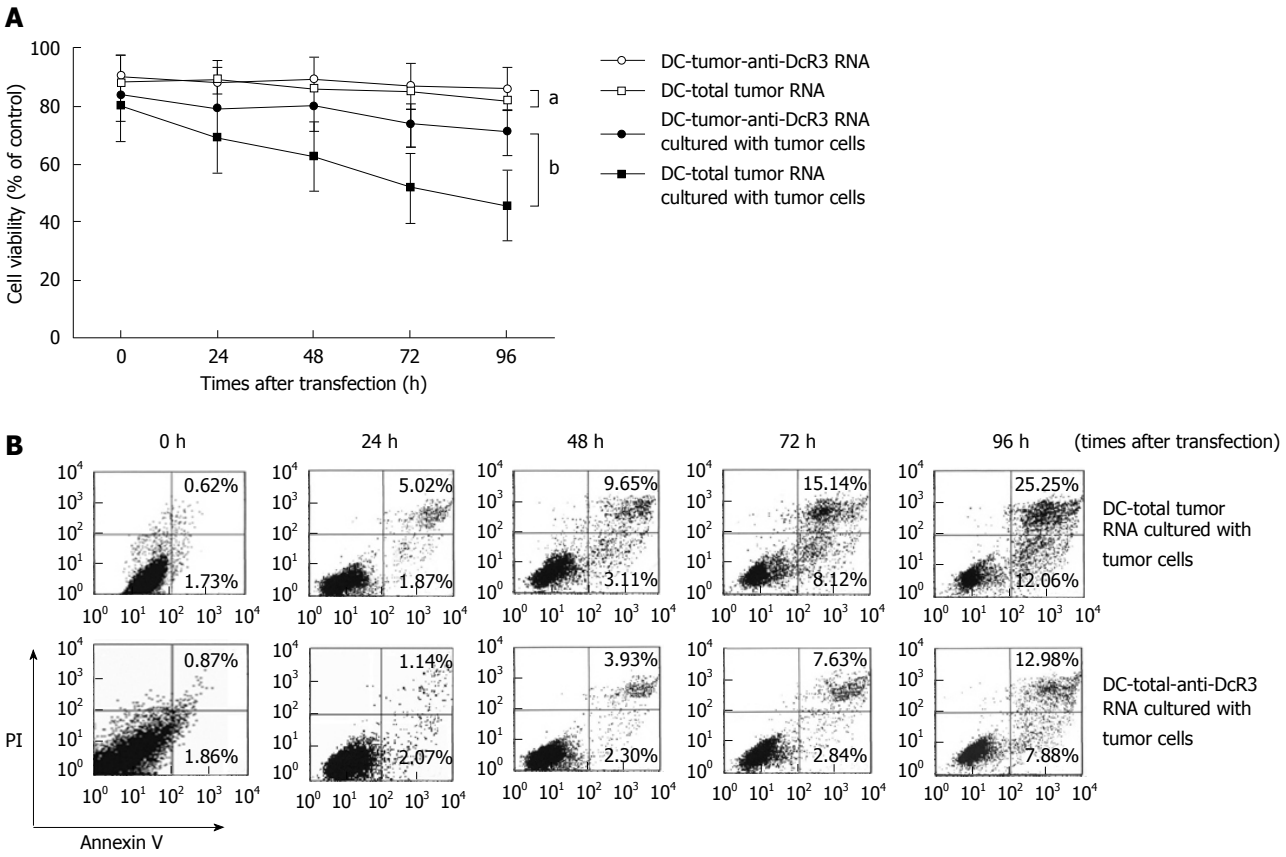

Figure 4 Effects of anti-DcR3 monoclonal antibody secreting dendritic cells on cell viability and apoptosis in tumor RNA-loaded dendritic cells. A: Dendritic cells (DCs) secreting anti-DcR3 mAb improved the viability of RNA-loaded DCs. The viabilities of DC-total tumor RNA and DC-tumor-anti-DcR3 RNA, cultured with or without autologous tumor cells, were measured using MTT assay after transfection for 0-96 h. The viability of DCs transfected with total tumor RNA alone or together with anti-DcR3 mAb mRNA did not change significantly at the designed time points, with approximately 85% survival throughout ($^{a}P > 0.05$). Within the same period, the viability of DC-total tumor RNA cultured with tumor cells was evidently lower than that of DC-tumor-anti-DcR3 RNA and decreased in a time-dependent manner ($^{b}P < 0.01$). Three representative experiments were run, and the data are shown as mean ± SD; B: DC-total tumor RNA and DC-tumor-anti-DcR3 RNA were co-cultured with autologous pancreatic cancer (PC) cells (1×10^{6}) for 0, 24, 48, 72 and 96 h. Cells stained with annexin V-FITC and propidium iodide were analyzed by flow cytometry. The percentage of annexin V-positive apoptotic cells in DCs transfected with total tumor RNA markedly increased in a time-dependent manner, whereas the apoptotic cells increased slowly in DCs co-transfected with total tumor RNA and anti-DcR3 mAb mRNA. mAb: Monoclonal antibody.

DISCUSSION

Tumor cells can produce several immunomodulatory molecules to induce immunosuppressive TME and inhibit the function of tumor-associated DCs[9,30]. Therefore, new DC-based strategies for producing tumor vaccines are necessary to abrogate immunosuppressive molecules in tumor tissue-induced mechanisms for suppressing the activation of CTL responses that can treat established cancers[8].

DcR3 is one of the candidate target tumor-derived factors[31]. As we initially demonstrated, most cultured primary tumor cells showed a DcR3-positive expression in the cytoplasm of PC patients, which is consistent with the findings of Zhou *et al*[17]. Tumor cells engineered to release high amounts of DcR3 are able to protect themselves from apoptosis, consequently resulting in a decreased immune response and suggesting that DcR3 is involved in the immune evasion of malignant tumors[19,32]. As a powerful immunomodulatory factor, DcR3 can suppress actin polymerization in mitogen-stimulated T cells, prevent the formation of pseudopodia, down-regulate the activation of DCs and macrophages, induce abnormal aggregation of T cells after antigen stimulation, reduce the interaction between T cells and DCs, inhibit T cell chemotaxis, and induce T cell apoptosis[19,33]. Therefore, neutralizing the DcR3 protein secreted by tumor cells is particularly important in cancer immunotherapy.

Different methods have been developed for neutralizing immunosuppressive factors released by tumor cells. The effects of systemic administration of antibodies have been examined by numerous studies. Their findings showed that mAbs can improve special immune responses when administered systemically[34]. Human studies, however, have revealed that the side-effects of mAbs are unavoidable when delivered systemically, and one major concern is induction of autoimmunity[35-37]. Thus, our present study shows an alternative strategy of delivering mAb by transfecting DCs with the RNA that encodes both PC tumor-antigens and a defined immunosuppressive molecule, namely humanized anti-DcR3 mAb. Using this strategy, DCs were used both to produce anti-DcR3 mAb and

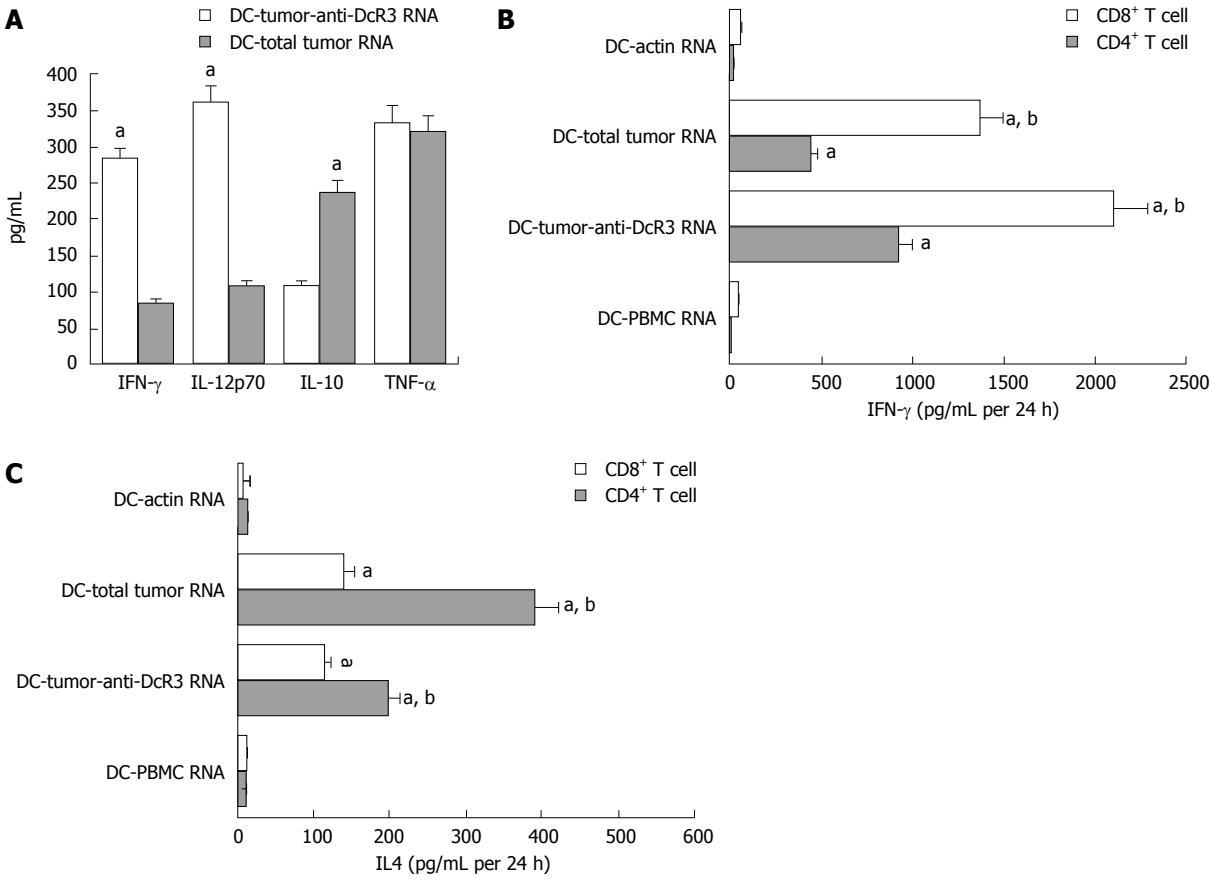

Figure 5 T cell responses induced by anti-DcR3 monoclonal antibody secreting dendritic cells. The T cells were co-cultured with RNA-dendritic cells (DCs) for 24 h. Then, the supernatants were collected, and the cytokine levels were measured by ELISA assay. A: ELISA test showed the cytokines of IL-12p70 and IFN-γ secreted by T cells pulsed by DC-tumor-anti-DcR3 RNA were higher than those secreted by T cells pulsed by DC-total tumor RNA without significant change of TNF-α. Meanwhile, IL-10 level was lower in DC-tumor-anti-DcR3 RNA than in DC-total tumor RNA ($^aP < 0.05$); B: $CD4^+$ T cells and $CD8^+$ T cells incubated with DCs encoding whole tumor antigens could produce extremely higher IFN-γ levels compared with those incubated with DCs as control or DCs treated with normal tissues ($^aP < 0.01$). Furthermore, the $CD4^+$ T and $CD8^+$ T cells incubated with DCs co-transfected total tumor RNA and anti-DcR3 mAb mRNA produced higher IFN-γ levels than those incubated with DC-total tumor RNA ($^bP < 0.01$); C: The $CD4^+$ T and $CD8^+$ T cells incubated with DCs loaded with whole tumor antigens showed a positive expression of IL-4, whereas a negative or weak expression of IL-4 was observed in DC-actin RNA and DC-PBMC RNA cells ($^aP < 0.01$). Moreover, decreased levels of IL-4 production were detected in $CD4^+$ T cells stimulated by DCs co-transfected with total tumor RNA and anti-DcR3 mAb mRNA compared with DCs transfected with total tumor RNA individually ($^bP < 0.01$). The experiment was repeated three times representatively, and results are shown as mean ± SD. IL: Interleukin; ELISA: Enzyme-linked immunosorbent assay; PBMC: Peripheral blood monocyte cell; mAb: Monoclonal antibody.

as vehicles for delivery to the site of T cell activation considering their well-documented function as antigen-presenting cells.

Our results showed that the supernatant of cultured DCs co-transfected with total tumor RNA and anti-DcR3 H+L mRNA could specifically bind the recombinant human DcR3 protein and generate a band with molecular weight that was slightly greater than 30 kDa, which was in line with the theoretical molecular weight of DcR3 protein. In addition, the DcR3 level in the supernatant of autologous PC cells co-cultured with DC-tumor-anti-DcR3 RNA was significantly lower than that of the control group. These data suggest that this approach is feasible and can generate sufficient anti-DcR3 mAb to neutralize the DcR3 secreted by PC cells *in vitro*.

One advantage of the local release of anti-DcR3 mAb that is provided *via* DC mRNA transfection is its secretion over a relatively short time span at the precise site where T cell activation is needed[8]. We previously showed that mRNA subunits have a brief half-life of less than 24 h after DC transfection[2], implying that mRNA translating into protein is an instantaneous event in DC. Here, we confirmed that for anti-DcR3 mAb, most target proteins were freed in 12 h after mRNA transfection, but the extra secreting of target could last for 24-48 h. Considering that human PC cells showed unregulated production of DcR3 within 12 h, we found that the time course of anti-DcR3 mAb release by DCs is ideal for obstructing the DcR3 expressed by PC cells[8].

In the present study, we found that DCs transfected with total tumor RNA alone or together with anti-DcR3 mAb mRNA could induce tumor-specific cytotoxic T cells to recognize and lyse tumor RNA-loaded DCs and tumor cells effectively. In comparison, no damage in K562 cells were found, which implies that the two manners can not only be PC-specific but also eliminated the possibility of NK cell activity [2]. These data also indicate that CTLs induced by DC-tumor-

anti-DcR3 RNA were more powerful at indicting lysis than the CTLs induced by DC-total tumor RNA. These results demonstrate that use of DCs co-transfected with RNA encoding humanized anti-DcR3 mAb and whole PC tumor-antigens may be a superior strategy for designing a DC-based tumor vaccine. In addition, it is known that, like Fas-Fc antibody, DcR3 can block apoptosis in Jurkat cells[21]. The more superior cytolytic function might be due to blockade of activation-induced cell death (AICD) in CTLs induced by DC-tumor-anti-DcR3 RNA rather than better CTL response generation due to superior priming of CTL precursors by engineered DCs. This should be addressed in future studies.

An HLA allele that matches the target cells and tumor-specific CTLs is necessary. All PC patients that were included in this study were HLA-A2+, and we are concerned with the function of HLA-A2 among different HLA alleles. We found that CTLs induced by DC-tumor-anti-DcR3 RNA and DC-total tumor RNA could deliver potent cytotoxicity towards Capan-2 cells. However, owing to HLA-A2 mismatching, AsPC-1 PC cells with HLA-A2- could not be lysed by HLAA2+ CTLs. This result indicates that HLA-A2 may be a key allele for presenting antigens, and PC-specific CTL immune response may be limited to MHC class I antigens.

The induction of autoimmunity is one potential problem that may limit the application of PC tumor and anti-DcR3 mAb RNA-transected DC vaccines, partly because RNAs loaded with both normal antigens and tumor antigens share the same antigen presentation pathway when they are delivered to DCs[26,38], and partly because RNA-encoding anti-DcR3 mAb in DCs may improve the risk of inducing autoimmunity just like systemic administration of neutralizing immunosuppressive factor antibody in TME[34]. Here, we adopted tissue cell enrichment by primary tumor cell culture, and found that CTLs stimulated by DC-total tumor RNA lysed tumor cells (autologous primary cultured tumor cells and PC cell lines) but not PBMCs (normal tissue cells). Meanwhile, using mRNA-transfected DCs to locally deliver anti-DcR3 mAb, no increase in non-specific background immune responses against control target cells or the normal tissue PBMCs *in vitro* was detected. On the basis of these results, we anticipate that local delivery of anti-DcR3 mAb by utilizing DCs transfected with RNA will bypass the adverse effects of autoimmune responses triggered by systemic delivery of mAb. At the same time, vaccine-induced anti-tumor immune response increased in patients. These findings indicate that harmful autoimmunity with pathological results may not be an issue with this method.

The mechanism by which DCs engineered to secrete anti-DcR3 mAb augments CTL response remains to be fully elucidated. In our study, we found that the cell viability of both DC-tumor-anti-DcR3 RNA and DC-total tumor RNA did not change significantly at the designated time points, with approximately 85% survival when cultured alone (without DcR3 influence). On the contrary, when co-cultured with autologous tumor cells (with DcR3 influence), the viability of DC-total tumor RNA was evidently lower than that of DC-tumor-anti-DcR3 RNA. Furthermore, when co-cultured with tumor cells for 0-96 h, apoptotic cells in DC-total tumor RNA evidently increased, whereas apoptotic cells in DC-tumor-anti-DcR3 RNA were found to increase slowly and mildly. Similar to the findings of You *et al*[39], results suggested that enhancement of CTL responses induced by anti-DcR3 mAb DCs may partly be due to its powerful DcR3 blocking capability, which down-regulated the apoptosis of DCs induced by DcR3 and increased DC viability at the site of T-cell activation in the process of whole tumor-antigen delivery.

The whole total tumor and anti-DcR3 mAb RNA electroporation likely acted as a powerful tumor vaccine which could effectively activate antigen-specific T cells against PCs, just like Th1 cells[2]. This interpretation is supported by our observations of a high percentage of killing of tumor cells and IFN-γ secretion of T cells. High expressions of cytokines, such as IL-12 and IFN-γ, and low expression of cytokine, such as IL-10, might be the reason why DC-tumor-anti-DcR3 RNA exhibited enhanced capability of inducing CTL responses. Th1 cells and Th2 cells are two important T regulatory (Treg) cells in the body. Transformation of Treg cells from Th1 to Th2 is a unique phenomenon in malignant tumors. Development of Th2 cells promotes long-term retention of cancer cells in the host body and protects them from immune surveillance and attack. Th1 cells and Th2 cells can both stimulate IFN-γ production, whereas Th2 cells preferentially induced production of IL-4 compared to Th1 cells[39,40]. In this study, we clearly demonstrated that CD4+ T cells induced by DCs co-transfected with total tumor RNA and anti-DcR3 mAb mRNA markedly decreased IL-4 secretion and increased IFN-γ production, indicating that DCs engineered to secrete anti-DcR3 mAb could increase the number of Th1 cells and decrease Th2 cells. Ojima *et al*[41] and Chen *et al*[2] reported that DCs that were loaded with TAA could induce antigen-specific CD4+Th1 cells and such CD4+ Th1 cells played a key role in the priming phase of CD8+ CTLs. In the present study, we showed that both DC-tumor-anti-DcR3 RNA and DC-total tumor RNA can activate not only tumor-specific CD4+ T cells but also CD8+ T cells assessed by IFN-γ release. Besides, CD4+ T cells and CD8+ T cells incubated with DC-tumor-anti-DcR3 RNA could produce more IFN-γ compared with those incubated with DC-total tumor RNA. These results indicate that adjusting the Th1/Th2 cytokine network and promoting the recovery of CD8+ anti-tumor cellular immunity may be the other two mechanisms for engineering DCs to secrete anti-DcR3 mAb to augment CTL response.

In summary, our results demonstrate that DCs engineered to secrete anti-DcR3 antibody can augment

CTL responses against PC *in vitro*. The observed immune-enhancing effects may be partly due to their capability of down-regulating DC apoptosis and adjusting the Th1/Th2 cytokine network. Therefore, use of DCs engineered to secrete anti-DcR3 antibody vaccine may be an attractive and promising therapeutic strategy for a patient with PC.

COMMENTS

Background

Pancreatic cancer (PC) is considered as a highly destructive human malignant tumor without effective treatments. Dendritic cell (DC) tumor vaccines have emerged as an alternative treatment manner for advanced PC. But tumor cells can produce some immunosuppressive molecules to inhibit the function of tumor-associated cells, such as T lymphocytes and DCs.

Research frontiers

DcR3, a soluble protein secreted by tumor cells, is overexpressed in carcinoma originating from the gastrointestinal tract system, including PC. DcR3 is regarded as an important immunosuppressive factor in immune effector cells' defect and it is particularly important to neutralize DcR3 protein secreted by tumor cells in cancer immunotherapy.

Innovations and breakthroughs

A new strategy of delivering mAb by transfecting DCs with the RNA encoding both anti-DcR3 mAb and the whole tumor-antigens was shown by this study. Furthermore, the DCs engineered to secrete anti-DcR3 mAb could augment cytotoxic T lymphocyte responses against PC *in vitro* and the immune-enhancing effects may be partly due to their capability of down-regulating apoptosis of DCs and adjusting the Th1/Th2 cytokine network.

Applications

This study lays a good foundation for further investigation of tumor DC vaccine targeting DcR3 protein against PC.

Terminology

As a tumor necrosis factor receptor superfamily's member, DcR3 works as decoy receptor for Fas ligand, LIGHT and TNF-like molecule 1A to neutralize their cytotoxic and regulatory functions.

Peer-review

This manuscript has shown that DCs engineered to secrete anti-DcR3 antibody can stimulate cytotoxic T lymphocyte responses against pancreatic cancer cells *in vitro*. It provided important contribution to development of immunotherapy for pancreatic cancers. The proposed method is convincing.

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ORIGINAL ARTICLE

Basic Study

Remote ischemic perconditioning prevents liver transplantation-induced ischemia/reperfusion injury in rats: Role of ROS/RNS and eNOS

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Ning He, Jun-Jun Jia, Li Jiang, Yan-Fei Zhou, Bing-Yi Lin, Yi-Fan Peng, Jun-Jie Chen, Tian-Chi Chen, Rong-Liang Tong, Jian-Hui Li, Hai-Yang Xie, Lin Zhou, Shu-Sen Zheng, Key Lab of Combined Multi-Organ Transplantation, Ministry of Public Health, Hangzhou 310003, Zhejiang Province, China

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Author contributions: He N and Jia JJ contributed equally to this work; Zhou L and Zheng SS conceived the experiments; He N, Jia JJ, Li JH, Zhou YF, Lin BY, Peng YF, Chen JJ, and Jiang L conducted the experiments; Xie HY contributed reagents/materials/analysis tools; Chen TC and Tong RL analyzed the results; He N and Jia JJ wrote the paper; All authors reviewed the manuscript to be published.

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Abstract

AIM

To investigate the underlying mechanisms of the

protective role of remote ischemic perconditioning (RIPerC) in rat liver transplantation.

METHODS

Sprague-Dawley rats were subjected to sham, orthotopic liver transplantation (OLT), ischemic postconditioning (IPostC) or RIPerC. After 3 h reperfusion, blood samples were taken for measurement of alanine aminotransferase, aspartate aminotransferase, creatinine (Cr) and creatinine kinase-myocardial band (CK-MB). The liver lobes were harvested for the following measurements: reactive oxygen species (ROS), H_2O_2, mitochondrial membrane potential ($\Delta\Psi m$) and total nitric oxide (NO). These measurements were determined using an ROS/H_2O_2, JC1 and Total NOx Assay Kit, respectively. Endothelial NO synthase (eNOS) was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) and western blotting, and peroxynitrite was semi-quantified by western blotting of 3-nitrotyrosine.

RESULTS

Compared with the OLT group, the grafts subjected to RIPerC showed significantly improved liver and remote organ functions ($P < 0.05$). ROS ($P < 0.001$) including H_2O_2 ($P < 0.05$) were largely elevated in the OLT group as compared with the sham group, and RIPerC ($P < 0.05$) reversed this trend. The collapse of $\Delta\Psi m$ induced by OLT ischemia/reperfusion (I/R) injury was significantly attenuated in the RIPerC group ($P < 0.001$). A marked increase of NO content and phosphoserine eNOS, both in protein and mRNA levels, was observed in liver graft of the RIPerC group as compared with the OLT group ($P < 0.05$). I/R-induced 3-nitrotyrosine content was significantly reduced in the RIPerC group as compared with the OLT group ($P < 0.05$). There were no significant differences between the RIPerC and IPostC groups for all the results except Cr. The Cr level was lower in the RIPerC group than in the IPostC group ($P < 0.01$).

CONCLUSION

Liver graft protection by RIPerC is similar to or better than that of IPostC, and involves inhibition of oxidative stress and up-regulation of the PI3K/Akt/eNOS/NO pathway.

Key words: Liver transplantation; Ischemia/reperfusion injury; Remote ischemic perconditioning; Endothelial nitric oxide synthase; Reactive oxygen species

Core tip: This study is believed to be the first to investigate remote ischemic conditioning using a novel model of remote ischemic perconditioning (RIPerC) in liver transplantation and to identify the PI3K/Akt/endothelial nitric oxide (NO) synthase/NO axis involved. Compared to the traditional method of ischemic postconditioning, RIPerC works similar to or better than it and overcomes the main concern of increasing total ischemic time, which may lead to problems. RIPerC appears as the most promising technique to avoid ischemia/reperfusion injury in liver transplantation and is convenient clinically.

He N, Jia JJ, Li JH, Zhou YF, Lin BY, Peng YF, Chen JJ, Chen TC, Tong RL, Jiang L, Xie HY, Zhou L, Zheng SS. Remote ischemic perconditioning prevents liver transplantation-induced ischemia/reperfusion injury in rats: Role of ROS/RNS and eNOS. *World J Gastroenterol* 2017; 23(5): 830-841 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i5/830.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i5.830

INTRODUCTION

Ischemia/reperfusion (I/R) injury is a frequent sequel of liver transplantation (LT) and significantly predisposes patients to graft dysfunction, which is related to increased risk of morbidity and mortality[1,2]. It is also a major obstacle to increasing the donor pool using marginal grafts, which are prone to a higher degree of I/R injury[3]. Evidence has shown that I/R induces vascular endothelial dysfunction, which is defined as abolished endothelium-dependent dilation[2,4]. Endothelial nitric oxide synthase (eNOS) protein expression, which is responsible for the basal production of endothelium-derived nitric oxide (NO), is markedly reduced along with the abolished dilation of endothelium[5,6]. It has been suggested that increasing NO availability notably improves the microcirculation and attenuates I/R injury, possibly due to the role of NO in reducing the generation of reactive oxygen species (ROS)/reactive nitrogen species (RNS)[7].

ROS/RNS play a pivotal role in signaling cascades that are essential for I/R injury[8]. ROS, including the superoxide anion (O_2^-), H_2O_2 and the hydroxyl radical, are generated with the reintroduction of O_2 to ischemic tissues. This is associated with mitochondrial depolarization, which is responsible for a positive feedback loop of ROS-induced ROS release[9-15]. RNS, which include NO· and peroxynitrite ($ONOO^-$), the latter originating from a reaction of O_2^- with transient initial excessive NO on reperfusion[16], together with ROS are responsible for oxidative/nitrative stress of I/R injury[17,18].

Remote ischemic conditioning (RIC), including remote ischemic preconditioning (RIPreC)[19], remote ischemic postconditioning (RIPostC)[20] and the recently described remote ischemic perconditioning (RIPerC)[21], was first described by Przyklenk *et al*[22], who demonstrated that short periods of ischemic reperfusion to a distant organ can protect the target organ[23]. To date, only three major studies have mentioned RIPerC in liver I/R injury[24-26]. There is only limited information about the relationship of oxidative/nitrative stress and NO during hepatic I/R, and about RIC *vs* classic surgical conditioning techniques of IPreC[27] and IPostC[28], which

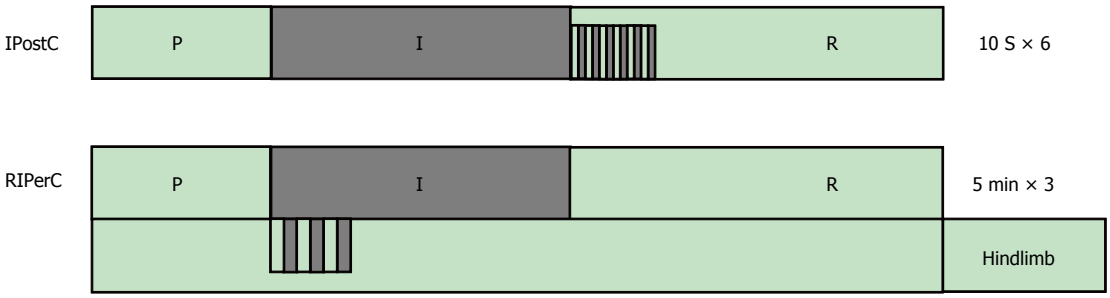

Figure 1 Ischemic postconditioning and remote ischemic perconditioning models. IPostC was performed by six 10-s cycles of reperfusion and 10 s reocclusion of the portal vein. RIPerC was performed by three 5-min cycles of reperfusion and 5 min reocclusion by tourniquet. IPostC: Ischemic postconditioning; RIPerC: Remote ischemic perconditioning.

reduce I/R injury in liver[29]. The effects of RIC on liver grafts have not been reported.

We have established an LT model of RIC/RIPerC and validated its protection against I/R injury[30], and here, we further investigate the underlying mechanisms. We postulated that the liver graft protection of RIPerC involved inhibition of oxidative/nitrative stress and up-regulation of the eNOS/NO pathway, and compared it with IPostC, which is also effective in our established LT I/R injury model.

MATERIALS AND METHODS

Animals and experimental design

Adult male Sprague-Dawley rats (250-300 g) were kept at 25-30 ℃ in a humidity-controlled environment and allowed access to a standard diet and water *ad libitum*. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital, Zhejiang University School of Medicine and were conducted in accordance with the ARRIVE (Animal Research: Reporting *in vivo* Experiments) guidelines (http://www.nc3rs.org/ARRIVE). Thirty-five rats (including 15 donors) were randomly assigned to four groups (n = 5 for each group) which were subjected to the following procedures. The Sham group (Group 1) underwent opening and closure of the abdomen under anesthesia, lasting approximately 75 min, which is the mean total ischemic time of orthotopic liver transplantation (OLT) in our center. The OLT group (Group 2) was subjected to standard OLT as above. The IPostC group (Group 3) underwent OLT with portal vein reperfusion and reocclusion for six 10-s cycles applied immediately at the onset of reperfusion (Figure 1). The RIPerC group (Group 4) underwent OLT with hindlimb ischemia and reperfusion for three 5-min cycles starting at the beginning of the anhepatic phase (Figure 1).

Rat OLT, IPostC and RIPerC models

The OLT model has been described previously[30]. The animals were anesthetized with 4% chloral hydrate (Shanghai No. 1 Biochemical and Pharmaceutical Co. Ltd, China). After isolation of the donor liver, the graft was perfused by cold saline containing 25 U/mL heparin through the portal vein, then placed into cold saline (0-4 ℃) for approximately 40 min before transplantation. After the completion of anastomosis of the suprahepatic vena cava, followed by inserting the cuffs into the recipient portal vein, the liver was reperfused to end the anhepatic period, which lasted approximately 15 min, with the hepatic artery being ligated. Subsequently, the same cuff procedure was carried out on the infrahepatic vena cava, and the common bile duct with a stent was also reconstructed. The abdominal incision of the recipient was closed. Immediately, 1.5 mL saline was injected through the penile vein. All rats were anesthetized with 4% chloral hydrate at 3 h after OLT for the collection of samples. The rats were killed at 3 h after the portal vein of the recipients was opened. During the operation, we manipulated the rats lightly and softly to ameliorate any suffering. The blood collected from the portal vein was immediately centrifuged to obtain a plasma supernatant and stored at -80 ℃ until it was assayed for organ function tests. The left and median liver lobes were obtained and stored at -80 ℃ for further analysis.

The IPostC model with six 10-s cycles of reperfusion and 10 s reocclusion of the portal vein was applied immediately at the onset of reperfusion. The RIPreC model consisted of clamping of the femoral vessels for 5 min followed by 5 min of reperfusion for a total of three cycles applied immediately at the onset of the anhepatic phase to the recipient hindlimb, using a standard tourniquet with 1 kg weight on both sides.

Biochemical assay of blood samples

Blood samples were taken for measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (Cr) and creatinine kinase-myocardial band (CK-MB) by standard laboratory methods using the Hitachi 7600 automatic analyzer (Tokyo, Japan).

Primary hepatocyte culture

Primary hepatocytes were cultured as described previously[31], with modification. The rat livers were dissected mechanically and disaggregated with collagenase under sterile conditions. The fragmented liver tissues were pipetted 10 times with 10 mL Dulbecco's modified Eagle's medium (Gibco, Grand

Island, NY, United States) through a nylon mesh. The cell suspension was centrifuged (50 × *g*, 5 min, 4 ℃) and the supernatant was removed. The precipitate was rinsed with Hank's solution and centrifuged again (50 × *g*, 5 min, 4 ℃), and this process was repeated three times. The cell suspension was seeded (6.25×10^4 cells/well) on a 96-well plate. All cells were maintained in a humidified atmosphere of 50 mL/L CO_2 at 37 ℃ and cultured according to standard cell culture techniques.

Mitochondrial membrane potential (ΔΨm), ROS, H_2O_2 and NOx assays

ΔΨm, ROS, H_2O_2 and NOx activity were determined using the JC-1 Mitochondrial Membrane Potential Detection Kit (Biotium, Fremont, CA, United States), ROS Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China), H_2O_2 Assay Kit (Beyotime Institute of Biotechnology) and Total NOx Assay (R and D Systems, Minneapolis, MN, United States), respectively. Fluorescence of JC-1 (5,5,6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide) was measured in the channel FL-1 and FL-2 by flow cytometry (FCM, Cytomics FC500; Beckman Coulter, Miami, FL, United States). In non-apoptotic cells, the mitochondria appeared red following aggregation of the JC-1 reagent, with an emission centered at 590 nm. In apoptotic cells, the dye remained in its monomeric form and appeared green with an emission centered at 530 nm. ROS were determined by measuring the oxidative conversion of cell-permeable 2',7'dichlorofluorescein (DCF) diacetate to cell-impermeable fluorescent DCF. Then, DCF fluorescence distribution was detected by confocal microscopy (FV1000; Olympus, Tokyo, Japan) analysis at an excitation/emission wavelength of 488/525 nm. H_2O_2 activity was determined in samples of tissue homogenate. H_2O_2-oxidized ferrous (Fe^{2+}) ions to ferric (Fe^{3+}) ions reacted with an indicator dye, xylenol orange, to form a purple complex that was detected at 560 nm using a microplate spectrophotometer (Biotek Instruments, Winooski, VT, United States). The amount of H_2O_2 released was calculated according to the standard curve originating from standard solutions from identical experiments. Total NO in the liver tissue was determined indirectly as the stable end products of nitrate and nitrite by the Griess reaction. NO^{3-} was converted to NO^{2-} with *Aspergillus* nitrite reductase and the total NO^{3-} plus NO^{2-} (NOx) was measured by measuring NO^{2-} with the Griess reagent. OD_{540} was determined (wavelength correction at 690 nm).

Detection of mRNA expression by reverse transcription-polymerase chain reaction

Total RNA was isolated from liver tissue homogenate using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, United States). One microgram of total RNA was isolated and reverse-transcribed to cDNA using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio, Shiga, Japan). The PCR system contained 0.8 μL forward primer (10 μmol/L), 0.8 μL reverse primer (10 μmol/L), 2 μL template cDNA, 0.4 μL ROX Reference Dye or Dye II (50 ×), 10 μL SYBR Premix Ex *Taq*II (Tli RNaseH Plus) (2 ×) and 6 μL distilled water to a total volume of 20 μL. The target gene expression was quantified using an ABI 7500 Fast Real-Time PCR (Foster City, CA, United States). Homogeneity was detected in each sample in all wells using dissociation curve analysis and the relative quantification was calculated using comparative cycle threshold (CT) method. The eNOS primers used were 5'-GTATTTGATGCTCGGGACTG-3' (forward) and 5-AGATTGCCTCGGTTTGTTG-3' (reverse). The amplification conditions for p-eNOS were: 30 s at 95 ℃ for one cycle and 5 s at 95 ℃, followed by 30 s at 60 ℃ for 40 cycles.

Western blot analysis for eNOS and 3-nitrotyrosine

Protein was isolated from liver tissue after incubation in RIPA Lysis Buffer (Beyotime Institute of Biotechnology) supplemented with protease inhibitor cocktail (Sigma-Aldrich, Dublin, Ireland) for 1 h on ice. After centrifugation (14000 × *g*, 4 ℃, 15 min), the supernatants were collected for protein concentration measurement using a Bicinchoninic Acid Protein Assay Kit (Thermo Fisher Scientific). Denatured protein was separated by SDS-PAGE (Invitrogen, Carlsbad, CA, United States) and transferred to nitrocellulose membranes. After blocking with 50 mL/L non-fat milk in Tris-buffered saline with Tween (TBST; 10 mmol/L Tris-HCl, 0.5 mL/L Tween 20 and 0.15 mol/L NaCl, pH 7.2) for 3 h, the membranes were incubated with primary antibodies against β-actin (1:1000; Abcam, Cambridge, MA, United States), total eNOS (1:1000; Abcam), p-eNOS (1:500; Abcam), 3-nitrotyrosine (1:1000; Abcam) for 15 h at 4 ℃. Blots were washed in TBST and incubated with appropriate horseradish-peroxidase-linked anti-mouse or anti-rabbit secondary antibodies (goat anti-mouse IgA for total eNOS, goat anti-rabbit IgG for p-eNOS and goat anti-mouse IgG2a for 3-nitrotyrosine; all supplied by Abcam) for 1 h at room temperature. Enhanced chemiluminescence was conducted using an ECL kit (Pierce Biotechnology, Rockford, IL, United States).

Statistical analysis

Data were expressed as mean ± SD. For comparison of statistical differences between experimental groups, analysis of one-way analysis of variance was used, followed by the Dunnett's test or Student's *t*-test for the histopathological outcomes. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of RIPerC treatment on liver graft and other remote organ functions

Compared with the Sham group, markers for acute damage of the liver, kidney and heart in all the other

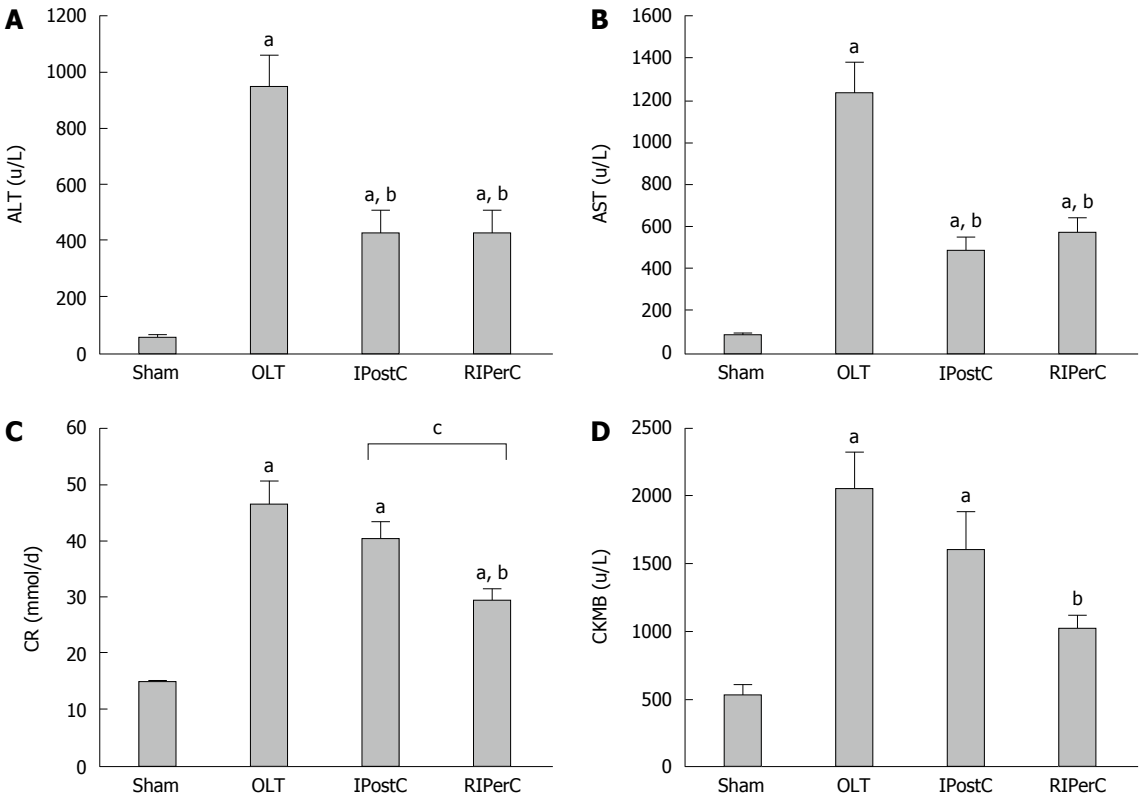

Figure 2 Effects of remote ischemic perconditioning treatment on liver graft and other organ functions. A and B: Effects of remote ischemic perconditioning (RIPerC) and ischemic postconditioning (IPostC) on alanine aminotransferase (ALT) and aspartate aminotransferase (AST), parameters for hepatocellular damage; C: Effects of RIPerC and IPostC on creatinine (Cr), a marker of acute kidney injury; D: Effects of RIPerC and IPostC on creatinine kinase-myocardial band (CK-MB), a marker for acute myocardial damage. Data represent mean ± SEM for 5 animals per group. [a]$P < 0.05$ *vs* sham group; [b]$P < 0.05$ *vs* orthotopic liver transplantation (OLT) group; [c]$P < 0.05$ RIPerC *vs* IPostC.

groups were elevated ($P < 0.05$). ALT, AST and parameters for hepatocellular damage were significantly lower in the RIPerC ($P < 0.001$) group as compared with OLT (Figure 2A and B) group. Serum Cr, a marker of acute kidney injury, was significantly decreased in the RIPerC group as compared with the OLT group ($P < 0.05$). CK-MB, a marker for acute myocardial damage, was significantly decreased in the RIPerC group as compared with the OLT group ($P < 0.05$) (Figure 2D).

Effects of RIPerC treatment on I/R-induced oxidative stress

Levels of ROS were significantly elevated by OLT I/R injury ($P < 0.001$) as compared with the Sham group. RIPerC decreased the ROS level ($P < 0.001$) (Figure 3A). Levels of H_2O_2 were also significantly elevated in the OLT group ($P < 0.05$) as compared with the Sham group. RIPerC reversed this trend ($P < 0.05$) (Figure 3B).

Effects of RIPerC treatment on I/R-induced nitrative stress

Expression of 3-nitrotyrosine was measured by western blot analysis. 3-nitrotyrosine levels were significantly elevated in reperfused hepatocytes ($P < 0.05$). I/R-induced 3-nitrotyrosine was significantly reduced in the RIPerC group ($P < 0.05$) (Figure 4).

Effects of RIPerC treatment on ΔΨm

JC-1 fluorescence shifted from red to green, indicating a collapse of ΔΨm. The ratio of green to red fluorescence was significantly increased in the liver graft after I/R injury ($P < 0.001$) as compared with the Sham group. The fluorescence shift induced by OLT I/R injury was significantly attenuated in the RIPerC group ($P < 0.001$) (Figure 5).

Effects of RIPerC on eNOS and NO expression

Expression of total eNOS was measured by western blotting and RT-PCR, and no significant alteration was found. There was a marked increase of eNOS phosphorylation on serine-1176 in the liver graft of the RIPerC group ($P < 0.05$) as compared with the OLT group (Figure 6A-C). NO expression, which was determined indirectly as the stable end products of nitrate and nitrite, was significantly increased in the RIPerC as compared with the OLT group ($P < 0.05$) (Figure 6D).

Effects of RIPerC vs IPostC

Compared with the OLT group, the IPostC group had significantly lower levels of ALT, AST ($P < 0.001$) (Figure 2A and B), ROS ($P < 0.001$), H_2O_2 ($P < 0.05$)

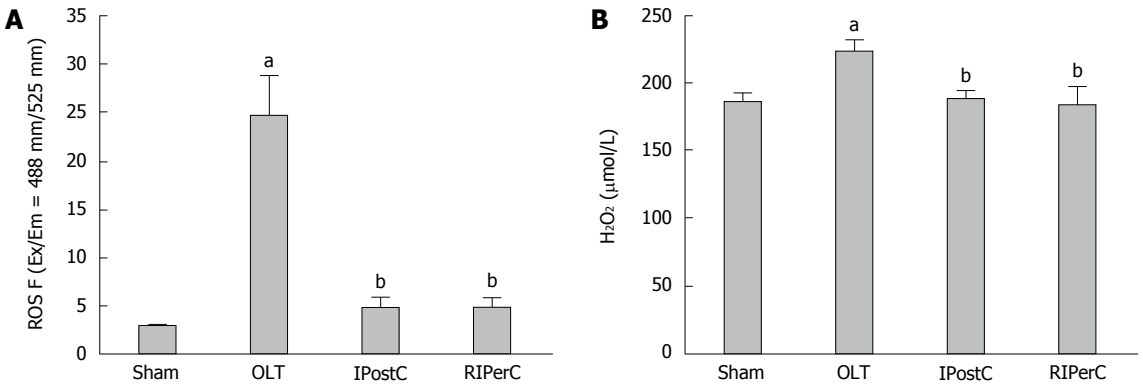

Figure 3 Effects of remote ischemic perconditioning on ischemia/reperfusion-induced oxidative stress. A: Effects of remote ischemic perconditioning (RIPerC) and ischemic postconditioning (IPostC) on ROS expression; B: Effects of RIPerC and IPostC on H_2O_2 expression. Data represent mean ± SEM for 5 animals per group. [a]*P* < 0.05 *vs* sham group; [b]*P* < 0.05 *vs* orthotopic liver transplantation (OLT) group.

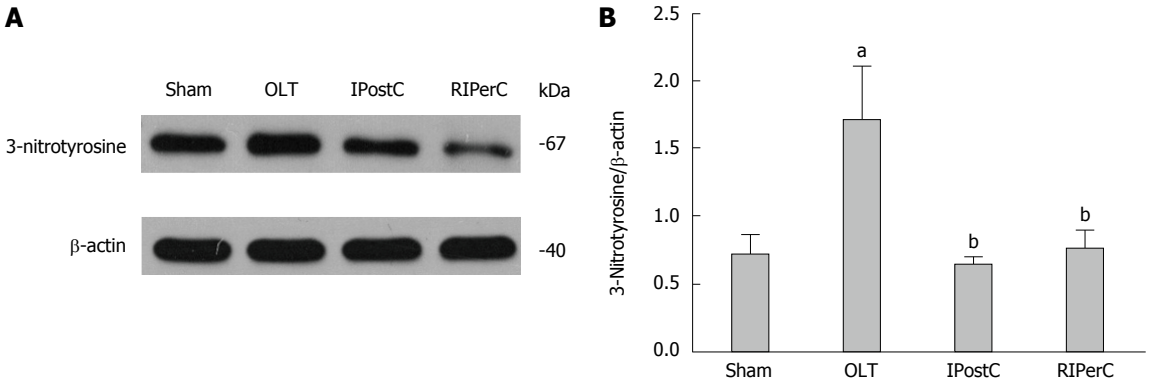

Figure 4 Effects of remote ischemic perconditioning on ischemia/reperfusion-induced nitrative stress. A: Protein expression levels of 3-nitrotyrosine were measured by western blot analysis (the gels were run under the same experimental conditions); B: Statistical analysis of 3-nitrotyrosine in liver tissues is shown. Data represent mean ± SEM for 3 animals per group. [a]*P* < 0.05 *vs* sham group; [b]*P* < 0.05 *vs* orthotopic liver transplantation (OLT) group. RIPerC: Remote ischemic perconditioning.

(Figure 3A and B) and 3-nitrotyrosine ($P < 0.05$), and significantly higher expression of Δm ($P < 0.001$) (Figure 5) and NO ($P < 0.05$) (Figure 6D). eNOS phosphorylation of serine-1176 in the IPostC group was increased by 1-8-fold compared with the OLT group, although this difference was not significant ($P = 0.106$) (Figure 6A-C). No significant differences were observed between the RIPerC and IPostC groups expect for Cr. The Cr level was lower in the RIPerC group than in the IPostC group ($P < 0.01$) (Figure 2C).

DISCUSSION

We previously established a new rat model of RIC and validated its protection against I/R injury by its anti-inflammatory and antioxidative activities and effects of activating the PI3K/Akt pathway[30]. Currently, we used the same model of hindlimb RIPerC (5 min × 3) to explore further the downstream effector proteins of the PI3K/Akt pathway, and compared it with IPostC under the same treatment conditions. We showed that RIPerC is similar to or better than IPostC, which prevented liver graft dysfunction and apoptosis induced by hepatic I/R injury by inhibiting oxidative/nitrative stress, as assessed by ROS and 3-nitrotyrosine, through the eNOS/NO pathway. We also found additional beneficial effects of RIPerC, such as reduced levels of Cr and CK-MB, compared with the OLT group, which indicates protection of other organs. To the best of our knowledge, this is the first investigation of RIC using a novel model in LT to identify the PI3K/Akt/eNOS/NO axis involved.

The present study provides new information about RIC after I/R injury beyond that in previous studies showing that protection associated with RIC mainly involves the heart. While most of the previous studies have only investigated the effects of RIPreC and RIPostC on organ protection, the present study focused on the novel model of RIPerC protection against hepatocyte I/R injury of LT. We found a significant reduction in plasma aminotransferase levels, which reflected liver function in the RIPerC group *vs* the I/R group, and exerted a protective effect by attenuating the congestion and hepatocyte necrosis shown in our previous study[31]. Several studies have also reported these protective effects of RIC, using different models from ours, in other organs, including the heart[32], brain[33] and kidneys[34,35]. However, those results

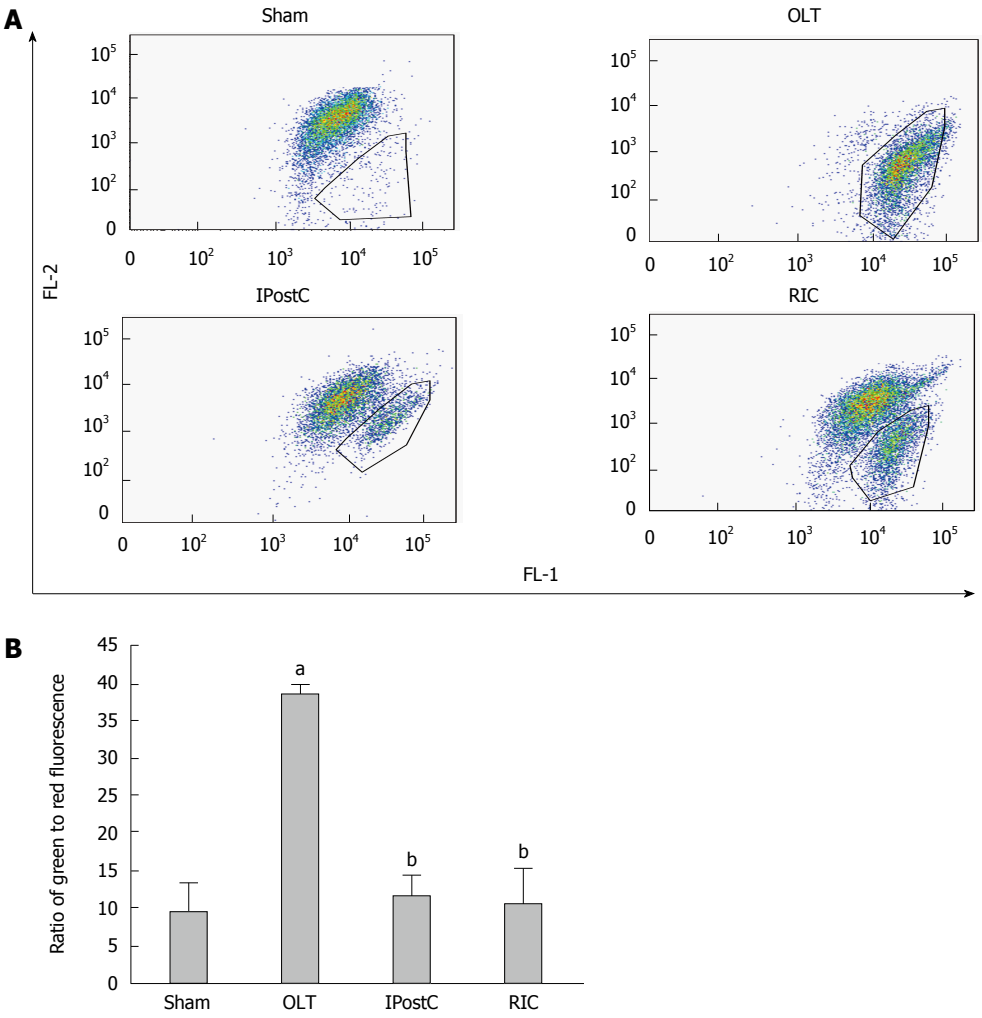

Figure 5 Effects of remote ischemic perconditioning on ΔΨm. A: Typical dot plot analysis of JC-1 of Δm. A dot plot of red fluorescence (FL2) *vs* green fluorescence (FL1) resolved live liver cells with intact ΔΨm (Sham group) from apoptotic and dead cells with lost ΔΨm (OLT group); B: The change of Δm was reported by the ratio of green to red fluorescence. Data represent mean ± SEM for 5 animals per group. $^{a}P < 0.05$ *vs* sham group; $^{b}P < 0.05$ *vs* OLT group. OLT: Orthotopic liver transplantation.

contrast with the lack of improved clinical outcomes of RIPreC in patients undergoing elective on-pump coronary artery bypass graft, with or without valve surgery[36] (Figure 2A and B).

Increased oxidative stress was one of the features observed in the liver and the isolated liver mitochondria during the initial phase of hepatic I/R[37]. We found that elevated production of ROS in the OLT and RIPerC groups could reverse this situation. In particular, H_2O_2, which is not a free radical, can be formed from O_2^- and generate hydroxyl radicals. Because H_2O_2 is very reactive, it is often discussed along with ROS[38,39]. H_2O_2 triggers release of proinflammatory cytokines and induces apoptosis, leading to tissue oxidative damage[40]. As there is non-specific suppression of ROS in the human body, H_2O_2-responsive co-polyoxalate containing vanillyl alcohol nanoparticles can serve as I/R-targeted nanotherapeutic agents[41]. Therefore, we also measured the H_2O_2 level alone, which reflected the same trend (Figure 3). Oxidant stress is an effective trigger of mitochondrial permeability transition pore opening in hepatocytes[42], leading to ΔΨm collapse, which is a hallmark of apoptosis[43]. The deleterious effects of ROS, such as apoptosis, are blocked using various antioxidants during I/R[44]. In the present study, RIPerC attenuated the related ΔΨm decrease induced by liver transplantation I/R injury, which was correlated with an increase in hepatocyte apoptosis (Figure 5). In early reperfusion, increased production of ROS is accompanied by reduced availability of NO[16]. Thus, we attempted to clarify the relationship between RIPerC protection against I/R injury and NO. The proposed mechanism is shown in Figure 7.

Experimental evidence indicates that eNOS-derived NO is hepatoprotective against liver I/R injury[45-47], and eNOS knockout mice exhibit significantly greater liver injury following I/R compared with wild-type counterparts[2]. It has also been demonstrated that genetic ablation of eNOS abolishes the cardioprotective effect in anesthetized mice and that stimulation of eNOS during RIPerC contributes to cardioprotection[48]. Phosphorylation of eNOS in the myocardium was higher

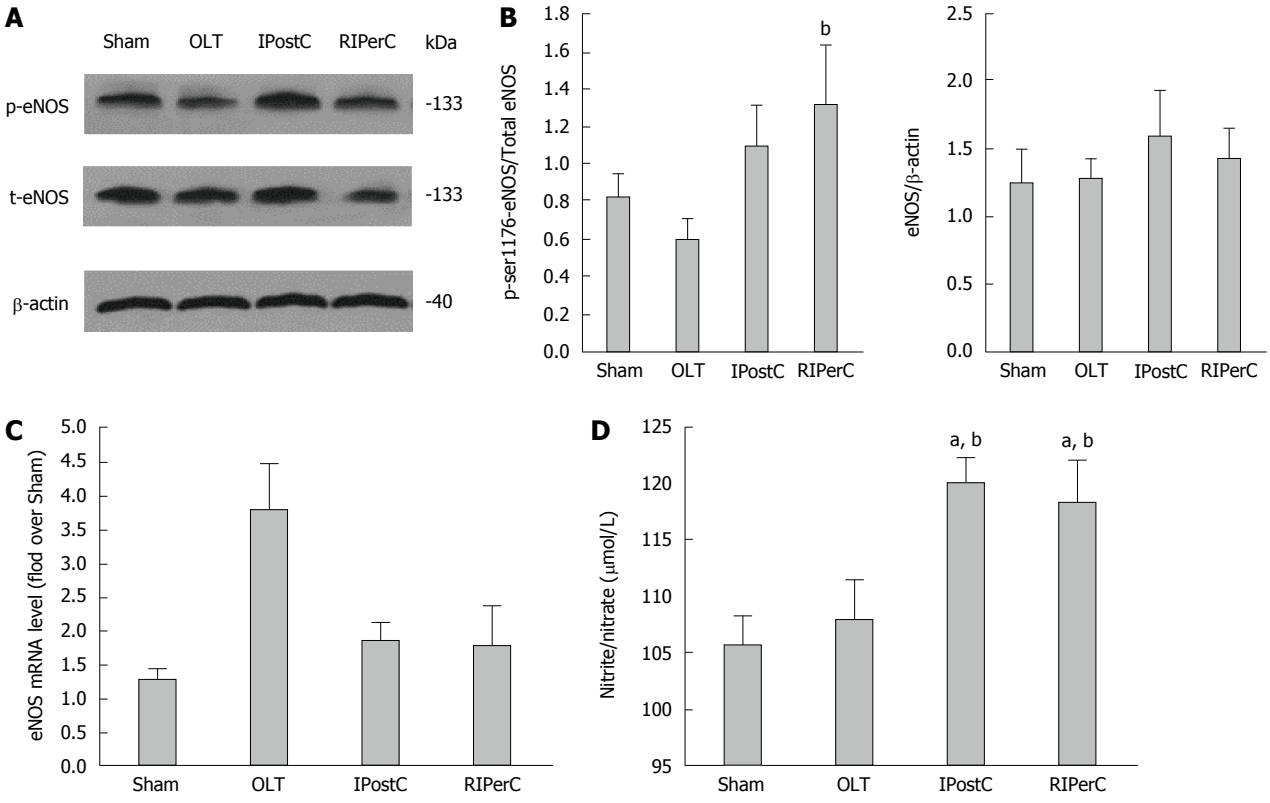

Figure 6 Effects of remote ischemic perconditioning on endothelial nitric oxide synthase and nitric oxide expression. A: Western blots of phospho-ser1176-endothelial nitric oxide synthase (eNOS) [p-eNOS] and total eNOS (t-eNOS) detection in liver tissues (gels run under the same experimental conditions); B: Statistical analysis of phospho-ser1176-eNOS and total eNOS in liver tissues; C: mRNA level of eNOS; D: Total nitrate/nitrite content in the four groups. Data represent mean ± SEM for 5 animals per group. $^{a}P < 0.05$ *vs* sham group; $^{b}P < 0.05$ *vs* orthotopic liver transplantation (OLT) group.

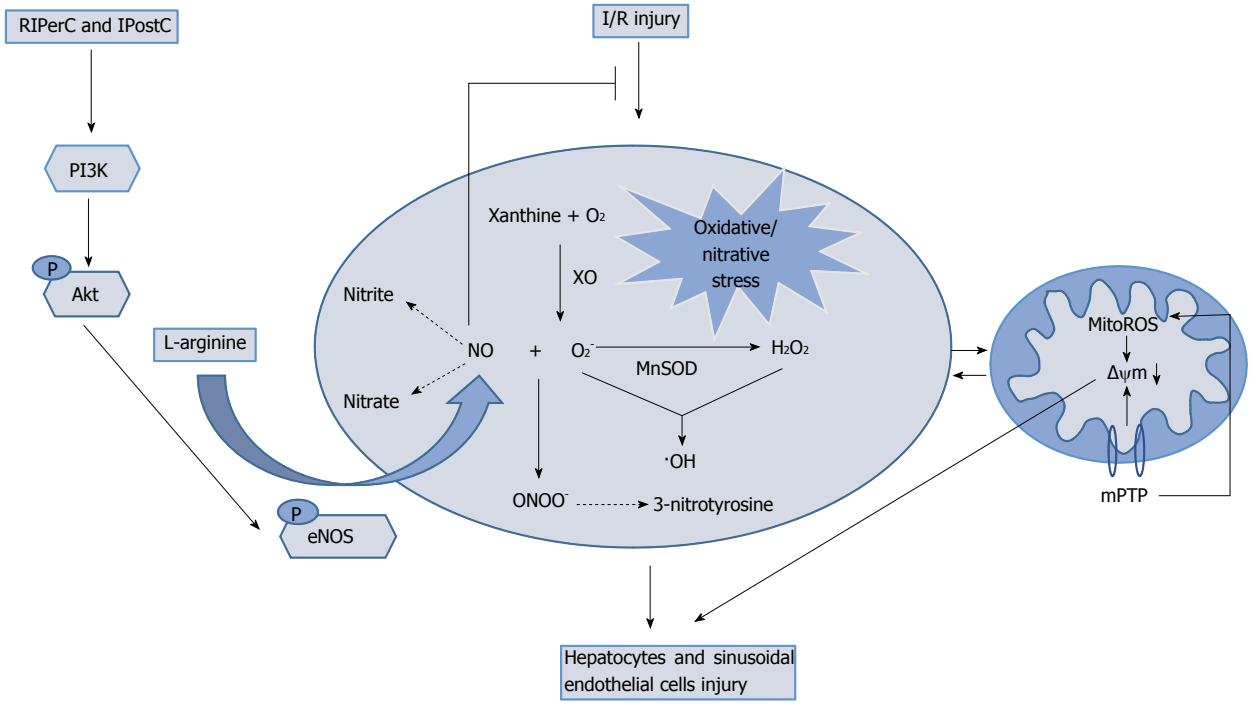

Figure 7 Simplified mechanisms of remote ischemic perconditioning protective effects against ischemia/reperfusion injury. Remote ischemic perconditioning (RIPerC) liver graft protection involves inhibition of oxidative/nitrative stress and up-regulation of the PI3K/Akt/eNOS/NO pathway. •OH: Hydroxyl radical; XO: Xanthine oxidase; MitoROS: Mitochondrial reactive oxygen species. IPostC: Ischemic postconditioning; I/R: Ischemia/reperfusion.

in the RIPerC group compared to the control group, parallel to infarct size reduction[49]. In agreement with the above findings, we found that phosphorylation of eNOS on serine-1176, through which RIPerC mediated protection against liver I/R injury, increased along with eNOS-derived NO, while there was no significant change in total eNOS (Figure 6). However, during early reperfusion, a transient initial excessive release of NO occurred in the presence of O_2^- *via* a diffusion-limited reaction to form $ONOO^-$[16], further impairing the mitochondrial[50] and cellular functions and increasing ROS generation[51]. These radicals further promote the post-translational modification of tyrosine to 3-nitrotyrosine[52], a marker for RNS and nitrative stress[37]. Consistent with these findings, our data showed that the liver tissue protein levels of 3-nitrotyrosine were higher after I/R injury compared to the sham-operated rats (Figure 4). Therefore, we propose that the tyrosine-nitrated proteins may experience a loss of function, attenuating the protective roles of eNOS, which contributes to I/R injury. Previous work has also shown that tyrosine nitration inactivates the PI3K/Akt pathway[51]. We determined Akt phosphorylation and observed significant increases in the RIPerC group[30], which indicated an inhibitory effect of tyrosine nitration. Akt activates phosphorylation of eNOS, resulting in increased production of NO in the endothelium[53]. We hypothesized that RIPerC exerts protection against LT I/R injury through the eNOS/NO pathway, as reported by previous studies that Akt is an important upstream regulator of eNOS activation[54-56]. The proposed mechanism is shown in Figure 7.

Compared to the local ischemic conditioning, RIC overcomes the main concern of increasing total ischemic time that may lead to sequence problems[30]. However, almost no work has been conducted comparing the protective effects between them. We found that the effect of RIPerC was similar to or better than that of IPostC in preventing liver graft I/R injury. RIPerC appears to be the most promising technique to avoid I/R injury of LT and is clinically convenient.

We revealed an increase in the levels of CK-MB and Cr 3 h after reperfusion of LT. A few studies have been conducted under similar conditions. A large single-center study showed for the first time that hepatic I/R injury plays a modifiable and critical role in the pathogenesis of acute kidney injury[57]. RIPerC induces multiple beneficial effects following valve replacement surgery, including reduced drainage and myocardial damage, attenuated hyperbilirubinemia, and acute lung injury[58]. Based on these studies, we attempted to demonstrate the hypothesis that RIPerC could mitigate the acute injury to other organs induced by liver I/R injury. Therefore, we measured CK-MB and Cr for acute heart and kidney injury, respectively, and verified our hypothesis. The underlying mechanism deserves further investigation.

We failed to verify the direct relationship between RIPerC-induced hepatic I/R injury protection and Akt/eNOS phosphorylation. Further studies using specific Akt or eNOS inhibitors could help to clarify the protection of this pathway in this setting. Nevertheless, this work provides clear clues for future studies.

In conclusion, our findings suggest that the liver graft protection of RIPerC involves inhibition of oxidative/nitrative stress and up-regulation of the PI3K/Akt/eNOS/NO pathway. The procedure of RIPerC for 5 min × 3 is simple to perform, particularly during LT, and is potentially applicable in clinical practice.

ACKNOWLEDGMENTS

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COMMENTS

Background

Remote ischemic conditioning (RIC) includes remote ischemic preconditioning (RIPreC), remote ischemic postconditioning (RIPostC) and the recently described remote ischemic perconditioning (RIPerC). To date, only three major studies have mentioned RIPerC in liver ischemia/reperfusion (I/R) injury. There is limited information available on the relationship between oxidative/nitrative stress and NO during hepatic I/R, and between RIC and classic surgical conditioning techniques of IPreC and ischemic postconditioning (IPostC), which are useful in reducing I/R injury in liver. The effects of RIC on liver grafts have also not been reported.

Research frontiers

Previous studies have demonstrated that the protection of RIC mainly revolves around the heart. Most of these studies have only investigated the organ-protective effects of RIPreC and RIPostC. The protective activity of RIPerC in liver I/R injury and the mechanism of action need to be explored.

Innovations and breakthroughs

This is believed to be the first study to investigate RIC using a novel model in liver transplantation and to identify PI3K/Akt/eNOS/NO axis involvement.

Applications

This study showed that the effect of RIPerC, which overcomes the main concern of increasing total ischemic time, was similar to or better than that of IPostC in preventing liver graft I/R injury. RIPerC appears to be the most promising technique to avoid I/R injury of liver transplantation and is clinically convenient.

Terminology

RIC was originally developed by Przyklenk *et al* in 1993, who showed that brief ischemia in one organ conferred protection on important distant organs without direct stress on the target organ. Compared to IPostC, RIC can be applied before ischemia of the target organ (RIPreC), after ischemia, and before perfusion (RIPerC) or at onset of reperfusion (remote ischemic postconditioning), without increasing the total ischemic time.

Peer-review

The authors established a new rat model of RIC and validated its protection against I/R injury by the PI3K/Akt/eNOS/NO axis. These results are interesting. The strategy of RIPerC 5 min × 3 is relatively simple to perform, particularly during liver transplantation, and may be considered as potentially applicable in

the clinical field.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Levothyroxine therapy and impaired clearance are the strongest contributors to small intestinal bacterial overgrowth: Results of a retrospective cohort study

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Abstract

AIM

To identify a set of contributors, and weight and rank them on a pathophysiological basis.

METHODS

Patients who have undergone a lactulose or glucose hydrogen breath test to rule out small intestinal bacterial overgrowth (SIBO) for various clinical symptoms, including diarrhoea, weight loss, abdominal pain, cramping or bloating, were seen as eligible for inclusion in a retrospective single-centre study. Clinical data such as co-morbidities, medication, laboratory parameters and other possible risk factors have been identified from the electronic data system. Cases lacking or with substantially incomplete clinical data were excluded from the analysis. Suspected contributors were summarised under four different pathophysiological pathways (impaired gastric acid barrier, impaired intestinal clearance, immunosuppression and miscellaneous factors including thyroid gland variables) and investigated using the χ^2 test, Student's *t*-test and logistic regression models.

RESULTS

A total of 1809 patients who had undergone hydrogen breath testing were analysed. Impairment of the gastric acid barrier (gastrectomy, odds ratio: OR = 3.5, PPI therapy OR = 1.4), impairment of intestinal clearance (any resecting gastric surgery OR = 2.6, any colonic

resection OR = 1.9, stenosis OR = 3.4, gastroparesis OR = 3.4, neuropathy 2.2), immunological factors (any drug-induced immunosuppression OR = 1.8), altered thyroid gland metabolism (hypothyroidism OR = 2.6, levothyroxine therapy OR = 3.0) and diabetes mellitus (OR = 1.9) were associated significantly to SIBO. Any abdominal surgery, ileocecal resection, vagotomy or IgA-deficiency did not have any influence, and a history of appendectomy decreased the risk of SIBO. Multivariate analysis revealed gastric surgery, stenoses, medical immunosuppression and levothyroxine to be the strongest predictors. Levothyroxine therapy was the strongest contributor in a simplified model (OR = 3.0).

CONCLUSION

The most important contributors for the development of SIBO in ascending order are immunosuppression, impairment of intestinal clearance and levothyroxine use, but they do not sufficiently explain its emergence.

Key words: Bacterial overgrowth syndrome; Hydrogen breath tests; Immunosuppression; Intestinal motility; Hypothyroidism

Core tip: Several contributors to small intestinal overgrowth have been described, but the impact of particular risk factors is poorly understood. We aimed to determine the influence of several pathomechanisms, such as impaired gastric acid barrier function, impaired intestinal clearance, impairment of defence mechanisms and miscellaneous factors, as well as to weight and rank a large set of potential contributors by means of a retrospective cohort study of 1809 consecutive patients who had undergone a hydrogen breath test to rule out small intestinal bacterial overgrowth. Overall, levothyroxine therapy, impaired intestinal clearance and immunosuppression are the strongest contributors, while an impaired gastric acid barrier only plays a minor role.

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INTRODUCTION

Small intestinal bacterial overgrowth (SIBO) is defined as an increase in the number of bacteria in the upper gastrointestinal tract. The aetiology and pathogenesis of SIBO are incompletely understood. It is believed that endogenous defence mechanisms prevent abundant microbial growth in the small intestine[1] under physiological conditions, so that the development of SIBO is usually seen to be associated with disorders of protective antimicrobial mechanisms, anatomical abnormalities or motility disorders. High recurrence rates after successful antibiotic treatment emphasise the need to identify aetiological factors in order to potentially remedy the situation[2].

Impairment of the gastric acid barrier function

Gastric acidity constitutes an effective barrier against the invasion of ingested microorganisms. Although the data is weak and contradictory, reduction of the acid barrier function, as suspected for atrophic gastritis[3,4], use of proton pump inhibitors[5] and gastrectomy[6,7], is thought to lead to higher microbial loads in the small intestine.

Impaired intestinal clearance

Although several reviews highlight anatomic pathologies associated with small intestinal obstruction and stagnation, for example, strictures, adhesions, tumours of the small bowel, duodenal and jejunal diverticula, previous abdominal surgery such as blind loop syndrome after Billroth-II or Roux-en-Y procedure, or bariatric bypass surgery, to be associated with SIBO, only very few or no data at all support these hypotheses. Overall abdominal surgery was not associated with SIBO in a small retrospective study[8]. However, gastrectomy and bariatric surgery in morbid obese patients often leads to the development of SIBO[6,7,9]; the existence of blind loops might be the common mechanism. Additionally, conditions that predispose stool reflux, such as ileocecal resection or low ileocecal valve pressure, are discussed as SIBO predisposing factors[10].

More evidence exists concerning impaired motility, such as small intestinal pseudo-obstruction and several neurological diseases or diabetes[1,11]. It has been demonstrated for a long time that a subset of patients with SIBO show reduced motility[12] with fewer phase III contractions of the migrating motor complexes and a mutual influence since eradication of bacterial overgrowth improved motility[13]. Gastroparesis, which has been shown to be associated with SIBO[14,15], might indicate gastrointestinal autonomous neuropathy. Otherwise, little is known about the effect of drugs used to deteriorate intestinal motility, even though "narcotics" have been identified as contributing towards SIBO[16].

Impairment of immunological mechanisms

Data about the role of the immune system are also scarce and contradictory. A higher bacterial load of jejunal aspirates have been shown in ten paediatric patients with IgA deficiency and seven with other immune syndromes[17], while data concerning adult patients are lacking. In fact, medical therapy is the

most common reason for immunosuppression in adults, though SIBO was not shown to be associated with immunosuppressive medication in patients with Crohn's disease (CD)[18,19], while steroids predisposed to SIBO in a more unselected cohort[20].

Miscellaneous factors

Various other diseases and disorders have been described as being associated with or complicated by SIBO, such as alcohol consumption[21], liver cirrhosis[22-25], non-alcoholic steatohepatitis[26], hypothyroidism[27] or chronic pancreatitis[28].

In summary, several contributors to the development of SIBO have been proposed, but only a few have been proven in clinical studies, which often refer to small and selected cohorts. Additionally, it is uncertain which pathomechanisms are more and which are less important contributors. We aimed, therefore, to (1) evaluate a larger set of potential risk factors; (2) arrange them in a pathogenetic model; and (3) rank the contributors referring to their particular weight in a largely unselected cohort of SIBO and non-SIBO patients in an extensive retrospective cohort study.

MATERIALS AND METHODS

Study population

We conducted a retrospective single-centre study of patients undergoing lactulose or glucose hydrogen breath testing between 1995 and 2010, who were referred to the Department of Gastroenterology at the University Hospital Bergmannsheil Bochum, Germany. Patients underwent breath tests to rule out SIBO for various clinical symptoms, including diarrhoea, weight loss, abdominal pain, cramping or bloating. All cases with an original examination report available were considered eligible for the study. Patients with both missing or incomplete clinical data, or incomplete or aborted examination were excluded. Patients with multiple hydrogen breath tests within the study period were considered only once.

Clinical work-up and reference standard

All patients underwent a hydrogen breath test with lactulose, glucose or both in combination. Additionally, patients underwent a routine diagnostic work-up following a clinical algorithm by a symptom-based diagnostic approach including clinical evaluation (history, symptomatology, clinical examination, complaints, clinical course), laboratory including stool testing, and endoscopy (ileocolonoscopy, esophagogastroduodenoscopy) including histopathology and transabdominal ultrasound. Further diagnostic tools may have been used, for example, small bowel magnetic resonance imaging, endoscopic ultrasound, small bowel endoscopy, manometry or extended function testing.

Hydrogen breath test

Breath tests were performed according to a standardised protocol with either 25 g lactulose or 75 g glucose in 300 mL water, respectively. Only one test was performed per day. Breath samples were collected using an AlveoSampler™ (Campro Scientific GmbH, Germany) every 10 min over a period of 2 h. In cases of failed hydrogen exhalation during a lactulose breath test, collecting periods were extended up to 180 min.

Analysis of breath test examinations

An increase in hydrogen of at least 20 ppm was considered positive; for a lactulose breath test, this increase had to have occurred at least 15 min before a sustained rise in hydrogen exhalation indicating colonic lactulose metabolism.

Clinical data acquisition

Additional clinical data was collected from the electronic database. Cases lacking or with substantially incomplete clinical data were excluded from the analysis.

Statistical analysis

Statistical analysis was performed with SPSS 23 (IBM, Armonk, United states). The χ^2 test was used to determine statistical significance with categorial variables, and the Student's *t*-test for metric variables. Regarding the multivariate analysis, binary logistic regression was performed in each pathophysiological pathway suspected separately (hypo-/achlorhydria, impaired clearance, immunosuppression, thyroid gland variables and miscellaneous factors) and with statistically significant variables in a summarising model. Finally, the highest ranked parameter of each particular section was chosen to calculate a simplified ranking model. The odds ratios (OR) and 95%CI were estimated for specific clinical factors using logistic regression models. Analysis was considered significant with a P value $\leqslant 0.05$.

Ethical considerations

The study was approved by the institutional review board (registration number 4864-13). Informed consent was obtained from the patients before particular examinations.

RESULTS

Basic characteristics

A total of 3715 hydrogen breath test examinations was considered eligible, 1586 of these were excluded due to missing or insufficient clinical information, and 320 records were excluded due to repetitive examinations of the same subject (Figure 1), therefore, our study population in summary contained a total of 1809 patients, with a slight, but not significant excess of women (Table 1). The age did not differ significantly between the gender groups. The overall basic characteristics of the patients were equally distributed as displayed, but patients with SIBO took slightly more

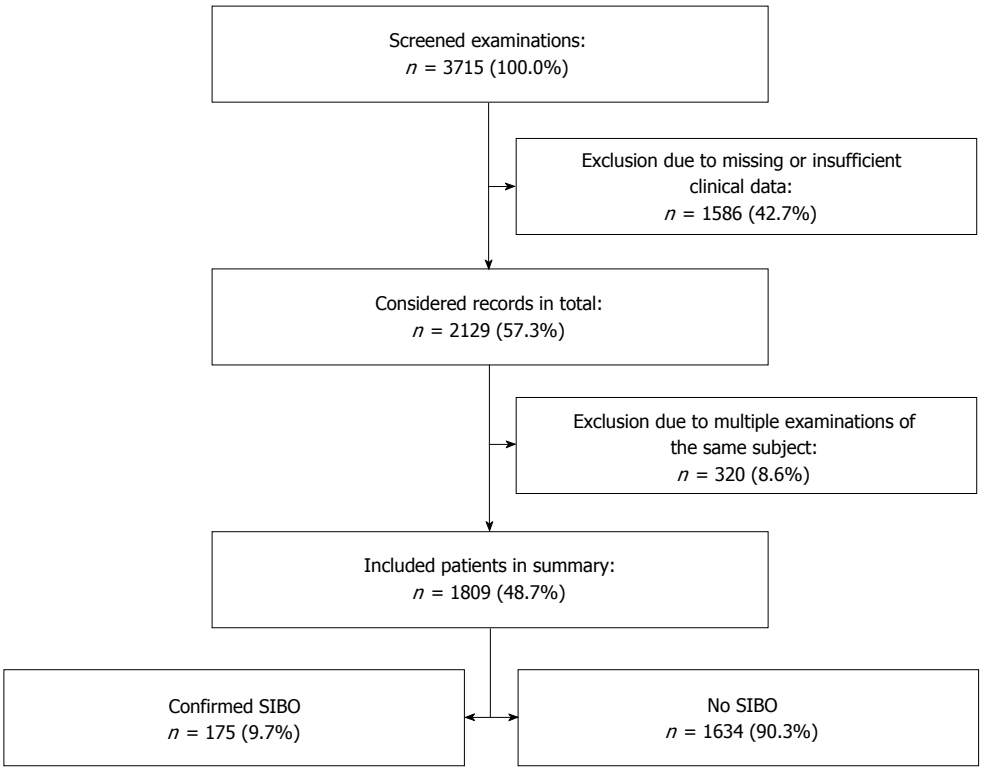

Figure 1 Study flow chart. A total of 3715 hydrogen breath examinations was eligible; 1586 were excluded due to missing or insufficient clinical data. From the remaining 2129 records, 320 were excluded because of multiple breath tests in the same subject, so that a summary total of 1809 patients were included in our study. 175 (9.7%) showed SIBO in terms, while 1634 did not (90.3%). SIBO: Small intestinal bacterial overgrowth.

Table 1 Basic characteristics

	SIBO		Non-SIBO		χ^2 or Student's *t*-test
	n = 175	%	*n* = 1634	%	*P* value
Age (yr)	48.7 ± 17.9	-	49.3 ± 18.0	-	0.70
Sex (female)	107	61.1	924	56.5	0.139
Pathological LHBT	102	58.3	212	13.0	< 0.001[1]
Pathological GHBT	146	83.4	9	0.6	< 0.001[1]
Diarrhoea	72	41.1	648	39.7	0.380
Constipation	4	2.3	38	2.3	0.616
Weight Loss	39	22.3	313	19.2	0.185
Malabsorption	9	5.1	53	3.2	0.138
Iron Deficiency Anaemia	2	1.1	38	2.3	0.240
Vitamin B12 Anaemia	1	0.6	4	0.2	0.399
Number of drugs used	1.61	-	1.32	-	0.005[1]
Supplementation of folic acid	3	1.7	22	1.3	0.442
Supplementation of iron	17	9.7	18	1.1	0.022[1]
Supplementation of vitamin B12	6	3.4	18	1.1	0.023[1]
Abdominal ultrasound	152	86.9	1508	92.3	0.022[1]
EGD	120	68.6	1187	72.6	0.146
Colonoscopy	100	57.1	1069	65.4	0.019[1]
Enteroclysis	45	25.7	357	21.8	0.143
Video capsule endoscopy	7	4.0	19	1.2	0.009[1]
Enteroscopy	1	0.6	4	0.2	0.399

[1]Statistically significant with *P* value < 0.05 in χ^2 or Student's *t*-test. As expected, the SIBO group showed a pathological hydrogen breath test. Symptoms did not differ significantly. Patients with SIBO took more drugs and were more likely to supplement vitamin B12 or iron. During the hospital stay, patients with SIBO underwent small bowel diagnostic procedures more often, while patients without SIBO were more likely to undergo other diagnostic procedures. SIBO: Small intestinal bacterial overgrowth; LHBT: Lactulose Hydrogen Breath Test; GHBT: Glucose Hydrogen Breath Test; EGD: Esophagogastroduodenoscopy.

drugs (1.32% *vs* 1.61%), especially spasmolytics and antiemetics (4.1% *vs* 14.9% and 8.8% *vs* 11.6%, respectively). Supplementation of iron and cobalamin was more common in SIBO patients.

The SIBO patients were more likely to undergo small bowel visualisation (video capsule endoscopy

Table 2 Hypo-/achlorhydria

	SIBO		Non-SIBO		χ^2 test	OR	RR
	n = 175	%	*n* = 1634	%	*P* value		
Gastrectomy	4	2.3	11	0.7	0.049[1]	3.451	0.295
Atrophic gastritis	2	1.1	5	0.3	0.141	-	-
PPI therapy	70	40.0	519	31.8	0.034[1]	1.432	0.794
Ulcer indicated PPI therapy	5	2.9	47	2.9	0.611	-	-
Gastritis indicated PPI therapy	20	11.4	195	11.9	0.481	-	-
GERD indicated PPI therapy	20	11.4	179	11.0	0.464	-	-
Indicated PPI therapy	42	24.0	395	24.2	0.522	-	-
Not indicated PPI therapy	58	33.1	370	22.6	0.002[1]	1.728	0.693

[1]Statistically significant with $P < 0.05$ in χ^2 test. Patients with SIBO had a history of gastrectomy more often and were more likely to take PPI. In subgroup analysis, those patients who received PPI due to peptic ulcer disease, gastritis or GERD had the same risk of developing SIBO, while those patients who did not exhibit such indications had a 1.7-fold increased risk of showing SIBO. SIBO: Small intestinal bacterial overgrowth; PPI: Proton pump inhibitor; GERD: Gastroesophageal reflux disease.

Table 3 Impaired clearance

	SIBO		Non-SIBO		χ^2 test	OR	RR
	n = 175	%	*n* = 1634	%	*P* value		
Any abdominal surgery	51	29.1	487	29.8	0.466	-	-
Obstetric surgery	2	1.1	45	2.8	0.135	-	-
Abdominal w/o obstetric surgery	49	28.0	444	27.2	0.438	-	-
Gastrectomy	4	2.3	11	0.7	0.049[1]	3.451	0.295
BII-resection	5	2.9	31	1.9	0.265	-	-
Existence of blind loops	8	4.6	41	2.5	0.094[2]	1.861	0.549
Any resecting gastric surgery	12	6.9	45	2.8	0.007[1]	2.600	0.402
Resection of Ileocecum	7	4.0	41	2.5	0.176	-	-
Appendectomy	7	4.0	134	8.2	0.027[1]	0.466	2.050
Functional appendectomy	14	8.0	175	10.7	0.163	-	-
Cholecystectomy	8	4.6	127	7.8	0.078[2]	0.568	1.700
Resection of small intestine	11	6.3	80	4.9	0.260	-	-
Any colonic resection	19	10.9	97	5.9	0.013[1]	1.930	0.547
Sigmoid resection	3	1.7	13	0.8	0.196	-	-
Vagotomy	2	1.1	6	0.4	0.177	-	-
Fistula	2	1.1	16	1.0	0.532	-	-
Stenosis	10	5.7	29	1.8	0.003[1]	3.354	0.311
Impaired motility	17	9.7	9	0.6	< 0.001[1]	5.157	0.202
Gastroparesis	5	2.9	24	1.5	0.030[1]	3.403	0.300
Neuropathy	9	5.1	39	2.4	0.037[1]	2.217	0.464
Opioid use	10	5.7	55	3.4	0.090[2]	1.740	0.589

[1]Statistical significant with *P* value $P < 0.05$; [2]Statistical tendency with *P* value $0.05 < P < 0.1$ χ^2 test each. Overall abdominal surgery was not associated with an increased risk of developing SIBO. Resecting gastric surgery in general and gastrectomy was associated with the development of SIBO. Patients with SIBO were less likely to have a history of appendectomy and - as a tendency - cholecystectomy. Ileocecal resection had no impact on the development of SIBO. As a further anatomical alteration, stenoses were more frequent in SIBO patients. Impaired motility, as shown by gastroparesis, led to SIBO; patients with neuropathy or opioid medication are patients at risk. SIBO: Small intestinal bacterial overgrowth.

4.0% *vs* 1.2%) while colonoscopy was performed more often in the non-SIBO group (57.1% *vs* 65.4%).

Suspected risk factor analysis

Hypo-/achlorhydria: Patients with a history of total gastrectomy were more likely to develop SIBO (2.3% *vs* 0.7%). The OR was 3.45 (Table 2). Current PPI therapy led to a higher SIBO rate (40.0% *vs* 31.8%), although the effect was small (OR = 1.43).

Impaired bowel clearance - anatomical alterations and surgery: Overall abdominal surgery was not associated with a higher risk of SIBO (Table 3). Patients after gastrectomy and patients with a history of any resecting gastric surgery had a higher prevalence of SIBO (6.9% *vs* 2.8%, OR = 2.60). By contrast, neither Bilroth-II resection nor the existence of blind intestinal loops exhibited a higher prevalence of SIBO, although the latter group showed a tendency (4.6% *vs* 2.5%). Loss of the ileocecal valve did not increase risk, while a history of appendectomy occurred more often in non-SIBO patients (8.2% *vs* 4.6%), indicating a protective factor with an OR of 0.46. On the other hand, functional appendectomy (including those patients with ileocecal resection) did not show such an effect. Obstetric surgery, sigmoid and small intestinal resection did not affect development of SIBO, while any colonic resection was associated with

Table 4 Immunological factors

	SIBO		Non-SIBO		χ^2 test	OR	RR
	n = 175	%	*n* = 1634	%	*P* value		
IgA-deficiency	0 of 6	0.0	13 of 75	17.3	0.337	-	-
IgG-deficiency	3 of 5	60.0	15 of 52	28.8	0.175	-	-
IgM-deficiency	0 of 5	0.0	15 of 54	21.7	0.311	-	-
5-Aminosalicylates	24	13.7	183	11.2	0.191	-	-
Steroid use	36	20.6	222	13.6	0.010[1]	1.647	0.660
Immunosuppressant use	8	4.6	31	1.9	0.029[1]	2.477	0.415
Azathioprin use	7	4.0	31	1.9	0.067[2]	2.155	0.474
Metotrexate use	3	1.7	0	0.0	0.001[1]	n/a	n/a
Any drug-induced immunosuppression	39	22.3	230	14.1	0.004[1]	1.751	0.632
Steroid plus immunosuppressant	7	4.0	23	1.4	0.021[1]	2.918	0.352

[1]Statistical significant with *P* value $P < 0.05$; [2]Statistical tendency with *P* value $0.05 < P < 0.1$ χ^2 test each. Deficiencies of immunoglobulins were not associated with a higher prevalence of SIBO. On the other hand, pharmacologically induced immunosuppression with steroids alone, with an immunosuppressant alone or with both in combination lead to a higher risk of developing of SIBO. SIBO: Small intestinal bacterial overgrowth.

an OR of 1.93 (10.9% *vs* 5.9%). Cholecystectomy tended to be protective (4.6% *vs* 7.8%). Stenoses of the intestinal tract were associated with SIBO (5.7% *vs* 1.8%) with an OR of 3.36.

Impaired bowel clearance - functionally impaired clearance: Impaired motility (9.7% *vs* 0.6%), gastroparesis (2.9% *vs* 1.5%) and neuropathy (5.1% *vs* 2.4%), but not vagotomy were associated with higher risks of SIBO; referring to Table 3, the ORs were 5.16, 3.40 and 2.22, respectively. Prevalence of SIBO tended to be higher under opioid medication, but did not achieve statistical significance (5.7% *vs* 3.4%).

Immunological factors: Immunoglobulin deficiency did not change the risk of developing SIBO (Table 4), while the use of steroids (20.6% *vs* 13.6%) or classical immunosuppressants (4.6% *vs* 1.9%), any immunosuppressive therapy (22.3% *vs* 14.1%) and the combination of steroids and immunosuppressants led to a higher risk of SIBO (4.0% *vs* 1.4%). The OR was highest in the combination group (steroid plus immunosuppressant: OR = 2.92).

Other factors

Thyroid gland metabolism: As referred to in Table 5, patients with hypothyroidism and patients with levothyroxine therapy showed a higher prevalence of SIBO (9.7% *vs* 4.0% and 17.1% *vs* 6.5%, respectively) while a history of thyroidectomy slightly failed to.

Miscellaneous: Diabetes mellitus was associated with a 1.90-fold increased risk of developing SIBO (14.3% *vs* 8.1%; Table 5). Sigmoid, but not colonic diverticulosis was associated with a lower prevalence of SIBO (4.6% *vs* 9.2%).

Multivariate analysis: The different pathomechanistic pathways were tested in a binary logistic regression analysis. The strongest particular independent variables were PPI therapy and gastrectomy for hypo- or achlorhydria. Both showed statistical significance with an OR of 1.45 and 3.64, respectively.

All variables which potentially impair intestinal clearance were studied in a further model. Any resecting gastric surgery, stenoses, gastroparesis and any colonic resection were significantly associated with the presence of SIBO ($P < 0.05$): the ORs were 6.49, 3.19, 3.25 and 1.85, respectively, while gastrectomy, neuropathy, existence of blind loops, appendectomy and cholecystectomy were not.

The model for immunosuppression did not show any significant parameters. Binary logistic regression for thyroid gland variables proved statistically significant for levothyroxine use with an OR of 2.8, while thyroidectomy and hypothyroidism did not. The only significant parameter in the model of miscellaneous variables was sigmoid diverticulosis with an OR of 0.453; the other factors were opioid use, smoking, diabetes and ulcerative colitis.

Finally, the variables PPI therapy, history of gastrectomy, history of any resecting gastric surgery, presence of stenoses, use of levothyroxine, presence of diabetes, neuropathy or gastroparesis, medical immunosuppression and therapy with opioids have been included in a summarising model (Table 6). The Omnibus test results are highly significant ($P < 0.001$). The Hosmer-Lemeshow test ($P = 0.500$) indicates that the independent variables form a good model to predict SIBO (54 cases observed, 56 cases expected); Nagelkerke's R^2 was 0.070. Variables with significant influence were any resecting gastric surgery ($P = 0.037$, OR = 2.40), stenoses ($P = 0.008$, OR = 2.81), any medical immunosuppression ($P = 0.036$, OR = 1.53), levothyroxine therapy ($P < 0.001$, OR = 2.92) and presence of sigmoid diverticulosis ($P = 0.028$, OR = 2.30).

Of the variables "impairment of acid barrier" (PPI therapy and gastrectomy), "impairment of intestinal clearance" (history of any resecting gastric surgery, presence of stenoses, gastroparesis), "impairment of immune response" (any medical suppression) or

Table 5 Miscellaneous variables

	SIBO		Non-SIBO		χ^2 test	OR	RR
	n = 175	%	*n* = 1634	%	*P* value		
Thyroid gland surgery	7	4.0	33	2.0	0.085[2]	2.021	0.505
Hypothyroidism	17	9.7	66	4.0	0.002[1]	2.556	0.416
Hyperthyroidism	1	0.6	24	1.5	0.287	-	-
Levothyroxine use	30	17.1	106	6.5	< 0.001[1]	2.982	0.378
Adipositas	31	17.7	307	18.8	0.410	-	-
Diabetes mellitus	25	14.3	132	8.1	0.006[1]	1.896	0.565
Steatosis hepatis	22	12.6	158	9.7	0.139	-	-
Hepatitis	7	4.0	61	3.7	0.492	-	-
Liver cirrhosis	2	1.1	35	2.1	0.289	-	-
Renal insufficiency	2	1.1	16	1.0	0.532	-	-
Colonic diverticulosis	6	3.4	37	2.3	0.229	-	-
Sigmoid diverticulosis	8	4.6	151	9.2	0.020[1]	0.470	2.021
Crohn's disease	19	10.9	134	8.2	0.146	1.363	0.755
Ulcerative colitis	2	1.1	52	3.2	0.092[2]	0.352	2.785
Alcoholism	3	1.7	36	2.2	0.468	-	-
Smokers	10	5.7	52	3.2	0.070[2]	1.844	0.557
NSAID use	29	16.6	284	17.4	0.442	-	-
Laxative use	3	1.7	58	3.5	0.142	-	-
Antidiarrhoics use	9	5.1	118	7.2	0.195	-	-
Spasmolytics use	9	5.1	257	15.7	< 0.001[1]	0.290	3.058
Antiemetics use	22	12.6	144	8.8	0.071[2]	1.488	0.701
Irritable bowel syndrome	8	4.6	322	19.7	< 0.001[1]	0.195	4.311

[1]Statistical significant with *P*-value $P < 0.05$; [2]Statistical tendency with *P*-value $0.05 < P < 0.1$ χ^2 test each. Patients with hypothyroidism and substitution of levothyroxine show a higher risk of SIBO. In the case of thyroidectomy, statistical significance was not achieved. The presence of diverticulosis of the sigmoid, but not of the entire colon was associated with a decreased risk of SIBO. Patients with Crohn's disease did not exhibit a higher prevalence of SIBO, and ulcerative colitis tended to be protective, but did not reach statistical significance. Patents without SIBO were more likely to use spasmolytics and to suffer from irritable bowel syndrome. SIBO: Small intestinal bacterial overgrowth; NSAID: Non-steroidal anti-inflammatory drugs.

Table 6 Multivariate analysis

Equation variables	Regression coefficient B	Wald	Significance	Exp (B)	95%CI for Exp (B)	
					Lower limit	Upper limit
PPI therapy	0.241	2.015	0.156	1.273	0.912	1.776
Gastrectomy	0.600	0.676	0.411	1.821	0.436	7.604
Any resecting gastric surgery	0.875	4.369	0.037	2.399	1.056	5.450
Stenoses	1.033	7.011	0.008	2.809	1.308	6.033
Gastroparesis	1.016	3.244	0.072	2.762	0.914	8.345
Any colon resection	0.479	3.012	0.083	1.614	0.940	2.772
Any medical immunosuppression	0.428	4.380	0.036	1.534	1.028	2.291
Levothyroxine therapy	1.070	20.980	0.000	2.916	1.845	4.609
Diabetes mellitus	-0.453	3.223	0.073	0.636	0.388	1.042
Sigmoid diverticulosis	0.832	4.835	0.028	2.298	1.095	4.823

Binary logistic regression analysis for risk factors for SIBO. All parameters found to be significant in previous analyses were used for analysis with binary logistic regression. A history of gastric surgery, stenoses, medical immunosuppression, levothyroxine therapy and sigmoid diverticulosis showed statistical significance as contributors to SIBO. SIBO: Small intestinal bacterial overgrowth; Exp: Exponent.

hypothyroidism (levothyroxine supplementation), the latter three were significantly associated with SIBO with odds ratios of 2.2, 1.6 and 3.0, respectively, in a simplified model in which every factor that was significant in the particular logistic regression was summarised (Table 7).

DISCUSSION

The pathogenesis of SIBO and underlying predisposing conditions are insufficiently understood. Several risk factors have been proposed, but most studies refer to one or very few variables in selected populations. Furthermore, no study investigated and ranked the underlying main pathomechanistic pathways. In this retrospective study, we sought to identify, categorise and, finally, rank the influence of potential contributors to SIBO in a large and widely unselected population. Three main pathogenetic pathways have been hypothesised: hypo- or achlorhydria, impaired intestinal clearance and immunosuppression. Further factors of unknown action, such as hypothyroidism, inflammatory bowel disease or sigmoid diverticulosis, were also considered.

Table 7 Binary logistic regression for categorised variables

Equation variables	Regression coefficient B	Wald	Significance	Exp (B)	95%CI for Exp (B)	
					Lower limit	Upper limit
Impaired acid barrier	0.277	2.780	0.095	1.319	0.953	1.827
Impaired clearance	0.772	14.463	0.000[1]	2.164	1.454	3.221
Impaired immune response	0.453	5.161	0.023[1]	1.572	1.064	2.324
Hypothyroidism	1.083	22.514	0.000[1]	2.953	1.888	4.620

[1]Statistically significant with P value < 0.05 in binary logistic regression. Regression analysis for categorised variables: CI: Confidence Interval. Exp (B): Exponent B. A simplified model, which included a leading factor of each pathophysiological pathway, revealed that, in ascending order, impaired immune response, impaired intestinal clearance and hypothyroidism are the key pathways for the development of small intestinal bacterial overgrowth.

Hypo-/achlorhydria

Both PPI therapy and, even more predominantly, gastrectomy were significantly associated with SIBO (Table 2). These findings coincide with results in the literature, although glucose hydrogen breath tests usually failed to find an association between SIBO and PPI therapy[5-7]. Consequently, gastrectomy was a stronger predictor of SIBO in binary regression analysis than PPI therapy (OR 3.6 *vs* 1.5). Moreover, the altered anatomical situation with the establishment of blind loops might play a role in the development of SIBO after gastrectomy, although our data does not confirm blind loops as a pathophysiological factor (Table 3). However, both variables are of minor relevance and lost their significance in multivariate models.

Impaired intestinal clearance

Anatomic modifications which might lead to impairment of intestinal clearance derive mostly from surgery, but, similar to a smaller retrospective study by Petrone *et al*[8], overall abdominal surgery was not associated with an increased risk of SIBO, neither were sigmoid nor ileocecal resection nor obstetric surgery (Table 3). Our data, therefore, does not confirm the previously assumed protective function of the ileocecal valve[10]. By contrast, total gastrectomy, any resecting gastric surgery and stenoses which lead to stasis of the chymus contributed to the development of SIBO with odds ratios between 1.9 and 3.6. Since blind loops of any reason were astonishingly not associated to SIBO, the combination of intestinal diversion and loss of acid barrier is of greater relevance.

Since small intestinal motility is difficult to quantify as a promoter of clearance, this effect might be estimated by measuring gastroparesis as a surrogate parameter. This association has already been described in other studies[14,15]. Our data shows that conditions believed to be associated with intestinal paralysis, such as polyneuropathy, are indeed associated with gastroparesis ($P < 0.001$, χ^2 test). Both are linked to SIBO, but multivariate analysis reveals that gastroparesis is the better parameter (Tables 3 and 6), by which it is easier to quantify than intestinal motility itself.

Any resecting gastric surgery, stenoses and gastroparesis were associated with SIBO in binary regression analysis, and the particular OR varied between 1.8 and 6.5, with the highest value for overall gastric surgery.

Immunosuppression

Data about the role of the immune system is scarce, biased and contradictory. Higher bacterial loads of jejunal aspirates have been shown in ten paediatric patients with IgA deficiency and seven with other immune syndromes[17]. Therapy with immunosuppressives in CD did not increase the occurrence of SIBO[18,19], while steroids did so in a more unselected cohort by lowered levels of IgA, as hypothesised by the authors[20]. Since neither IgA nor IgG deficiency were associated to SIBO in our analysis, we conclude that IgA deficiency does not - either directly or indirectly triggered by steroids - contribute to SIBO in adult patients. On the other hand, therapy with classical immunosuppressants, such as azathioprine or methotrexate, with steroids alone or in combination with immunosuppressants leads to a higher risk of SIBO (Table 4), while other anti-inflammatory drugs, such as 5-aminosalicylates, that usually serve as reference did not change the risk, suggesting that immunosuppression is the underlying mechanism. The association was quite loose, with odds ratios between 1.6 and 2.5, but was still found to be a minor contributor to SIBO in the multivariate analysis therapy with any immunosuppressant drug, *i.e.*, azathioprine, methotrexate or steroids.

Thyroid gland metabolism

Hypothyroidism and levothyroxine therapy are the most strongly associated to SIBO in our cohort. A case control study by Lauritano *et al*[27] has already revealed a high prevalence of SIBO in patients with autoimmune thyroiditis and hypothyroidism, but the influence of the autoimmune process was a questionable biasing factor. Multivariate analysis confirmed that levothyroxine therapy is a stronger predictor of SIBO than hypothyroidism. The underlying mechanism is unclear. One might speculate that hypothyroidism leads to hypomotility, but, surprisingly, levothyroxine therapy was even more associated to SIBO and not able to reverse the effect of hypothyroidism.

Miscellaneous variables

Although SIBO was more frequent in patients with

cholecystectomy in one study[29], our data propose that cholecystectomy is a protective factor. This finding is supported by another retrospective study[21]. We assume that the prolonged contact time and the optimised environment due to constant excretion of the bile allow its antimicrobial effects to better develop as a potential underlying mechanism.

Though reported as a slight risk factor for the development[20] and recurrence[2] of SIBO, in our cohort, appendectomy was prevalent more often in non-SIBO patients (8.2% *vs* 4.6%, $P = 0.027$, χ^2 test), indicating that it is a protective factor with an OR of 0.455. The potential underlying mechanism is unclear, but an effect on other bowel diseases, especially ulcerative colitis, has already been shown[30].

As in previous studies[11], diabetes was associated with SIBO, but lost its effect in multivariate analysis (Tables 5 and 6), proposing that diabetes does not contribute directly, but as a consequence of complications such as neuropathy, which is supported by a strong association between diabetes and gastroparesis ($P < 0.001$, χ^2 test).

Weighting of the particular etiological pathways

Binary logistic regression reveals the history of any resecting gastric surgery, stenoses, medical immunosuppression, levothyroxine supplementation or presence of sigmoid diverticulosis to be associated to a higher risk of SIBO (Table 6). In this model, a reduced gastric acid barrier lost its significance as a pathogenetic factor. The highest odds ratios were seen for factors that, similar to stenoses, lead to impaired clearance, on the one hand, and supplementation of levothyroxine, on the other hand (Table 6). In this model, several independent factors of impaired clearance, but only one of immunosuppression and thyroid gland metabolism were confirmed.

In a simplified model in which any impairment of the acid barrier, of intestinal clearance, or of immune response and hypothyroidism were analysed, the impaired acid barrier again lost its significance (Table 7). Hypothyroidism or, respectively, levothyroxine therapy was most the important single factor with an odds ratio of 3.0; the second most important was impaired intestinal clearance (OR = 2.2) and the third most important was impaired immune response (OR = 1.6).

Limitations

Due to selective information on outpatients, our study population consists mostly of inpatients, which might have led to a certain bias. Furthermore, the retrospective study design itself is associated with certain limitations, such as primary lack or secondary loss of information, uncertainty of adherence to protocols or unequal power between groups. We, therefore, included only a subset of possible contributors that were available for retrospective analysis. Diagnosis of SIBO referred to clinical work-up and is mainly based on the hydrogen breath test. That approach covers advantages such as independence from jejunal cultures, which is neither well standardised nor able to culture all respective flora, but also disadvantages such as unclear reliability. Finally, not every potential contributor who underwent clinical work-up was carefully ruled out in every subject. Therefore, some suspected contributors, such as gastroparesis or polyneuropathy, might be underreported.

To the best of our knowledge, this is the largest study that investigated contributors to SIBO in a widely spread population. The results confirm that impairment of acid barrier, impaired intestinal clearance, immunosuppression and especially levothyroxine therapy/hypothyroidism to be important pathomechanistic pathways for the development of SIBO. The strongest effects derive from levothyroxine supplementation/hypothyroidism, although the relevant mechanism of action remains unclear, and intestinal stasis.

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COMMENTS

Background

Small intestinal bacterial overgrowth (SIBO) is characterised by an excessive colonisation of the small bowel. Pathogenesis is incompletely understood, but recurrence rates after successful antibiotic treatment are high. Several risk factors that contribute to the development have been proposed, and some potential contributors have been shown in mostly small studies with selected populations.

Research frontiers

SIBO is defined as an increase in the number of bacteria in the upper gastrointestinal tract. Related symptoms are nonspecific and include bloating, abdominal pain or diarrhea, and thus overlap widely with symptoms of irritable bowel syndrome. Diagnosis of SIBO still is a challenging clinical problem. Several methods are available, but up to now, a standard of choice is not yet defined. Furthermore, the pathogenesis of SIBO is incompletely understood and studies are needed to identify etiology and pathogenesis. Therapy of SIBO is unsatisfactory; empiric and often broad-spectrum antibiotic treatment fails frequently and suffers from high recurrence rates.

Innovations and breakthroughs

The aim of the study was to identify a set of risk factors, weight and rank them on a pathophysiological basis in a large and widely unselected population. Potential contributors were summarised under four different pathophysiological pathways. Impairment of the gastric acid barrier (gastrectomy, odds ratio: OR = 3.5, PPI therapy OR = 1.4), impairment of intestinal clearance (any resecting gastric surgery OR = 2.6, any colonic resection OR = 1.9, stenosis OR = 3.4, gastroparesis OR = 3.4, neuropathy 2.2), immunological factors (any drug-induced immunosuppression OR = 1.8), altered thyroid gland

metabolism (hypothyroidism OR = 2.6, levothyroxine therapy OR = 3.0) and diabetes mellitus (OR = 1.9) were associated significantly to SIBO. Any abdominal surgery, ileocecal resection, vagotomy or IgA-deficiency did not have any influence, and a history of appendectomy decreased the risk of SIBO. Multivariate analysis revealed gastric surgery, stenoses, medical immunosuppression and levothyroxine to be the strongest predictors. Levothyroxine therapy was the strongest contributor in a simplified model (OR = 3.0).

Applications

The most important contributors for the development of SIBO in ascending order are immunosuppression, impairment of intestinal clearance and levothyroxine use, but they do not sufficiently explain its emergence. The knowledge of respective contributors potentially enables to intervene in the pathogenesis and therefore minimise the risk of recurrence.

Terminology

SIBO is characterised by an increase of bacterial colonisation of the small bowel.

Peer-review

It is an interesting and well performed study.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Preoperative albumin level is a marker of alveolar echinococcosis recurrence after hepatectomy

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Abstract

AIM

To identify a preoperative blood marker predictive of alveolar echinococcosis (AE) recurrence after hepatectomy.

METHODS

All consecutive patients who underwent operation for liver AE at the Lausanne University Hospital (CHUV) between January 1992 and December 2015 were included in this retrospective study. Preoperative laboratory values of leukocytes, mean corpuscular volume (MCV), red blood cell distribution width (RDW), thrombocytes, C-reactive protein (CRP) and albumin were collected and analyzed. Univariate and multivariate Cox regression analyses were performed to determine the risk factors for AE recurrence after liver resection. A receiver operating characteristic (ROC) curve was used to define the best discrimination threshold of the blood marker. Moreover, recurrence-free survival curves were calculated using the Kaplan-Meier method.

RESULTS

The cohort included 68 adult patients (37 females) with median age of 61 years [interquartile range (IQR): 46-71]. Eight of the patients (12%) presented a recurrence over a median follow-up time of 76 mo (IQR: 34-128). Median time to recurrence was 10 mo (IQR: 6-11). Median preoperative leukocyte, MCV, RDW,

thrombocyte and CRP levels were similar between recurrent and non-recurrent cases. Median preoperative albumin level was 43 g/L (IQR: 41-45) for non-recurrent cases and 36 g/L (IQR: 33-42) for recurrent cases (P = 0.005). The area under the ROC curve for preoperative albumin level to predict recurrence was 0.840 (95%CI: 0.642-1, P = 0.002). The cut-off albumin level value was 37.5 g/L for sensitivity of 94.5% and specificity of 75%. In multivariate analysis, preoperative albumin and surgical resection margins were independent predictors of AE recurrence (HR = 0.099, P = 0.007 and HR = 0.182, P = 0.045 respectively).

CONCLUSION

Low preoperative albumin level was associated with AE recurrence in the present cohort. Thus, preoperative albumin may be a useful biomarker to guide follow-up.

Key words: Liver surgery; Alveolar echinococcosis; Albumin; Predictive marker; Recurrence

Core tip: This study assessed different blood markers as potential preoperative predictors of recurrence of alveolar echinococcosis (AE) after liver resection. A preoperative serum albumin level of < 37.5 g/L was found to be associated with AE recurrence after liver resection. Preoperative albumin level is easy to obtain and could represent a useful tool to guide postoperative follow-up after hepatectomy for AE.

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INTRODUCTION

Liver alveolar echinococcosis (AE) is a rare parasitic infection caused by *Echinococcus multilocularis*[1]. As this infection behaves like a malignant tumor, surgical resection is the treatment of choice when total ablation of the parasitic lesions can be achieved[2]. Even in cases of surgical liver resection with negative histological margins (R0), 2%-5% of the patients will present with recurrence during lifetime[3].

As the global incidence of AE is low and AE is endemic only in certain parts of the world[4], no specific risk factors for recurrence after hepatectomy have been described yet. Furthermore, no preoperative blood markers predictive of AE recurrence have been reported.

Predictive blood markers should be easy to measure and interpret for clinical use[5,6]. Typical markers of inflammation, including leukocytes, thrombocytes and C-reactive protein (CRP), represent such candidate blood markers. Mean corpuscular volume (MCV) and red blood cell distribution width (RDW) are included as well, based upon findings from several recent studies that have shown their levels to be correlated with liver inflammation[7,8]. Serum albumin has also been assessed following new data from liver surgery that have indicated a correlation with inflammation, peri-operative stress and postoperative complications[9-11].

The aim of the present study was to assess potential preoperative biological blood markers to predict recurrence in patients operated for liver AE.

MATERIALS AND METHODS

Patients and data collection

Medical records of all consecutive patients with liver AE who were operated on in the Department of Visceral Surgery, University Hospital CHUV, Lausanne, Switzerland, between January 1992 and December 2015, were retrospectively reviewed for data on demographics, peri-operative details and postoperative outcomes. Recurrent cases were chosen for the analysis. The study was recorded in the Research Registry (UIN: researchregistry1033) and approved by the local ethics committee. Informed consent was obtained from each participant included in the study. The study protocol conforms to the provisions of the Declaration of Helsinki.

Diagnostic methods

All patients underwent thoraco-abdominal computed tomography (CT) scan to assess hepatic involvement and presence of extrahepatic lesions. In case of CT-suspected AE, serology using western blotting and enzyme-linked immunosorbent assay, and liver magnetic resonance imaging (MRI) were performed. In case of preoperative jaundice, percutaneous or endoscopic biliary drainage was placed. If the estimated future remnant liver was < 30% of total liver volume, preoperative portal vein embolization was systematically performed. AE lesions were staged according to the PNM classification of the World Health Organization (WHO)[12]. All patients with liver AE or high suspicion of AE, independent of the lesion size, were candidates for hepatectomy. Anatomical resections were performed. Contra-indications to surgery were Child-Pugh C cirrhosis, comorbidities precluding general anesthesia, or estimated future remnant liver < 30% of total liver volume after portal vein embolization. No surgical strategy change occurred during the study period.

Peri-operative outcomes

Major hepatectomy was defined as liver resection of ⩾ 3 Couinaud's segments, whereas minor hepatectomy consisted of resection of 1 or 2 segments. Postoperative

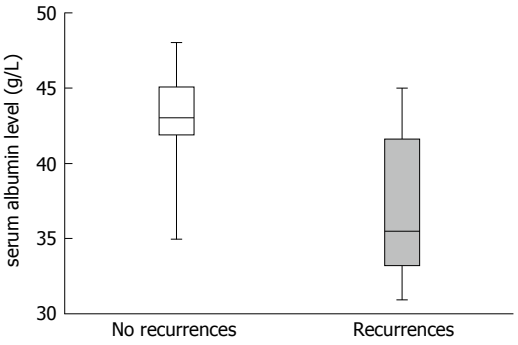

Figure 1 Box-and-whisker plot of preoperative serum albumin level in patients with and without alveolar echinococcosis recurrence (*P* = 0.005).

complications were graded according to the Clavien classification[13]. R0 resection was defined as negative histological margins (> 1 mm) and R1 resection as positive histological margins. During the study period, 3 senior surgeons specialized in AE performed all liver resections.

Blood tests

Preoperative laboratory values of leukocytes, MCV, RDW, thrombocytes, CRP and albumin were determined at the time of hospital admission. These blood tests were performed the day before the operation for all patients.

Follow-up and recurrence

Benzimidazole was given for 2 years after R0 resections and lifelong after R1 resections, according to WHO guidelines[14]. Patients were seen at the outpatient clinic 1 mo postoperatively. They were also seen twice by the surgeon in a clinical encounter at 6 mo and 1 year after the operation. Moreover, patients had regular (monthly) appointments with the infectiologist for follow-up of the albendazole blood levels and standard serologies. In case of clinical recurrence suspicion or positive serologies, complementary imaging (CT/MRI) was performed. Recurrence was defined as the appearance of new intrahepatic or extrahepatic disease after R0 resection or intrahepatic disease progression after R1 resection.

Statistical analysis

For continuous variables, a Mann-Whitney *U* test or Student's *t*-test was used depending on the normality of the distribution and homogeneity of the variances. Survival curves were calculated using the Kaplan-Meier method, and the log-rank test was used to compare survivals. To assess the discriminative power of the marker, a receiver operating characteristic (ROC) curve analysis was performed. $P < 0.05$ was considered significant. Univariate and multivariate analyses were performed using Cox regressions. Only those factors with $P < 0.1$ (10%) on univariate analysis were included in the multivariate analysis. All statistical analyses were performed using GraphPad Prism 5.0© for Mac OS X and SPSS 19.0© for Mac OS X. The statistical methods of this study were reviewed by Dr. Jocelyn Bellier from the University Hospital CHUV, Lausanne, Switzerland.

RESULTS

Patient characteristics and peri-operative outcomes

During the study period, a total of 68 patients (31 men, 37 women) were diagnosed with AE and underwent liver resection. Between 1992 and 2003, major hepatectomies were performed in 12 patients and minor hepatectomies in 10 patients. Between 2004 and 2015, 27 patients underwent major hepatectomies and 19 patients minor hepatectomies. The median age was 61 years [interquartile range (IQR) 46-71]. Eight patients also had additional extrahepatic lesions. Minor hepatectomy was performed in 29 patients (43%) and major hepatectomy in 39 (57%). Postoperative complications occurred in 23 patients (34%). Minor (grade Ⅰ-Ⅱ) and major (grade Ⅲ-Ⅳ) complications were observed in 16 (24%) and 7 patients (10%) respectively. One death (grade Ⅴ) occurred during the postoperative period and was due to septic shock. The 90-d mortality was 1/68 (1.5%). The median overall survival (OS) was 69 mo for the entire cohort (IQR: 30-111).

Recurrent cases

Eight of the total 68 patients presented with recurrence over a median follow-up of 76 mo (IQR: 34-128). Median time to recurrence was 10 mo (IQR: 6-11). There were no statistically significant differences between patients with and without recurrence in terms of demographics and preoperative characteristics. For cases with recurrence the median OS was 81 mo (IQR: 34-108), compared to 69 mo (IQR: 30-113, $P = 0.932$) for cases without recurrence. Regarding the treatments of the 8 patients with recurrence, 4 underwent a repeat hepatectomy, 1 a palliative biliodigestive bypass, 1 a thoracic wedge resection, and 2 pursued long-term medical treatment with albendazole.

Preoperative blood tests

For recurrent and non-recurrent cases, the median laboratory values were: leukocytes: 8.1 G/L (IQR: 4-9) *vs* 6 G/L (IQR: 5-8, $P = 0.554$); MCV: 88 fl (IQR: 88-92) *vs* 89 fl (IQR: 86-92, $P = 0.851$); RDW: 14% (IQR: 13-15) *vs* 14% (IQR: 13-15, $P = 0.979$); thrombocytes: 249 G/L (IQR: 192-266) *vs* 244 G/L (IQR: 215-302, $P = 0.627$); CRP: 18 mg/L (IQR: 8-41) *vs* 4 mg/L (IQR: 2-26, $P = 0.118$); albumin: 36 g/L (IQR: 33-42) *vs* 43 g/L (IQR: 41-45, $P = 0.005$) (Figure 1). The area under the ROC curve for preoperative albumin predicting recurrence (Figure 2) was 0.840 (95%CI: 0.642-1, $P = 0.002$). The cut-off albumin level for a sensitivity of 94.5%, a specificity of 75%, a positive predictive value of 60%, a negative predictive value of 96% and a likelihood ratio of 3.7 was 37.5 g/L.

Table 1 Univariate and multivariate analyses of potential risk factors for alveolar echinococcosis recurrence (Cox regressions)

	Univariate			Multivariate		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Leukocytes > 9 g/L	1.572	0.171-14.454	0.690			
MCV > 90 fl	0.298	0.034-2.647	0.278			
RDW > 14 %	0.444	0.080-2.461	0.353			
Thrombocytes > 300 g/L	0.044	0.001-68'792	0.692			
CRP > 20 mg/mL	1.061	0.171-6.567	0.949			
Hemoglobin > 130 g/L	0.789	0.132-4.738	0.796			
Bilirubin > 10 μmol/L	0.242	0.040-1.472	0.124			
Albumin > 37.5 g/L	0.078	0.015-0.415	0.003	0.099	0.018-0.530	0.007
R0 resection	0.137	0.027-0.711	0.018	0.182	0.034-0.962	0.045
Lesion size > 5 cm	3.342	0.385-28.998	0.274			
PV invasion[1]	3.564	0.769-16.511	0.104			

[1]Defined as presence of microscopic invasion of the PV by alveolar echinococcosis on histopathological reports. CRP: C-reactive protein; MCV: Mean corpuscular volume; PV: Portal vein; RDW: Red blood cell distribution width.

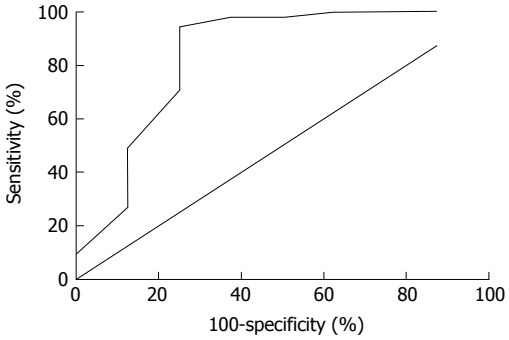

Figure 2 Receiver operating characteristic curve for preoperative serum albumin level to predict recurrence of alveolar echinococcosis (AUC = 0.840, 95%CI: 0.642-1, *P* = 0.002).

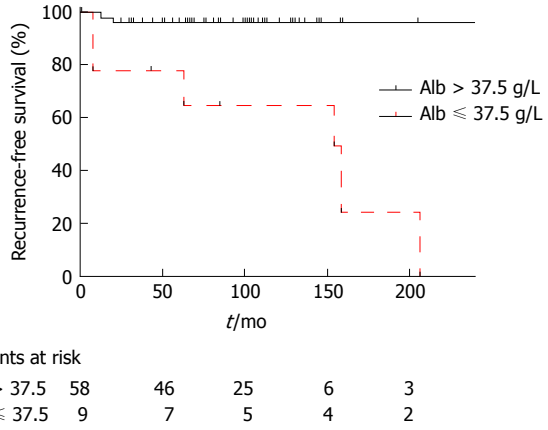

Figure 3 Kaplan-Meier curves of recurrence-free survival (log-rank test: *P* = 0.0004). Alb: Albumin.

Recurrence-free survival was better for patients with preoperative serum albumin level > 37.5 g/L (log-rank test: *P* = 0.0004; Figure 3).

Multivariate analysis

Table 1 summarizes the univariate and multivariate Cox regressions for the preoperative values, surgical resection margins, lesion size and portal vein invasion. Only serum albumin level and surgical resection margins showed significance in the univariate analysis (HR = 0.078, *P* = 0.003 and HR = 0.137, *P* = 0.018); these two variables retained significance in the multivariate analysis (Table 1). The disease-free survival of patients according to the different included peri-operative items is presented in Table 2.

DISCUSSION

The results of this study suggest that preoperative serum albumin level has a good discriminative power to predict recurrence in patients operated for liver AE. No predictive markers of recurrence after hepatectomy for AE have been reported in the literature to date. The present study identified low preoperative serum albumin as associated with AE recurrence. A recent study by Wang *et al*[15] showed that a low level of CD44 proteins in AE resected liver was associated with the development of AE metastasis; however, in the multivariate analysis, a low level of CD44 proteins did not correlate with OS. The level of CD44 proteins in a pathological specimen is not easily assessed, and immunohistochemical staining of a resected liver sample is necessary. On the contrary, serum albumin can be determined easily in the preoperative period. Moreover, as a preoperative test, serum albumin can provide important information regarding management and follow-up. Indeed, in the case of preoperative hypoalbuminemia, follow-up can be adapted and tailored (*e.g.*, closer follow-up visits with serologies or regular postoperative imaging examinations) since the patient will be at higher risk for recurrence.

Recently, albumin has been shown to be linked to peri-operative stress[9]. Several studies of liver surgery and other major surgeries have shown that a drop in albumin level correlates with postoperative complications[10]. Moreover, preoperative serum albumin level has been characterized as an independent factor of recurrence and OS after resection of hepatocellular carcinoma[16] and of OS in patients with hilar cholangiocarcinoma[17]. The tumor-like behavior of AE

Table 2 Disease-free survival of patients according to various peri-operative criteria

	Median DFS, mo (IQR)	*P* value
Leukocytes > 9 g/L	65 (6-81)	0.545
Leukocytes ≤ 9 g/L	58 (23-107)	
MCV > 90 fl	69 (29-121)	0.116
MCV ≤ 90 fl	58 (15-92)	
RDW > 14%	68 (28-117)	0.154
RDW ≤ 14%	56 (8-81)	
Thrombocytes > 300 g/L	68 (30-128)	0.510
Thrombocytes ≤ 300 g/L	58 (18-102)	
CRP > 20 mg/mL	68 (13-105)	0.325
CRP ≤ 20 mg/mL	50 (9-74)	
Hemoglobin > 130 g/L	65 (25-111)	0.440
Hemoglobin ≤ 130 g/L	41 (13-107)	
Bilirubin > 10 μmol/L	60 (12-129)	0.488
Bilirubin ≤ 10 μmol/L	44 (25-81)	
Albumin > 37.5 g/L	56 (26-79)	0.048
Albumin ≤ 37.5 g/L	25 (13-57)	
R0 resection	69 (27-129)	0.024
R1 resection	41 (12-92)	
Lesion size > 5 cm	65 (8-102)	0.733
Lesion size ≤ 5 cm	50 (25-114)	
PV invasion[1]	56 (7-130)	0.933
No PV invasion	60 (25-105)	

[1]Defined as presence of microscopic invasion of the PV by alveolar echinococcosis on histopathological reports. CRP: C-reactive protein; DFS: Disease-free survival; IQR: Interquartile range; MCV: Mean corpuscular volume; PV: Portal vein; RDW: Red blood cell distribution width.

is probably more similar to a cancer such as cholangiocarcinoma than to those cancers frequently related to underlying liver diseases, such as hepatocellular carcinoma which commonly presents with hepatitis and/or cirrhosis.

The findings of the present study suggest that the inflammatory state that accompanies AE likely plays a role in the production of albumin in the liver. It can be postulated that the hepatic parasitic load of *Echinococcus multilocularis* disturbs the standard albumin production by the hepatocytes. Therefore, albumin level could predict the risk of AE recurrence as a reflection of the inflammatory state of the liver. Moreover, intrahepatic dissemination of the parasites could decrease albumin synthesis, but these explanations are speculative. The underlying pathological mechanisms still need to be investigated. In the present study's cohort, no patient received intravenous albumin supplementation, even in cases of preoperative hypoalbuminemia. If surgeons routinely use albumin supplementation preoperatively, it can be suggested that the albumin level that would correlate to the recurrence risk would be the serum albumin level before supplementation. As no data exist on albumin supplementation and AE, further studies need to be performed to clarify the role of albumin supplementation preoperatively for patients with AE.

A surgical resection with positive histological margins was also identified as an independent risk factor for AE recurrence in the present study. This finding confirms the importance of a complete resection of the parasitic lesion in order to reduce the risk of recurrence. Although, the best distance for achieving a safe surgical resection margin is not clearly established and remains controversial. The WHO expert consensus has recommended a margin of 2 cm[12]. A previous paper from our group showed that the margin distance recommended for hepatocellular carcinoma (*i.e.*, 1 mm) reached the same long-term postoperative outcomes[3]. The resection surgical margin distance needs to be further studied to confirm the safety of a margin defined as < 2 cm.

The present study has several limitations that must be addressed. First, its retrospective nature has inherent biases related to chart review and missing data. Second, the number of patients was relatively small. However, the rarity of the disease and the low incidence of recurrence have to be taken into consideration. The patient cohort involved in the present study represents one of the largest series of patients operated for AE in the literature. Finally, the long study period can induce a historical bias due to improvement of experience and expertise of the team over the years.

In conclusion, low preoperative serum albumin may be a predictive factor for AE recurrence after primary hepatectomy. This easily obtainable blood marker could help in guiding postoperative follow-up of AE patients and should be a recommended preoperative test.

COMMENTS

Background

Alveolar echinococcosis (AE) is a rare parasitic infection, primarily involving the liver. As AE behaves like a malignant tumor (liver parenchyma invasion), surgical resection is the treatment of choice to remove all lesions. The recurrence rate after surgical resection is around 5%. No predictive blood markers for AE recurrence after hepatectomy have been described in the literature to date.

Research frontiers

New studies on AE have aimed to describe tailoring of the features of postoperative follow-up for AE patients [*e.g.*, length of albendazole treatment, feasibility of positron emission tomography-computed tomography (PET-CT) scan, or predictive markers of recurrence].

Innovations and breakthroughs

Recent articles on AE have evaluated the feasibility of postoperative PET-CT scan for the follow-up of operated AE patients. No preoperative markers of AE recurrence have yet been described in the literature.

Applications

The results of this study can be used in daily clinical practice. Indeed, dosing of preoperative serum albumin level in patients with liver AE could help in guiding the postoperative follow-up since patients with low albumin level are at higher risk for AE recurrence.

Terminology

AE is a parasitic infection that can disseminate within the liver or give rise to distant extrahepatic lesions. *Echinococcus multilocularis* is the parasitic agent causing AE.

Peer-review

This is a single-center retrospective study of 68 patients who underwent liver resection for AE. The authors found that preoperative albumin level is a

significant risk factor for recurrence of AE. This is an interesting result from a highly experienced European center for AE.

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ORIGINAL ARTICLE

Retrospective Study

Magnetic resonance elastography is accurate in detecting advanced fibrosis in autoimmune hepatitis

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Abstract

AIM

To assess the value of magnetic resonance elastography (MRE) in detecting advanced fibrosis/cirrhosis in autoimmune hepatitis (AIH).

METHODS

In this retrospective study, 36 patients (19 treated and 17 untreated) with histologically confirmed AIH and liver biopsy performed within 3 mo of MRE were identified at a tertiary care referral center. Liver stiffness (LS) with MRE was calculated by a radiologist, and inflammation grade and fibrosis stage in liver biopsy was assessed by a pathologist in a blinded fashion. Two radiologists

evaluated morphological features of cirrhosis on conventional magnetic resonance imaging (MRI). Accuracy of MRE was compared to laboratory markers and MRI for detection of advanced fibrosis/cirrhosis.

RESULTS

Liver fibrosis stages of 0, 1, 2, 3 and 4 were present in 4, 6, 7, 6 and 13 patients respectively. There were no significant differences in distribution of fibrosis stage and inflammation grade between treated and untreated patient groups. LS with MRE demonstrated stronger correlation with liver fibrosis stage in comparison to laboratory markers for chronic liver disease (r = 0.88 *vs* -0.48-0.70). A trend of decreased mean LS in treated patients compared to untreated patients was observed (3.7 kPa *vs* 3.84 kPa) but was not statistically significant. MRE had an accuracy/sensitivity/specificity/positive predictive value/negative predictive value of 0.97/90%/100%/100%/90% and 0.98/92.3%/96%/92.3%/96% for detection of advanced fibrosis and cirrhosis, respectively. The performance of MRE was significantly better than laboratory tests for detection of advanced fibrosis (0.97 *vs* 0.53-0.80, P < 0.01), and cirrhosis (0.98 *vs* 0.58-0.80, P < 0.01) and better than conventional MRI for diagnosis of cirrhosis (0.98 *vs* 0.78, P = 0.002).

CONCLUSION

MRE is a promising modality for detection of advanced fibrosis and cirrhosis in patients with AIH with superior diagnostic accuracy compared to laboratory assessment and MRI.

Key words: Autoimmune hepatitis; Advanced fibrosis; Magnetic resonance elastography; Liver stiffness; Cirrhosis

Core tip: Magnetic resonance elastography (MRE) provides a non-invasive imaging-based biomarker with excellent diagnostic accuracy for detecting advanced fibrosis and cirrhosis in patients with autoimmune hepatitis (AIH). The diagnostic performance of MRE is superior compared to conventional laboratory tests and morphology assessment with conventional magnetic resonance imaging. MRE may have utility in assessing disease progression during therapy, anticipating complications of cirrhosis, and evaluation of the risk of hepatocellular carcinoma in patients with AIH.

Wang J, Malik N, Yin M, Smyrk TC, Czaja AJ, Ehman RL, Venkatesh SK. Magnetic resonance elastography is accurate in detecting advanced fibrosis in autoimmune hepatitis. *World J Gastroenterol* 2017; 23(5): 859-868 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i5/859.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i5.859

INTRODUCTION

Autoimmune hepatitis (AIH) is a chronic inflammatory liver disease which can progress to advanced fibrosis and cirrhosis[1,2]. Hepatic fibrosis scores increase in 25% of patients despite corticosteroid therapy[3]. Cirrhosis develops in 3% of treated patients per year[4], and 1%-6% of individuals with cirrhosis develop hepatocellular carcinoma (HCC)[5,6]. The prevention and reversal of hepatic fibrosis are key objectives in AIH, and the safe and reliable assessment of hepatic fibrosis is essential[7].

Histological evaluation is the gold standard for assessing hepatic fibrosis, but is suboptimal for monitoring disease progression due to its invasiveness, sampling error, and inter-observer variation[8-10]. Noninvasive tests of hepatic fibrosis include laboratory and radiological tests, which have been validated in chronic viral hepatitis, but have not been rigorously assessed in AIH. Laboratory-based methods for staging liver fibrosis include the FibroTest®[11], the serum aspartate aminotransferase/platelet ratio index (APRI)[12], the Fibrosis 4 (FIB-4) test[13], and the enhanced liver fibrosis test[14]. These tests may detect cirrhosis, but their ability to reflect the stages of fibrosis in AIH is uncertain[15,16].

The radiological tests of hepatic fibrosis include transient elastography by ultrasonography (TE), acoustic radiation force impulse (ARFI) imaging, and magnetic resonance elastography (MRE). TE has had high sensitivity and specificity for advanced stages of fibrosis and cirrhosis in chronic viral hepatitis, but its performance may differ in AIH[17,18]. Serum alanine aminotransferase (ALT) levels greater than twice the normal limit have reduced the accuracy of TE in detecting early stages of fibrosis in chronic hepatitis B, and AIH is characterized by chronic inflammation of fluctuating intensity[19,20]. Acute liver damage, as may occur in AIH, can also increase liver stiffness (LS) to levels suggestive of cirrhosis, only to resolve spontaneously with recovery[21]. Obesity can reduce the accuracy of TE and can be an important consequence of corticosteroid-treated AIH[22,23]. The technical specifications of TE may also limit its utility in patients with ascites[24]. The correlation between LS and acute liver inflammation has expanded the clinical applications of TE to include the diagnosis of acute cellular rejection after liver transplantation[25].

Early studies with TE in AIH have reported that TE is an accurate and reliable non-invasive tool in assessing liver fibrosis in AIH[26,27]. However one study by Hartl *et al*[26] and another case series by Romanque *et al*[28] demonstrated that inflammation impacts the accuracy of TE in evaluation of fibrosis. The same confounding factors that limit TE also affect the performance of ARFI. Although ARFI can differentiate normal from fibrosis secondary to chronic immune-mediated liver

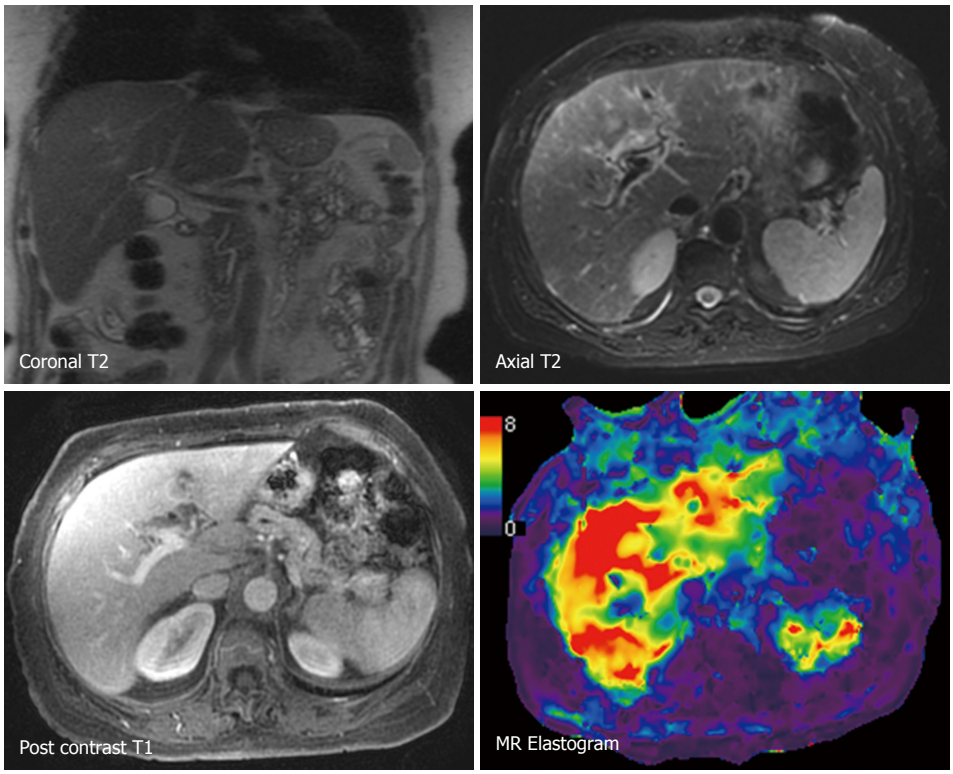

Figure 1 Magnetic resonance elastography in untreated autoimmune hepatitis. An 84-year-old female with grade 4 inflammation and cirrhosis. The liver has normal contour with no morphological features of cirrhosis. Lab tests were: AST 473, ALT 406, APRI 6.26 and FIB-4 10.31. LS was 6.4 kPa consistent with cirrhosis. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; APRI: AST to platelet ratio index; FIB-4: Fibrosis 4 test; LS: Liver stiffness.

disease[29], it has been outperformed by TE in diagnosing early fibrosis and distinguishing normal from fibrosis stage 1[30-32]. The attributes that could support current diagnostic, prognostic, and therapeutic efforts to improve outcomes in AIH may reside in MRE.

MRE (Figure 1) has had excellent performance parameters for all stages of fibrosis in diverse liver diseases[33-37], and it has outperformed TE for staging liver fibrosis in patients with diverse chronic liver diseases[38]. Furthermore, MRE is unaffected by body habitus or hepatic steatosis[39,40] and it can distinguish early from late stages of fibrosis and late stages of fibrosis from cirrhosis in liver diseases outside of AIH. It also may have prognostic implications *via* the assessment of splenic stiffness and the prediction of portal hypertension and esophageal varices[41].

Our goals were to determine the accuracy of MRE in the diagnosis of advanced hepatic fibrosis or cirrhosis in patients with AIH and to compare the findings to those of APRI, FIB-4, and magnetic resonance imaging (MRI).

MATERIALS AND METHODS

Patient selection

This retrospective study was approved by the Institution Review Board and informed consent was waived. We performed a search in the hospital database for patients who underwent MRE between 2007-2015 and had a diagnosis of AIH based on histology and by International AIH Group criteria[42-45]. One hundred and thirty-eight patients met these criteria, of whom 62 were excluded as the interval between liver biopsy and MRE exceeded 3 mo. Another 40 patients were excluded due to overlapping features of another chronic liver disease. The final study group comprised of 36 patients. Of these, 17 patients were treatment-naïve and 19 patients had received immunosuppression treatment either at our institution or elsewhere. The treatment naïve patients had MRE performed within 3 mo of liver biopsy (mean 5 d; range 0 to 42 d). The treated patients had diagnosis of AIH and received treatment for variable period ranging from 1 mo to 25 years with a mean duration of 5.5 years. The time interval between liver biopsy and MRE in this group was 8.2 d (range 0 to 85 d).

Laboratory parameters

Laboratory tests performed within two weeks of MRE were recorded for each patient, and included international normalized ratio (INR), platelet count, serum aspartate amino transferase (AST) and ALT levels, AST/ALT ratio, AST to Platelet Ratio Index (APRI), and FIB-4 score. The APRI was calculated using the equation (AST × 100)/platelet count (10^9/L)[46]. The FIB-4 score as calculated using the equation patient age [(years) × AST (U/L)]/[platelet count (10^9/L) × ALT (U/L)][7,47,48].

Histological assessment

Liver biopsy specimens were reviewed and scored by an experienced hepatopathologist who was blinded to patient data and MRE results. Portal-periportal and lobular inflammation activity grade and fibrosis stage were scored according to Batts *et al*[49]. Fibrosis was staged on a 0-4 scale on Masson Trichome stain. Interface hepatitis was defined as a portal-periportal inflammation score of ⩾ 2. Liver fibrosis stage was scored on a 5-point ordinal scale (0, 1, 2, 3, and 4). All patients with liver biopsy evidence of stage 3 (bridging fibrosis) or stage 4 (cirrhosis) were classified as having advanced fibrosis.

MRE

MRE of the liver was performed according to technique described previously[37]. A pneumatic passive driver was placed overlying the liver which transmitted acoustic vibrations generated at 60 Hz to produce propagating shear waves in the liver which were imaged using a standard MRE sequence as described previously[50]. Four slices were obtained through the largest cross section of the liver in each patient. Total acquisition time was approximately 2 min.

MRE data were processed by an inversion algorithm installed on the scanner to produce stiffness maps and wave images. Regions of interest were drawn by a single experienced abdominal radiologist over the liver and excluded artifacts, vessels > 3 mm in size, liver edges and fissures. LS levels above 2.5 kPa were interpreted as elevated[33].

MRI morphologic features

Two radiologists in consensus evaluated the liver on T2-weighted, T1-weighted, diffusion weighted and post gadolinium enhanced MRI images, and the results required consensus. The following features were assessed: (1) liver parenchyma signal: homogeneous, heterogeneous, patchy/segmental; (2) fatty change; (3) parenchymal enhancement: homogeneous, heterogeneous; (4) surface nodularity: absent, equivocal, present; (5) narrowed hepatic veins: yes/no; (6) presence/absence of the following signs: expanded gall bladder fossa sign, increased hilar periportal space (> 10 mm), hepatic notch sign, creeping mesenteric fat sign; (7) splenomegaly; (8) collaterals; (9) caudate-to-right lobe liver ratio; (10) modified caudate-to-right lobe liver ratio; and (11) ascites. An overall impression of the presence of cirrhosis was entered as absent, equivocal, or present.

Statistical analyses

Statistical analyses were performed using MedCalc for Windows, version 15.0 (MedCalc Software, Ostend, Belgium). Statistical analysis was performed by one author (Venkatesh SK) experienced in using MedCalc statistical software. Summary statistics are presented as mean ± SD for continuous variables and as numbers and percentages for categorical variables. The relationship between MRE and serum tests was evaluated using Pearson's correlation coefficient test. The relationships between serum tests, MRE, inflammation grade, and fibrosis stage were assessed using Spearman's correlation coefficient. Partial correlation analysis was used to evaluate the correlation between fibrosis stage and MRE correcting for inflammation grade. Kruskal-Wallis test was performed on serum tests and MRE to determine significant differences between fibrosis stages.

The overall performance of MRE for the diagnosis of advanced fibrosis and cirrhosis was determined by analyzing the area under the receiver operating characteristic (ROC) curve. Optimal cut-off values with accuracies, sensitivities, specificities, positive and negative predictive values were reported for predicting advanced fibrosis and cirrhosis. The performance parameters of all variables were compared by analyzing ROC curves. A two-tailed *P* value of < 0.05 was considered statistically significant for all analyses.

RESULTS

Clinical features

The study population had mean age of 51.6 ± 20.6 years and mean body mass index (BMI) of 27.8 ± 6.4 kg/m^2. The mean FIB-4 score was significantly lower in the treated group compared to the untreated group (2.72 *vs* 5.99, *P* = 0.025). A trend of higher levels of serum AST and ALT levels at the time of MRE and liver biopsy was found in the untreated group but was not statistically significant. There were no significant differences in BMI, mean LS, APRI, platelet and INR values between two groups (Table 1).

Histology findings

Liver biopsy was performed within 3 mo of MRE study with a mean interval of 11.7 d (95%CI: 2-76 d). Histological evaluation revealed fibrosis stages of 0, 1, 2, 3 and 4 in 4, 6, 7, 6 and 13 patients, respectively. Fibrosis (⩾ F1) was present in 32 patients (88.9%); significant fibrosis (⩾ F2) in 27 patients (75%); advanced fibrosis (⩾ F3) in 19 patients (52.8%) and cirrhosis (F4) in 13 patients (36.1%). Inflammation grade 0, 1, 2, 3, and 4 in 2, 7, 15, 9 and 3 patients respectively. The distribution of fibrosis stage and inflammation grade between treated and untreated patients was similar

Correlations between histological findings and laboratory tests

Spearman rank correlation analysis showed significant correlation between fibrosis stage and all serum tests except AST and ALT levels (Table 2). Both APRI and INR showed significant correlations with inflammation grade. No significant differences in ALT (*P* = 0.68), AST (*P* = 0.25), AST/ALT ratio (*P* = 0.07), and APRI

Table 1 Comparison of untreated and treated patients with autoimmune hepatitis

Characteristic	Untreated group (n = 17)		Treated group (n = 19)		P value
	mean ± SD	95%CI	mean ± SD	95%CI	
Age (yr)	62.9 ± 18.6	53.4-72.5	41.4 ± 16.8	33.30-49.5	0.001
BMI (kg/m^2)	27.2 ± 6.3	24.0-30.5	28.2 ± 6.8	24.9-31.5	0.65
Serum albumin	3.81 ± 0.8	3.39-4.23	4.0 ± 0.43	3.78-4.2	0.4
Serum ALP	117.6 ± 74.7	76.3-159.0	109.4 ± 45.6	85.9-132.9	0.19
Serum ALT	298.8 ± 459.9	62.3-535.3	144.5 ± 217.8	39.5-249.4	0.22
Serum AST	238.2 ± 313.1	77.2-399.1	110.0 ± 144.4	42.6-178.0	0.12
AST/ALT	1.0 ± 0.4	0.8-1.27	0.9 ± 0.41	0.8-1.0	0.38
APRI	2.9 ± 3.47	1.1-4.67	3.2 ± 5.8	0.36-6.0	0.85
FIB-4	5.99 ± 4.94	3.4-8.53	2.7 ± 3.3	1.1-4.3	0.025
Platelet	178.9 ± 78.0	138.8-219	193.3 ± 99.0	145.6-241.0	0.63
INR	1.1 ± 0.2	1.0-1.24	1.14 ± 0.3	1.0-1.3	0.96
Total bilirubin	1.5 ± 2.3	0.3-2.69	1.5 ± 1.9	0.5-2.5	0.96
Gamma globulin	2.3 ± 0.9	1.9-2.9	2.2 ± 0.9	1.6-2.8	0.61
Mean LS (kPa)	4.1 ± 1.6	3.2-4.9	4.5 ± 2.0	3.5-5.4	0.51

ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; APRI: AST to platelet ratio index; BMI: Body mass index; FIB-4: Fibrosis 4 test; INR: International normalization ratio; LS: Liver stiffness.

Table 2 Spearman rank correlation analysis results between variables and histological fibrosis stage and inflammation grade

Test	Fibrosis stage			Inflammation grade		
	Correlation	95%CI	P value	Correlation	95%CI	P value
AST	0.21	-0.13-0.50	0.2236	0.29	-0.043-0.56	0.0870
ALT	0.02	-0.31-0.35	0.8916	0.31	-0.02-0.58	0.0660
APRI	0.44	0.14-0.68	0.0064	0.39	0.07-0.64	0.0184
AST/ALT	0.40	0.08-0.65	0.0143	0.01	-0.32-0.34	0.9432
FIB-4	0.52	0.23-0.72	0.0012	0.24	-0.09-0.53	0.1497
Platelet	-0.48	-0.69--0.18	0.0032	-0.04	-0.37-0.29	0.7972
INR	0.49	0.19-0.71	0.0022	0.36	0.04-0.62	0.0294
Total Bil	0.36	0.030-0.63	0.0338	0.31	-0.03-0.58	0.0784
LS	0.83	0.69-0.91	< 0.0001	0.19	-0.14-0.49	0.2465

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; APRI: AST to platelet ratio index; FIB-4: Fibrosis 4 test; INR: International normalization ratio; LS: Liver stiffness.

(P = 0.09) were found between different stages of fibrosis by the Kruskal-Wallis test. INR values for stage 4 fibrosis were significantly higher than for stage 1 and 2 fibrosis (1.2 *vs* 1.0, P < 0.05). Total bilirubin levels were different between fibrosis stage 2 and 4 (P = 0.049), and platelet counts were significantly higher in fibrosis stage 0 and 2 than in stage 4 and between fibrosis stage 2 and 3. Fib-4 scores were significantly higher for fibrosis stage 4 than stages 0-2.

Correlations between histological findings and radiological tests

MRE correlated closely with fibrosis stage (r = 0.83, P < 0.001), and it performed better than MRI. The correlation between LS and fibrosis stages remained significant after correction for age and BMI (r = 0.75, P < 0.001), inflammation grade (r = 0.76, P < 0.001), and all laboratory tests (r = 0.68, P < 0.0001). LS was significantly higher in fibrosis stage 4 than stage 0-3; similarly stage 3 had significantly higher stiffness than stages 0-2. There were no significant differences in LS between stages 0-2 (Figure 2).

Untreated patients had a slightly higher mean LS as compared to treated patients (3.83 kPa *vs* 3.7 kPa), but this was not statistically significant. This trend was seen at each fibrosis stage (stage 0, 3.1 kPa *vs* 2.61 kPa; stage 1, 2.94 kPa *vs* 2.74 kPa; stage 2, 3.2 kPa *vs* 2.63 kPa; stage 3, 4.1 kPa *vs* 3.99 kPa). The only exception was cirrhotic patients where the treated patients had a higher LS compared to the untreated group (6.5 kPa *vs* 5.9 kPa).

ROC analysis showed that MRE (cut off, 4.1 kPa) predicted advanced fibrosis (≥ stage 3) with 0.97 accuracy (95%CI: 0.85-0.99), 89.5% sensitivity (95%CI: 67%-99%), 100% specificity (95%CI: 80.5%-100%), 100% positive predictive value (PPV, 95%CI: 80.5%-100%), and 89.5% negative predictive value (NPV, 95%CI: 67%-99%) NPV. Similarly, a cut-off of 4.5 kPa predicted cirrhosis with 0.98 accuracy (95%CI: 0.87-1.00), 92.31% sensitivity (95%CI: 85%-99%) and 96% specificity (95%CI: 78%-99.9%), 92.3% PPV (95%CI: 64%-99.8%) and 88% NPV (95%CI: 68.8%-97.5%).

Comparison between radiological tests and laboratory tests

Comparison of ROC curves for MRE and laboratory tests showed that MRE performed significantly better

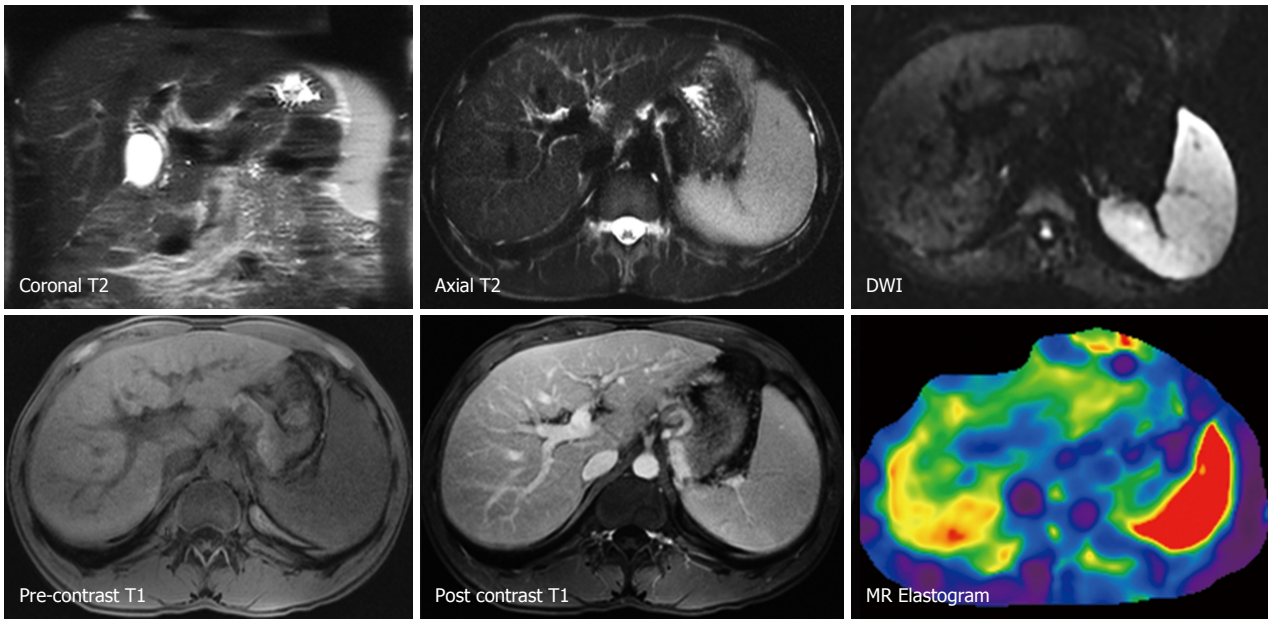

Figure 2 Magnetic resonance elastography in treated autoimmune hepatitis. A 43-year-old male with grade 2 inflammation and advanced fibrosis. MRI images show no features to suggest advanced fibrosis. Note prominent spleen. Lab tests were AST 81, ALT 147, FIB-4 2.95 and APRI 1.98. LS was 5.1 kPa consistent with advanced fibrosis. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; APRI: AST to platelet ratio index; FIB-4: Fibrosis 4 test; LS: Liver stiffness.

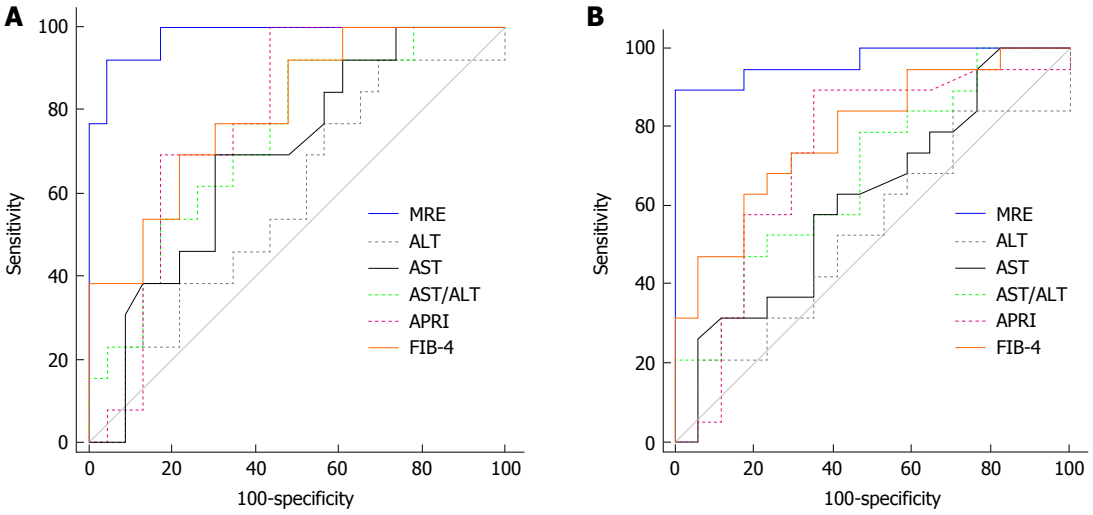

Figure 3 Graph showing area under the receiver operating characteristic curves of magnetic resonance elastography and lab tests for prediction of advanced fibrosis (A) and cirrhosis (B) in autoimmune hepatitis.

than ALT, AST, AST/ALT, APRI, FIB-4, INR and platelet counts for the detection of advanced fibrosis (Table 3, Figure 3A). FIB-4 performed better than AST, ALT and APRI for detecting advanced fibrosis, and all the laboratory tests performed better than the serum ALT level in making this distinction. Similarly for cirrhosis, MRE performed significantly better than all laboratory tests (Table 3, Figure 3B). FIB-4 only performed better than the serum ALT level in detecting cirrhosis, and the serum ALT level was worse than all other laboratory tests in making this distinction. We also analyzed diagnostic performance of MRE and laboratory tests for two study groups. In the untreated group of 17 patients MRE performance was better than laboratory tests for both advanced fibrosis (0.93 *vs* 0.51-0.86) and cirrhosis (0.95 *vs* 0.57-0.95). In the treated group of 19 patients, MRE performance was also better than serum tests for advanced fibrosis (0.98 *vs* 0.59-0.87) and cirrhosis (1.0 *vs* 0.64-0.89).

DISCUSSION

A non-invasive, accurate method of detecting advanced fibrosis and cirrhosis in patients with AIH is required to assess disease progression during therapy, anticipate complications of cirrhosis, and evaluate the risk of HCC. Our study demonstrates high accuracy of MRE in detecting advanced fibrosis and cirrhosis in patients with

Table 3 Area under the receiver operating characteristic curves of magnetic resonance elastography and laboratory tests for prediction of advanced fibrosis and cirrhosis in autoimmune hepatitis

	Advanced fibrosis			Cirrhosis		
	AUC	SE	95%CI	AUC	SE	95%CI
LS	0.966	0.0278	0.845-0.998	0.980	0.0175	0.867-1.000
ALT	0.526	0.0998	0.354-0.695	0.582	0.1010	0.406-0.744
AST	0.618	0.0964	0.441-0.774	0.691	0.0909	0.515-0.834
AST/ALT	0.681	0.0904	0.505-0.826	0.736	0.0860	0.563-0.868
APRI	0.728	0.0932	0.554-0.862	0.776	0.0789	0.606-0.898
FIB_4	0.786	0.0760	0.618-0.905	0.803	0.0750	0.636-0.916
INR	0.770	0.0770	0.596-0.891	0.800	0.0880	0.635-0.915
Platelet	0.802	0.0780	0.636-0.916	0.763	0.0904	0.592-0.888

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; APRI: AST to platelet ratio index; FIB-4: Fibrosis 4 test; INR: International normalization ratio; LS: Liver stiffness.

AIH, and its superiority to laboratory assessment and conventional MRI. Our findings are consistent with other studies that demonstrate greater diagnostic accuracy of MRE over laboratory assessment in detecting advanced fibrosis and cirrhosis in patients with diverse chronic liver diseases[34,38,40,50,51]. Furthermore, our study indicates that the laboratory and histological indices of liver inflammation do not compromise the accuracy of MRE in assessing hepatic fibrosis in AIH.

Untreated patients showed mildly higher LS as compared to treated patients which was not statistically significant, likely related to the presence of inflammation in the untreated group, and the subset of untreated AIH patients did have higher inflammation grades. This finding suggests that hepatic inflammation could have an impact on determinations of LS by MRE, and it was similar to that in patients with chronic viral hepatitis in whom the presence of chronic inflammation has been shown to increase LS by MRE[52]. In our study, there was no significant difference in the distribution of inflammation grades between the treated and untreated groups, and fibrosis stages were detected with similar accuracy in the treated and untreated patients. Our study also showed that cirrhotic livers in treated patients had higher mean stiffness as compared to cirrhotic livers in untreated patients. The exact reason is not known, however it is possible that the fibrosis content in treated patients is likely to be more as the duration of disease was longer in these patients. This needs to be confirmed in studies with a larger number of participants.

Recent studies performed with TE and ARFI in AIH have shown that both techniques are useful in assessment of significant fibrosis and cirrhosis in AIH. In one study with nearly 100 patients, Hartl *et al*[26] showed excellent diagnostic performance of TE for diagnosis of cirrhosis. They also showed that liver inflammation has a major impact on LS in first few months of AIH treatment and its diagnostic performance improves after 6 mo of immunosuppression treatment. In our study we also showed that untreated patients had higher stiffness compared to treated patients. In addition the diagnostic performance of MRE in treated patients was slightly better than that in untreated patients, however the numbers of patients in our study groups are too small to draw conclusions. In another study of only 15 patients, Efe *et al*[53] showed that ARFI is able to accurately differentiate significant fibrosis from non-significant fibrosis. There are no comparison studies between MRE, TE and ARFI and future studies combining all three modalities may be useful for determining their utility in different clinical scenarios.

Our study has limitations. First, the study was retrospective. This was unavoidable as patients frequently received treatment at outside medical centers. This also precludes assessment of the time interval between initial diagnosis and treatment to liver biopsy and MRE. Second, our sample size is small because the timing of liver tissue examinations and the performance of MRE was variable, and overlap syndromes were excluded. Third, the reference standard was histological assessment, which is limited by sampling error and inter-observer variability[8-10]. This was mitigated by applying a standardized scoring system for fibrosis and inflammation, requiring all specimens to be stained for fibrosis, and having each tissue sample re-reviewed by a pathologist specialized in autoimmune liver diseases[54]. Fourth, our study group comprised treated and untreated patients, which was unavoidable due to the rarity of AIH and retrospective nature of the study. Fifth, patients were assessed at varying intervals during the course of their disease, and were not studied sequentially to assess for detection of small gradations of change.

MRE is a non-invasive imaging-based biomarker with superior diagnostic accuracy for detecting advanced fibrosis and cirrhosis in patients with AIH compared to conventional laboratory and MRI assessment. MRE may become useful as a non-invasive tool for staging fibrosis in AIH, evaluating response to treatment, and decision-making regarding drug administration, dose adjustment, and duration of therapy. Our study provides a foundation for future prospective studies that evaluate the role of MRE to detect changes in LS that can be used safely and repeatedly in patients with AIH of all ages, habitus, and disease severity.

COMMENTS

Background

Autoimmune hepatitis (AIH) is a chronic inflammatory liver disease which can progress to advanced fibrosis and cirrhosis. Histological evaluation is the gold standard for assessing hepatic fibrosis, but is suboptimal for monitoring disease progression due to its invasiveness, sampling error, and inter-observer variation. A non-invasive, accurate method of detecting advanced fibrosis and cirrhosis in patients with AIH is required to assess disease progression during therapy, anticipate complications of cirrhosis, and evaluate the risk of hepatocellular carcinoma. Magnetic resonance elastography (MRE) has the potential to fulfill this function.

Research frontiers

MRE is a non-invasive imaging-based biomarker that has far reaching applications in the diagnosis, management, and treatment of patients with AIH.

Innovations and breakthroughs

This study provides a foundation for future prospective studies that evaluate the role of MRE to detect changes in liver stiffness that can be used safely and repeatedly in patients with AIH of all ages, habitus, and disease severity.

Applications

MRE may become useful as a non-invasive tool for staging fibrosis in AIH, evaluating response to treatment, and decision-making regarding drug administration, dose adjustment, and duration of therapy.

Terminology

MRE is a magnetic resonance imaging based technique that non-invasively assesses tissue stiffness.

Peer-review

These findings represent a first effort at defining the role of MRE in the evaluation of AIH. There is robust information supporting the usefulness of this technique in accurately assessing liver fibrosis in other liver diseases, such as hepatitis C, hepatitis B and non-alcoholic fatty liver disease.

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ORIGINAL ARTICLE

Retrospective Study

Risk of alcohol use relapse after liver transplantation for alcoholic liver disease

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Abstract

AIM

To investigate factors, including psychosocial factors, associated with alcoholic use relapse after liver transplantation (LT) for alcoholic liver disease (ALD).

METHODS

The clinical records of 102 patients with ALD who were referred to Nagoya University Hospital for LT between May 2003 and March 2015 were retrospectively evaluated. History of alcohol intake was obtained from their clinical records and scored according to the High-Risk Alcoholism Relapse scale, which includes duration of heavy drinking, types and amount of alcohol usually

consumed, and previous inpatient treatment history for alcoholism. All patients were assessed for eligibility for LT according to comprehensive criteria, including Child-Pugh score, Model for End-Stage Liver Disease score, and psychosocial criteria.

RESULTS

Of the 102 patients with ALD referred for LT, seven (6.9%) underwent LT. One (14.3%) of these seven patients returned to heavy drinking, but that patient was able to successfully quit drinking following an immediate intervention, consisting of psychotherapeutic education and supportive psychotherapy, by a psychiatrist. A comparison between the transplantation/registration (T/R) group, consisting of the seven patients who underwent LT and 10 patients listed for deceased donor LT, and 50 patients who did not undergo LT and were not listed for deceased donor LT (non-T/R group), showed statistically significant differences in duration of abstinence period ($P < 0.01$), duration of heavy drinking ($P < 0.05$), adherence to medical treatment ($P < 0.01$), and declaration of abstinence ($P < 0.05$).

CONCLUSION

Patients with ALD referred for LT require comprehensive evaluation, including evaluation of psychosocial criteria, to prevent alcoholic recidivism.

Key words: Liver transplantation; Risk assessment; Alcoholic liver disease; Psychosocial evaluation criteria; Liaison psychiatry; Alcohol use relapse

Core tip: Although alcoholic liver disease (ALD) is the second most common indication for liver transplantation (LT), post-transplant relapse of alcohol use can have a negative impact on patient outcomes. It is therefore important to preoperatively assess the risk of post-transplant alcohol use. To date, however, psychosocial evaluation criteria of LT for ALD have not been established, indicating a real need for useful criteria to assess the risks of post-transplant alcohol use. This study describes a set of psychosocial evaluation criteria that may be useful in assessing the risk of relapse in patients who undergo LT for ALD.

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INTRODUCTION

Alcoholic liver disease (ALD) is one of the most common causes of advanced liver cirrhosis and has become the second most common indication for liver transplantation (LT), after cirrhosis caused by viral hepatitis[1]. However, 20%-30% of patients who undergo LT for ALD will return to heavy drinking after LT[2]. Post-transplant relapse of alcohol use is extremely crucial, because alcoholic recidivism has a negative impact on post-transplant compliance and long-term outcomes of LT recipients[3]. Post-transplant relapse of alcohol use has been associated with increased damage to transplanted liver allografts[4,5] and may be associated with reduced survival after LT[3,6,7]. Thus, in evaluating candidates for LT, it is crucial to preoperatively predict and precisely assess the risk of post-transplant relapse of alcohol use in patients with ALD[8].

Various criteria and screening procedures have been reported to predict relapsed alcohol use[8]. Most transplant centers worldwide require a minimum of 6 mo of alcohol abstinence prior to LT. Patients who can maintain this 6-mo abstinence have been reported to be at lower risk of alcohol use relapse than those who are abstinent for less than 6 mo[9,10]. However, a method for selecting LT candidates based on the 6-mo rule alone has been criticized, because this method does not account for other factors that may influence alcoholic behavior[5,11,12]. Unlike physical evaluation criteria, psychosocial criteria evaluating the suitability of LT for patients with ALD have not yet been determined, because highly valid and reliable psychosocial criteria are more difficult to establish. There is therefore a real need for useful criteria to assess the risks of post-transplant relapse of alcohol use beforehand. This study was therefore performed to comprehensively investigate factors, including psychosocial factors, associated with alcoholic use relapse after LT for ALD. Based on these findings, we propose and present a set of psychosocial evaluation criteria that may be useful in assessing relapse risk in patients with ALD who are candidates for LT, and provide a framework that can be of use clinically.

MATERIALS AND METHODS

At Nagoya University Hospital, approximately 20 LTs are performed annually. A transplantation medical team, consisting of transplant surgeons, gastroenterologists, hepatologists, psychiatrists, transplant coordinators, and psychologists, was launched in 2004, and the team continues to hold interdisciplinary conferences at least once weekly[13]. Patients were treated by psychiatrists, if necessary. However, in our institution, the psychiatry and self-help groups work in a coordinated manner. Our style is a so-called "team medicine".

Between May 2003 and March 2015, 102 patients with ALD were referred to Nagoya University Hospital for LT. A definitive diagnosis of ALD was based on a history of habitual and excessive alcohol consumption. The clinical records of these patients were retrospectively reviewed. Pre-transplant levels of alcohol consumption

Table 1 Psychosocial evaluation criteria of liver transplantation for alcoholic liver disease

Criteria A
Abstinence period lasting at least 6 mo
An oath of the abstinence from alcoholic drinking for the future
Patients with alcoholic liver disease are needed to fulfill the criteria A.
Criteria B
No presence of psychiatric comorbidity except alcohol-related mental disease
A dherence of medical treatment
Understanding and agreement of transplant and a support by the family
Being at work or ready to work
The high-risk alcoholism relapse scale can be scored 0, 1, or 2
Criteria C
Re-evaluation one month later in case who is difficult to evaluate risk of alcohol use relapse in the initial interview

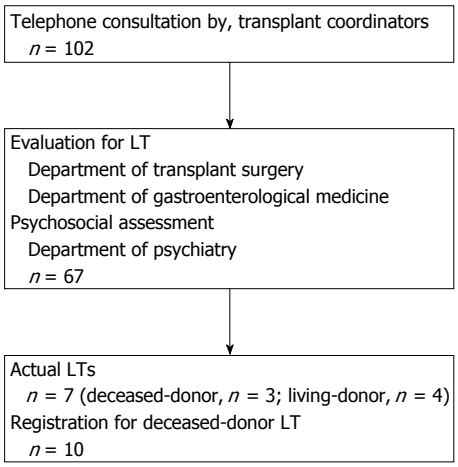

Figure 1 Flowchart of the study. LT: Liver transplantation.

were assessed using the High-Risk Alcoholism Relapse (HRAR) scale, which was developed from a study of relapse following inpatient treatment for alcoholism of a cohort of male US veterans[14]. This scale includes three items: duration of heavy drinking, usual number of drinks per day, and number of previous inpatient admissions for treatment of alcoholism[15,16]. Each item is scored 0, 1, or 2, resulting in total possible scores ranging from 0 to 6; high scores, ranging from 3 to 6, have been found to correlate positively with the risk of relapse.

The psychosocial evaluation criteria for LT candidates with ALD are shown in Table 1. Patients with ALD psychosocially considered likely candidates for LT were those at lower risk of alcohol relapse. Alcohol relapse after LT was based on interviews with patients and/or family members.

Sixty-seven patients with ALD were evaluated medically for LT by members of the Departments of Transplantation Surgery and/or Gastroenterological Medicine, and were evaluated psychosocially by members of the Department of Psychiatry. Medical factors evaluated included hepatic encephalopathy, ascites, serum concentrations of bilirubin and albumin, international normalized ratio of prothrombin time, plasma creatinine concentration, Model for End-stage Liver Disease score and Child-Pugh score. Alcohol-associated criteria included duration of heavy drinking, adherence to medical treatment, employment or willingness to work, understanding and agreeing to LT, support from family members, occurrence of psychiatric comorbidities except for alcohol-related mental disease, usual number of daily drinks, and HRAR score. The mean follow-up period after LT was 5.1 years.

To present the alcohol drinking, we used the unit of a standard drink in Japan contained 10 g of alcohol, in this study.

Statistical analysis

Continuous variables were compared by Student's *t*-tests and categorical variables by Fisher's exact test. A P value < 0.05 was regarded as statistically significant.

Ethical approval

The study protocol was approved by the Ethics Review Committee of Nagoya University Graduate School of Medicine (Approved No. 15), which waived the requirement for informed consent due to the retrospective design of this study. This study was fully supported by a Grant-in-Aid for Scientific Research (C, No. 24591875) from the Japanese Ministry of Education, Culture, Sports, Science and Technology, and by a grant from the Japanese Society for the Promotion of Science.

RESULTS

The 102 ALD patients referred to our center for possible LT were evaluated by telephone interviews with our transplant coordinators. Of these patients, 67 (65.6%) underwent both LT evaluation and psychosocial assessment (Table 2), and seven (6.9%) underwent LT (Table 3 and Figure 1). In addition, 10 patients (9.8%) were registered by the Japan Organ Transplant Network as candidates for deceased donor LT. Of the seven patients who met our criteria and underwent LT, six did not return to alcohol drinking after LT, whereas one did (Patient No. 4 in Table 3). This patient met both our medical and psychosocial criteria. He had no psychiatric comorbidity except for alcohol-related mental disease; he adhered to medical treatment, understood and agreed to undergo LT, had support from his family, was employed, and had a score of 2 on the HRAR scale. Therefore, it was difficult to predict his alcohol relapse preoperatively. Interestingly, however, this patient completely quit alcohol following an immediate intervention by psychiatrists, consisting of psychological education and supportive psychotherapy.

Data from the seven patients who underwent LT and the 10, who were listed for deceased donor LT, defined as the transplantation/registration (T/R) group, were compared with the data of the 50 patients who did not

Table 2 Sociodemographic and clinical characteristics of 67 alcoholic liver disease patients

	Mean	Range or standard deviation			No./No. responded	Percentage
Age[1] (yr)	50.2	28-69	Adherence of medical treatment	Present	45/67	67
Gender[2] (male/female)	48/19			Absent	20/67	30
Hepatic encephalopathy[2] (point)	1.2	0.4		Unknown	2/67	3
Ascites[2] (point)	2.0	0.8	Being at work or ready to work	Present	47/67	70
Bilirubin[2] (mg/dL)	6.1	6.0		Absent	18/67	27
Albumin[2] (g/dL)	2.8	0.5		Unknown	2/67	3
International normalized ratio of prothrombin time[2]	1.83	0.7	Understanding and agreement of transplant and a support by the family	Present	61/67	91
Creatinine[2] (mg/dL)	0.9	0.6		Absent	6/67	9
Model for end-stage liver disease score[2] (point)	1.9	7.0	Presence of psychiatric comorbidity except alcohol-related mental disease	Present	2/67	3
Child-Pugh score[2] (point)	10.1	2.0		Absent	65/67	97
Duration of heavy drinking[2] (yr)	21.7	10.4	Declaration of abstinence	Present	55/67	82
Usual number of daily drinks[2] (L)	2.1	0.8		Absent	12/67	18
The HRAR scale[2] (point)	2.3	1.0	Psychiatric hospitalizations	Present	1/67	1
Prothrombin time[2] (%)	34.3	16.4		Absent	66/67	99
Abstinence period[2] (mo)	12.1	15.8				

[1]Result was shown as mean and range; [2]Results were shown as mean and SD. HRAR: High-Risk Alcoholism Relapse.

Table 3 The characteristics of 7 patients with decompensated liver cirrhosis due to alcoholic liver disease who underwent liver transplantations

Case	Age at the first drinking	Age at the first examination	Age at the LT	Gender	Comorbidity	Daily intake of alcohol[1]	LT	Follow-up (yr)	Alcohol relapse	Self-help groups
1	24	32	36	Male	None	17.6	Deceased-donor	8.3	None	None
2	27	38	38	Female	None	6.5	Deceased-donor	9.9	None	None
3	15	42	44	Male	Non-B Non-C Liver cirrhosis	16.0	Deceased-donor	7.3	None	Participation (spouse only)
4	17	44	46	Male	Liver cirrhosis (type C)	20.0	Deceased-donor	4.8	Relapse[2] (3 yr after)	None
5	13	28	28	Male	None	20.0	Living-donor (relation: father)	3.3	None	Participation
6	17	51	51	Female	Liver cirrhosis (type C) Hepatocellular carcinoma	15.0	Living-donor (relation: daughter)	1.3	None	None
7	9	46	46	Female	None	20.0	Living-donor (relation: younger brother)	0.5	None	None

[1]The unit of a standard drink in Japan contained 10 g of alcohol; [2]The patient quit alcohol with the immediate psychiatric intervention (psychoeducation and supportive psychotherapy) by psychiatrists.

undergo LT and were not listed for deceased donor LT, defined as the non-T/R group (Table 4). The abstinence period was significantly longer ($P < 0.01$), while the duration of heavy drinking was significantly shorter ($P < 0.05$), in the T/R group than in the non-T/R group. In addition, the adherence to medical treatment ($P < 0.01$), and the declaration of abstinence ($P < 0.05$) were better in the T/R group than in the non-T/R group (Table 4).

One thought-provoking case

Although our comprehensive LT criteria for ALD seemed to be effective, we encountered one thought-provoking case. The patient was a 44-year-old man, married at age 23 years and with two children. He inherited a business from his father and was essentially self-employed. Because of overwork, he started drinking heavily at age 30 years. Although his family was aware of his drinking problem, he denied his drinking. He got divorced at age 37 years and lost his son in a traffic accident at age 40 years, after which he began drinking more heavily. At age 41 years, his family doctor warned him that he would die in the near future if he did not stop drinking. Although he stopped drinking immediately, his liver condition worsened and he required LT. Following psychosocial evaluation, he was registered for deceased donor LT. After being on the waiting list for 3 years, he underwent a deceased donor LT. During follow-up after LT, his transplant surgeons suspected that he might have returned to heavy drinking. Because he admitted that he had

Table 4 Statistical results

	T/R group (n = 17) Mean (range or SD)	Non-T/R group (n = 50) Mean (range or SD)	t-statistic	Degree of freedom	Statistical significance			T/R group (n = 17) No/no responded (percentage)		Non-T/R group (n = 50) No/no responded (percentage)		Statistical significance
Age[1] (yr)	45.5 (28-62)	51.8 (31-69)				Adherence of medical treatment	Present	17/17	(100)	28/50	(56)	$P < 0.01$
Gender (male/female)	11/6	37/13					Absent	0/17	(0)	20/50	(40)	
Abstinence period[2] (mo)	21.2 (17.4)	8.8 (13.6)	2.84	59	$P < 0.01$		Unknown	0/17	(0)	2/50	(4)	
Amount of drinking[2] (point)	2.3 (0.7)	2.1 (0.8)	1.14	58	NS	Being at work or ready to work	Present	15/17	(88)	32/50	(64)	NS
Duration of heavy drinking[2] (yr)	16.4 (7.5)	23.8 (10.5)	2.62	58	$P < 0.05$		Absent	2/17	(12)	16/50	(32)	
							Unknown	0/17	(0)	2/50	(4)	
						Understanding and agreement of transplant and a support by the family	Present	16/17	(94)	45/50	(90)	NS
							Absent	1/17	(6)	5/50	(10)	
						Presence of psychiatric comorbidity except alcohol-related mental disease	Present	0/17	(0)	2/50	(4)	NS
							Absent	17/17	(100)	48/50	(96)	
						Declaration of abstinence	Present	17/17	(100)	38/50	(76)	$P < 0.05$
							Absent	0/17	(0)	12/50	(24)	
						Psychiatric hospitalizations	Present	1/67	(6)	0/50	(0)	NS
							Absent	16/67	(94)	50/50	(100)	

[1]Results were shown as mean and range; [2]Results were shown as mean and SD.

actually returned to heavy drinking with his friends, he was referred to a psychiatrist. He underwent psychiatric treatment, which included psychological education and supportive therapy, and decided on abstinence. He has been followed-up regularly by surgeons and recipient coordinators, as well as by frequent psychiatric supervision. He has successfully continued to abstain from alcohol for 6 years.

DISCUSSION

The ALD is a major indication for LT, accounting for approximate 40% of all primary LTs in Europe[17] and about 25% in the United States[18]. The 1-, 3-, and 5-year survival rates after LT in ALD patients have been reported to be 84%, 78% and 73%, respectively, in Europe and 92%, 86% and 86%, respectively, in the United States[17,19]. Although LT for ALD compares favorably with other etiologies of liver cirrhosis[20], recidivism after LT for ALD negatively influences survival[3,4]. Early identification and monitoring of alcohol relapse are essential determinants of long-term outcomes after LT. Although psychosocial evaluation is mandatory for all transplant candidates, it is especially important in patients with ALD. To date, however, there has been a lack of firm consensus regarding psychosocial criteria for LT in patients with ALD. Based on our findings, we propose a set of comprehensive psychosocial criteria to preoperatively predict the risk of relapse after LT.

Although the minimum duration of sobriety before LT has not been determined conclusively, many transplant centers have adopted a minimum alcohol abstinence period of 6 mo as a criterion for transplantation. Abstinence for 6 mo may allow the clinical condition of ALD patients to stabilize or improve prior to LT[21] and has been associated with lower rates of post-transplant relapse[9,10]. However, few studies to date have assessed the accuracy of the 6-mo rule in predicting recidivism[22,23]. A recent survey in Japan showed that a pre-transplant sobriety cutoff of 18 mo was practical in identifying high-risk patients susceptible to harmful relapse and in

selecting patients for deceased donor LT[22]. We regard our selection criteria, consisting of abstinence for 6 mo and promise to abstain throughout life, as essential pre-requisites for LT.

In addition, it is important to evaluate psychosocial factors, with each institution establishing its own criteria. The psychosocial criteria for LT at our institution consist of five items: (1) absence of psychiatric comorbidity except for alcohol-related mental disease[15,24-27]; (2) adherence to medical treatment[26,28-30]; (3) understanding of and agreeing to transplant and support by the patient's family[15,24,26,31,32]; (4) being employed or willing to work[15,16,27]; and (5) having an HRAR score $\leqslant$ 2 points, indicating a lower risk of return to drinking[15,16,27]. In our institution, we did not employ the measurement of blood concentrations of alcohol-related substances, such as ethanol and carbohydrate deficient transferrin.

Because patients with ALD may not fulfill all five criteria, it is necessary to evaluate whether they meet these criteria in a comprehensive way. These five items include risk factors for post-transplant relapse, including psychiatric comorbidities other than alcohol-related mental disease and higher HRAR score[27]. Although a previous study reported that HRAR score alone was not predictive of relapse[22], its inclusion as one of several criteria may be useful. Non-adherence to medical treatment has been reported to predict relapse[26,28,29]. The HRAR score higher than 3 will be associated with relapse into harmful drinking[15]. However, we suggest that evaluation based on HRAR score alone is not enough, and consider that ALD patients should be comprehensively evaluated.

The psychopathology of ALD frequently includes denial, both by patients and their families. Consequently, it may be difficult to evaluate risks of alcohol relapse following an initial interview with ALD patients. Patients should therefore be reevaluated for risk of alcohol relapse one month after their initial evaluation, with further reevaluations required if the risk of alcohol relapse remains difficult to evaluate. Unnecessary delays in making a decision should be avoided. However, repeated patient follow-up may reveal any alcohol-related pathology within the family, including the autonomous intention of the patient and whether the family is supportive.

Psychiatric follow-up after LT is also required[33]. In addition to pre-transplant evaluation, pre- and post-transplant counseling may minimize the relapse of alcoholism after LT. In our hospital, follow-up in the psychiatry outpatient department is mandatory for all patients, although symptoms such as insomnia or irritation may not be recognized. During follow-up, one of seven patients returned to heavy drinking after LT. However, this patient quit drinking after an immediate intervention by psychiatrists. The rate of alcohol relapse in this study, 14.3%, was lower than in previous studies[2,22,29].

The target in treating alcoholism is not merely abstention from alcohol. Rather, everyday life instruction is necessary to prevent resumption of drinking after LT. Patients should also be gradually introduced to a self-help group such as Alcoholics Anonymous. Many patients with ALD and their families deny having a problem with alcohol, as denial is a psychological mechanism to exclude painful thoughts. Only two of the seven patients who underwent LT in this study participated in a self-help group.

In conclusion, we propose a set of psychosocial evaluation criteria that may be useful in assessing risk of alcohol relapse in patients with ALD who are candidates for LT. These psychosocial evaluation criteria will result in improvements in the selection of ALD patients for transplantation and may increase the LT success rate. Additional well-designed studies evaluating our criteria are required to predict risk of alcohol relapse in ALD patients after LT and to determine the optimal timing of LT in patients with ALD.

COMMENTS

Background

Alcoholic liver disease (ALD) is one of the most common causes of advanced liver cirrhosis. The ALD is the second most common indication for liver transplantation (LT).

Research frontiers

Post-transplant relapse of alcohol use can have a negative impact on patient outcomes. It is important to preoperatively assess the risk of post-transplant alcohol use.

Innovations and breakthroughs

To date, however, psychosocial evaluation criteria of LT for ALD have not been established. This psychosocial evaluation criteria may be useful in assessing the risk of relapse in patients who undergo LT for ALD.

Applications

Patients with ALD referred for LT require comprehensive evaluation, including evaluation of psychosocial criteria, to prevent alcoholic recidivism. This psychosocial evaluation criteria may be useful in assessing risk of alcohol relapse in patients with ALD who are candidates for LT.

Terminology

This psychosocial evaluation criteria will result in improvements in the selection of ALD patients for transplantation and may increase the LT success rate.

Peer-review

To investigate factors, including psychosocial factors, associated with alcoholic use relapse after LT for ALD still remains a Achilles points to avoid relapse after LT procedure and comprehensive evaluation, including evaluation of psychosocial criteria, to prevent alcoholic recidivism can be necessary.

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ORIGINAL ARTICLE

Clinical Trials Study

Effects of sex and generation on hepatitis B viral load in families with hepatocellular carcinoma

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Abstract

AIM

To explore factors associated with persistent hepatitis B virus (HBV) infection in a cohort of hepatocellular carcinoma (HCC)-affected families and then investigate factors that correlate with individual viral load among hepatitis B surface antigen (HBsAg)-positive relatives.

METHODS

We evaluated non-genetic factors associated with HBV replication in relatives of patients with HCC. Relatives of 355 HCC cases were interviewed using a structured

questionnaire. Demographics, relationship to index case, HBsAg status of mothers and index cases were evaluated for association with the HBV persistent infection or viral load by generalized estimating equation analysis.

RESULTS

Among 729 relatives enrolled, parent generation (P = 0.0076), index generation (P = 0.0044), mothers positive for HBsAg (P = 0.0007), and HBsAg-positive index cases (P = 5.98 × 10^{-8}) were associated with persistent HBV infection. Factors associated with HBV viral load were evaluated among 303 HBsAg-positive relatives. Parent generation (P = 0.0359) and sex (P = 0.0007) were independent factors associated with HBV viral load. The intra-family HBV viral load was evaluated in families clustered with HBsAg-positive siblings. An intra-family trend of similar HBV viral load was found for 27 of 46 (58.7%) families. Male offspring of HBsAg-positive mothers (P = 0.024) and older siblings were associated with high viral load.

CONCLUSION

Sex and generation play important roles on HBV viral load. Maternal birth age and nutritional changes could be the reasons of viral load difference between generations.

Key words: Familial generation; Sex; Hepatitis B virus; Perinatal infection; Viral replication

Core tip: Familial clustering of chronic hepatitis B infection is identified in this study. Most of the hepatitis B surface antigen (HBsAg) carriers in this cohort are in families of an HBsAg-positive index case. A high prevalence of HBsAg is found in the siblings' generation and in offspring of an HBsAg-positive mother. The HBsAg status of index cases and HBsAg status of the mother are important factors for determining the persistence of hepatitis B virus (HBV) infection in hepatocellular carcinoma families. Sex and generation are factors associated with HBV replication. Perinatal infection has a great influence on male offspring's HBV replication.

Hsieh AR, Fann CSJ, Yeh CT, Lin HC, Wan SY, Chen YC, Hsu CL, Tai J, Lin SM, Tai DI. Effects of sex and generation on hepatitis B viral load in families with hepatocellular carcinoma. *World J Gastroenterol* 2017; 23(5): 876-884 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i5/876.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i5.876

INTRODUCTION

In the families of hepatitis B virus (HBV)-infected individuals, clustering of chronic hepatitis B surface antigen (HBsAg) carriers and hepatocellular carcinoma (HCC) are common[1-6]. HBV is highly infectious[7,8], and a substantial number of individuals who are exposed to HBV early in life become chronic HBsAg carriers[4,9-11]. Furthermore, intra-familial transmission of HBV could underlie the high incidence of HCC among family members[3,4].

In addition to sex-related behavioral factors[12,13], genome-wide association studies in Japan indicated that the human leukocyte antigen subunits DP and DQ are associated with HBsAg persistence[14,15]. However, the genes identified as being responsible for clinical progression among chronic HBsAg carriers differ among several genome-wide association studies carried out in China and Taiwan[16-20]. Hence, it is possible that non-genetic factors may play a non-negligible role in determining HBV replication. For example, an increased risk of liver cancer among first-degree relatives of HCC patients was shown to be associated with a prolonged HBV replication phase[1,2]. Therefore, before evaluating genetic factors associated with HBV replication, non-genetic factors that may be associated with HBV viral load should be clarified[2-4,6,9-11,21].

Given the familial clustering of chronic HBsAg carriers in HCC families[2,5,6,9,21] with maternal status, those relatives having a similar genetic background may be instrumental in helping clinicians determine any non-genetic factors that may be associated with persistent HBV infection and viral replication. In this respect, we explored factors associated with persistent HBV infection in a cohort of HCC-affected families and then investigated factors that correlated with individual viral load among HBsAg-positive relatives.

MATERIALS AND METHODS

Patients

Patients with HCC who were diagnosed at Chang Gung Memorial Hospital, Lin-Kou Medical Center were included as index cases. From 2003 to 2007, relatives of these patients were prospectively invited to complete a survey concerning liver diseases. Spouses of index cases or spouses of their relatives were excluded.

This study was approved by the Institutional Review Board of Chang Gung Memorial Hospital, Taiwan (IRB: 91-124), and written informed consent was obtained from all participants before the study. All experiments and data comparisons were carried out in compliance with relevant laws and guidelines and in accordance with the ethical standards of the Declaration of Helsinki.

Survey

At entry, basic information that included national citizen identification number, sex, race, alcohol and smoking habits, profession, location of residency at birth, level of education, and family history were obtained through questionnaires and structured interviews.

Each relative that was enrolled in the study underwent liver biochemistry tests for α-fetoprotein and

viral markers, as well as a liver ultrasound. Serum HBsAg and hepatitis C virus antibody (anti-HCV) were measured by enzyme-linked immunosorbent assay (Abbott Diagnostics, Chicago, IL, United States). Maternal HBsAg was assayed at enrollment or obtained by reviewing our hospital records.

HBV viral load and HBV genotyping

A quantitative HBV DNA assay was carried out initially with the Digene Hybridization System (Digene Diagnostics, Inc., Beltsville, MD, United States; lower limit of detection, 1.4×10^5 cps/mL). Those with HBV DNA lower than the detectable limit were further assayed using the COBAS Amplicor HBV Monitor Test (Roche Diagnostics, Branchburg, NJ, United States; lower limit of detection, 200 cps/mL). Our previous long-term follow-up study revealed that nearly 40% of HBsAg carriers with persistent normal alanine aminotransferase levels have a level of HBV DNA of $> 1.0 \times 10^4$ cps/mL[22]. Therefore, relatives with HBV DNA levels of $\geqslant 1.0 \times 10^5$ cps/mL were considered as having high HBV replication, and those with levels $< 1.0 \times 10^5$ cps/mL were considered as having low HBV replication.

HBV genotype was initially determined with the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method[23], but we later changed to a more sensitive SMITEST HBV Genotyping kit (Medical and Biological Laboratories Co., Ltd., Nagoya, Japan) for all subjects. For those subjects with low HBV DNA level, the S region of the genome was amplified by nested PCR followed by direct sequencing (CEQ 8000 Genetic Analysis System; Beckman Coulter, Brea, CA, United States).

Body height in relation to birth year

Thomas *et al*[24] reported that body height at adulthood may predict the nutritional status of a population in a particular birth year. Hence, we estimated the nutritional status of Taiwan based on body height data according to birth year for subjects who received a general checkup between year 2000 and 2004 at Chang Gung Memorial Hospital[9] and in the cohort of HCC families.

Statistical analysis

The analysis of cohort data was divided into two stages. In the first stage, we searched for factors associated with chronic HBsAg carriers. In the second stage, we examined factors associated with HBV viral load in HBsAg-positive relatives only.

The relatives included in the study were individuals from the same household. Because both individual and familial responses from the same household should be evaluated, we used the generalized estimating equation (GEE) method to determine correlations between the data and each binary response (*e.g.*, for HBsAg status or HBV DNA level) using the exchangeable working correlation structure[25,26] in our first and second stages of the analyses. Univariate and *multivariate analyses* in the two stages were assessed using the GEE method with the PROC GENMOD procedure in SAS 9.3 (SAS Institute Inc., Cary, NC, United States).

The role of sex hormones in the development and progression of HBV-associated HCC has been reported[12,13]. Therefore, we added a new familial view on HBV replication status in this cohort. We examined intra-familial HBV replication among HBsAg-positive siblings of the same sex in each family. A sex difference with respect to HBV viral load in families clustered with HBsAg-positive siblings. We used logistic regression to explore the sex effect for families in which the mother was positive for HBsAg as well as in all families.

RESULTS

Index cases

A total of 355 families participated in this study. Of the 330 index cases with data on HBV, 203 (61.5%) were seropositive for HBsAg, 29 (8.8%) were seropositive for both HBsAg and anti-HCV, 75 (22.7%) were seropositive for anti-HCV, and 23 (7.0%) were seronegative for both HBsAg and anti-HCV. The diagnosis of HCC was based on cytology or histology for 180 (50.7%) patients. The others were diagnosed clinically based on a serum α-fetoprotein level and/or imaging studies[27].

Relatives

There were 806 relatives and 205 spouses in the study. Twenty-five relatives were diagnosed with liver cirrhosis by ultrasound at screening. None of the study relatives had HCC detected on initial screening. Three siblings and three children of the indexed HCC patients developed HCC during the subsequent follow-up study.

First-stage: Persistent HBV infection analysis

Of the 806 relatives who participated in this study, 77 were born after 1984 when the nationwide vaccination program against HBV started in Taiwan; these 77 subjects were excluded from the first-stage analysis (Figure 1). The dataset used for the first-stage analysis thus contained 729 individuals.

The risk factor of chronically expressing HBsAg was examined in the first stage. The following factors were evaluated: sex, index case sex, age, relation to the index case, HBsAg status of the mother (maternal HBsAg), and HBsAg status of the index case (index HBsAg). Index HBsAg, maternal HBsAg, and index generation were significantly associated with persistent HBV infection ($P < 0.0001$; Table 1). After controlling for sex, these associations remained statistically significant ($P < 0.0001$; Table 1).

In the multivariate GEE analysis, persistent HBV infection was lower for parents of index cases (OR = 0.24, $P = 0.0076$; Table 2). The risk was higher for subjects in the index generation (OR = 2.25, $P =$

Table 1 Association between demographics and hepatitis B surface antigen status among relatives of patients with hepatocellular carcinoma *n* (%)

Category	HBsAg		OR (95%CI)	Adjusted OR (95%CI)[1]
	Positive	Negative		
Total family members	314	415		
Sex				
Male	171 (54.46)	196 (47.23)	1.25 (0.97-1.61)	
Female	143 (45.54)	219 (52.77)		
Index sex				
Male	229 (72.93)	302 (72.77)	1.07 (0.70-1.62)	1.25 (0.97-1.60)
Female	85 (27.07)	113 (27.23)		
Age, mean ± SD	40.49 ± 10.89	37.87 ± 11.69	1.01 (1.00-1.03)	1.28 (1.00-1.64)
Relation to index				
Parent	10 (3.18)	20 (4.82)	0.78 (0.37-1.64)	0.81 (0.38-1.71)
Index generation	86 (27.39)	36 (8.67)	3.89 (2.32-6.51)[a]	3.97 (2.38-6.63)[a]
Child	206 (65.61)	347 (83.61)		
Grandchild	12 (3.82)	12 (2.89)	1.43 (0.66-3.13)	1.39 (0.65-3.00)
Maternal HBsAg				
Negative	86 (27.38)	244 (58.80)		
Positive	129 (41.08)	53 (12.77)	5.03 (3.16-8.01)[a]	5.00 (3.13-7.97)[a]
Unknown	99 (31.53)	118 (28.43)	2.01 (1.30-3.38)[a]	2.04 (1.33-3.13)[a]
Index HBsAg[2]				
Negative	48 (15.43)	203 (49.03)		
Positive	263 (84.57)	211 (50.97)	5.57 (3.56-8.71)[a]	5.51 (3.53-8.61)[a]

[1]Adjusted by sex; [2]Four index cases. HBsAg status unknown. [a]$P < 0.0001$. HBsAg: Hepatitis B surface antigen.

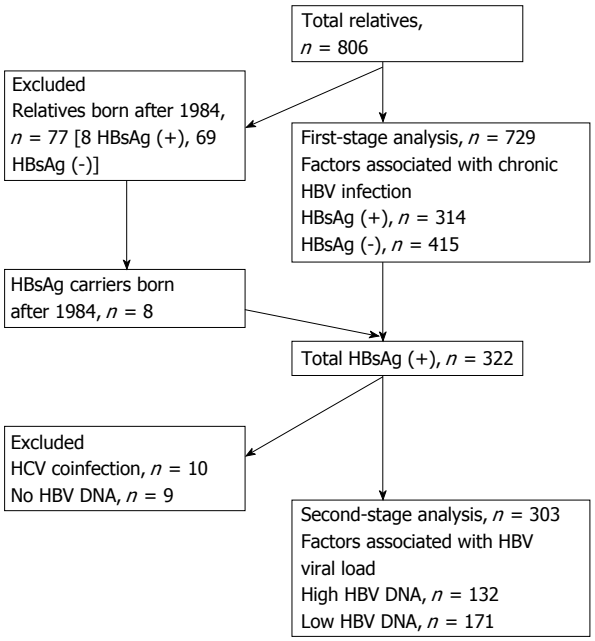

Figure 1 Flow chart depicting the collection and potential exclusion of subjects for our cohort and the stages of analysis.

0.0044; Table 2), those who had an HBsAg-positive mother (OR = 2.65, $P = 0.0007$; Table 2), those related to an HBsAg-positive index case (OR = 4.19, $P = 5.98 \times 10^{-8}$), and those of older age (OR = 1.03, $P = 0.0037$; Table 2).

Second-stage: HBV viral load association analysis

Among the 314 HBsAg-positive relatives born before 1984 and 8 relatives born after 1984, for this second-stage analysis we excluded 10 relatives with dual HBV and HCV infections and 9 relatives who did not have an HBV DNA assay (Figure 1). A total of 303 individuals were thus included in the HBV viral load association analysis.

Table 2 Multivariate analyses using generalized estimating equation to find predictive factors for hepatitis B surface antigen status

Factor	Item	OR (95%CI)	*P* value
Sex	Male	1.26 (0.94-1.70)	
Index sex	Male	1.28 (0.78-2.10)	
Age		1.03 (1.01-1.05)	0.0037
Relation to index	Parent	0.24 (0.09-0.69)	0.0076
	Index generation	2.25 (1.29-3.94)	0.0044
	Grandchild	2.06 (0.78-5.45)	
Maternal HBsAg	Positive	2.65 (1.51-4.67)	0.0007
	Unknown	1.21 (0.72-2.03)	
Index HBsAg	Positive	4.19 (2.50-7.04)	5.98×10^{-8}

HBsAg: Hepatitis B surface antigen.

The associations between HBV DNA level and sex, index sex, age, relation to index case, maternal HBsAg, index HBsAg, and HBV genotype were examined. A positive association was found between high HBV DNA level and male sex (OR = 2.12, $P = 0.0013$; Table 3). A significant association with HBV viral load was noted between parents of index cases and child plus grandchild generations (OR = 4.77, $P = 0.0348$; Table 3). Index HBsAg status was significantly associated with HBV DNA level (OR = 2.32, $P = 0.0221$; Table 3). A significant association with HBV viral load was also noted between HBV genotype C and HBV genotype B (OR = 1.71, $P = 0.008$; Table 3); after controlling for sex, however, the association was of marginal statistical

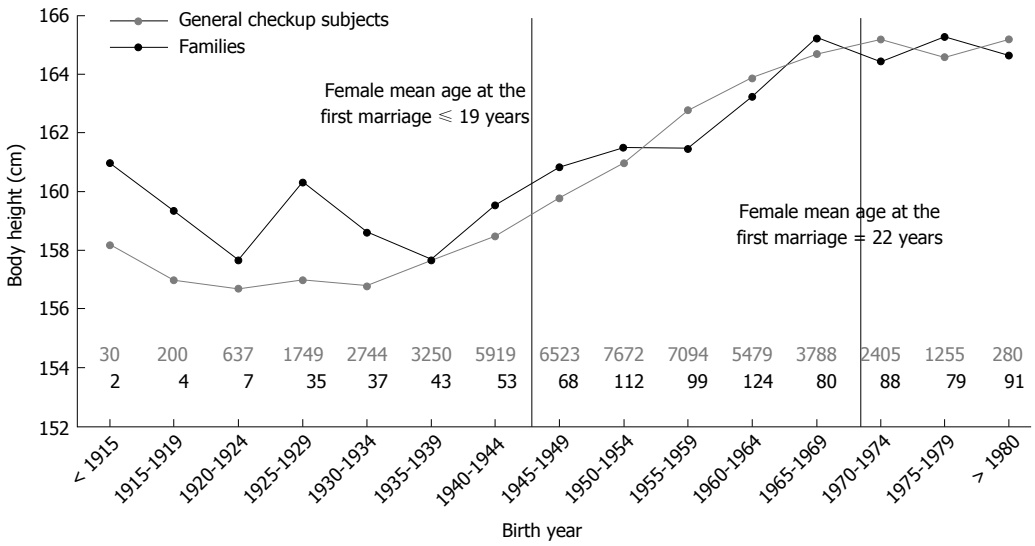

Figure 2 Body height changes according to birth year for subjects of our cohort who underwent a general checkup (gray line) and hepatocellular carcinoma families (black line). The two horizontal lines indicate the female mean age at first marriage for each birth-year period. The mean age at first marriage before 1945 was ≤ 19 years and was 22 years in 1970.

Table 3 Association between demographics and hepatitis B virus viral load in 303 hepatitis B surface antigen-positive relatives *n* (%)

Factor	HBV DNA		OR (95%CI)	*P* value	Adjusted OR (95% CI)[1]	*P* value
	≥ 100000 cps/mL	< 100000 cps/mL				
Total family members	132	171				
Sex						
Male	84 (63.64)	79 (46.20)	2.12 (1.34-3.39)	0.0013		
Female	48 (36.36)	92 (53.80)				
Index sex						
Male	99 (75)	121 (70.76)	1.83 (0.69-2.04)		1.17 (0.68-2.01)	
Female	33 (25)	50 (29.24)				
Age, mean ± SD	40.51 ± 12.18	39.15 ± 10.55	1.01 (0.99-1.03)		1.02 (0.99-1.04)	
Relation to index						
Child and grandchild	83 (62.88)	128 (74.85)				
Parent	7 (5.30)	2 (1.17)	4.77 (1.12-20.31)	0.0348	4.57 (1.15-18.14)	0.0307
Index generation	42 (31.82)	41 (23.98)	1.51 (0.87-2.62)		0.64 (0.36-1.14)	
Maternal HBsAg						
Negative	33 (25)	51 (29.82)				
Positive	61 (46.21)	64 (37.43)	1.55 (0.84-2.87)		1.57 (0.84-2.92)	
Unknown	38 (28.79)	56 (32.75)	1.08 (0.57-2.06)		1.20 (0.62-2.33)	
Index HBsAg						
Negative	12 (9.16)	32 (18.93)				
Positive	119 (90.84)	137 (81.07)	2.32 (1.13-4.76)	0.0221	2.47 (1.19-5.15)	0.0158
HBV genotype[2]				0.0017		
N[3]	2 (1.53)	21 (12.88)	0.11 (0.03-0.44)		0.09 (0.02-0.39)	0.0011
B	97 (74.62)	120 (73.62)				
C	31 (23.85)	22 (13.50)	1.71(0.94-3.14)	0.008	1.80 (0.97-3.36)	0.0640

[1]Adjusted by sex; [2]There are 10 missing HBV genotypes; [3]Genotyping failed due to low HBV DNA. HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen.

significance (*P* = 0.064; Table 3).

In the multivariate GEE analysis, HBV viral load was independently associated with sex (OR = 2.65, *P* = 0.0007; Table 4) and being the parent of an index case (OR = 6.49, *P* = 0.0359; Table 4).

Body height in relation to birth year

Figure 2 presents data for body height change according to birth year in general checkup subjects and HCC families. The body height of the general checkup subjects and of HCC families increased similarly according to birth year.

Intra-family comparison of HBV viral load among HBsAg-positive siblings

Forty-six families were found to have at least two HBsAg-positive siblings of the same sex. Among them, 28 were male sibling families and 18 were female

Table 4 Multivariate analyses using generalized estimating equation to find predictive factors for hepatitis B virus viral load

Factor	Item	OR (95%CI)	*P* value
Sex	Male	2.65 (1.51-4.64)	0.0007
Index sex	Male	1.47 (0.73-2.95)	
Age		1.01 (0.98-1.03)	
Relation to index	Parent	6.49 (1.13-37.27)	0.0359
	Index generation	1.19 (0.60-2.37)	
Maternal HBsAg	Positive	1.50 (0.71-3.17)	
	Unknown	1.02 (0.49-2.15)	
Index HBsAg	Positive	1.51 (0.68-3.38)	
HBV genotype	N	0.12 (0.03-0.56)	0.0066
	C	1.22 (0.59-2.51)	

HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen.

sibling families (Table 5). All siblings had a high HBV viral load in 13 (28.26%) families, and all siblings had a low HBV viral load in 14 (30.43%) families. These two groups (58.69%) revealed a familial trend of HBV replication status; among those siblings, male sibling families generally had a high HBV viral load, whereas female sibling families had a low HBV viral load (OR = 29.96, P = 0.007; Table 5). Maternal HBsAg positivity had a large influence on male offspring in that most of male offspring were in the high HBV viral load group; on the other hand, female offspring were generally in the low HBV viral load group (OR = 21, P = 0.024; Table 5).

For 11 families (23.91%), older siblings had a higher level of HBV DNA than their younger siblings; this trend was opposite for only 5 families (10.87%). Older siblings tended to have a higher HBV DNA level than their younger siblings, but the difference was not statistically significant owing to the small number of cases. Because all siblings were generally infected at an early stage of life[4,9-11], this phenomenon contradicts the general trend that HBV replication declines with increasing age[28,29].

DISCUSSION

This study reveals a familial clustering of chronic HBV infection. As shown in Table 1, most of the chronically HBV-infected carriers (84.57%) in this cohort were families of an HBsAg-positive index case. A high prevalence of HBsAg was apparent for the siblings' generation (86/122 or 70.49%, P < 0.0001) and for offspring of an HBsAg-positive mother (129/182 or 70.88%, P < 0.0001). These findings remained significant in the multivariate analysis. Notably, the majority of index cases were male (72.93%), indicating that both vertical and horizontal infections were present in HCC families.

HBV replication phase or viral load plays roles in determining the prognosis of chronic persistent HBV infection[2,30]. In our study, we found that sex and generation played independent roles in determining HBV DNA level (Tables 3 and 4). HBV viral load was higher for subjects with HBV genotype C than genotype B in the univariate analysis (P = 0.008; Table 3), but this difference was not statistically significant in the multivariate analysis (Table 4).

Sex is a well-known factor associated with chronic HBV infection[9]. We therefore added a new family view on HBV replication status in this cohort, and we identified a sex difference with respect to HBV viral load in families that had HBsAg-positive siblings (Table 5). HBV viral load was generally higher in male than female siblings (OR = 29.96, P = 0.007). In addition, male siblings in families of an HBsAg-positive mother tended to be in the high HBV DNA group, whereas female siblings were generally in the low HBV DNA group (OR = 21, P = 0.024). Male offspring are more vulnerable to the influence of maternal HBsAg status, whereas female offspring may overcome the maternal influence of persistent HBV replication.

Relatively high HBV replication in older generations has not been well documented in the literature. A study of pregnant women between 1990 and 1995 revealed a progressively decreasing prevalence of hepatitis B e antigen (HBeAg) among chronically HBV-infected carriers[31]. This finding was confirmed in a longer study spanning 1985 to 2000[32], in which the prevalence of HBsAg remained nearly the same, but the prevalence of HBeAg declined progressively from 40% in 1986 to 18% in 2000. This difference between HBsAg and HBeAg prevalence remained apparent even when the ages of the pregnant women were considered[32].

In our previous study of HCC families, we found that older siblings frequently cleared HBeAg later than did their younger siblings[21], and an HBV phylogenetic study yielded similar findings[33]. Among 13 families with an HBsAg-positive mother, the 11 oldest siblings were HBeAg positive whereas only 3 of the youngest siblings were HBeAg positive. These observations provided a clue that maternal age at birth might influence HBV replication in offspring.

The mean age of women entering their first marriage in Taiwan was 18 years before 1917 and remained at about 19 years between 1918 and 1945 (Figure 2)[34]. In the 1970s, however, this mean age had risen to 22 years (http://nccur.lib.nccu.edu.tw/handle/140.119/34632) and increased rather rapidly to 29.2 years by 2010 (http://www.moi.gov.tw/stat/news_content.aspx?sn=5261). Thus, mothers in younger generations of this period between 1918 and 2010 may be 3-5 years older than mothers of the older generations.

A 2014 review article by Bertoletti *et al*[35] presented an interesting viewpoint that immune responses change during the life of an individual, based on the observed higher mortality of influenza infection at age 30 than at age 20. This implies that a more vigorous immune response produces a more fulminant disease by age 30, whereas a weaker immune response produces a

Table 5 Intra-family comparison of hepatitis B virus viral load among hepatitis B surface antigen-positive siblings *n* (%)

HBV DNA level[1]	Maternal HBsAg			Total
	Positive	Unknown	Negative	
Total male siblings	12	9	7	28
All high level	7 (58.33)[2]	2 (22.22)	2 (28.57)	11 (39.3)[3]
All low level	1 (8.33)[2]	2 (22.22)	1 (14.29)	4 (14.3)[3]
Older > younger	3 (25.00)	3 (33.33)	3 (42.86)	9 (32.1)
Younger > older	1 (8.33)	1 (11.11)	1 (14.29)	3 (10.7)
Other	0 (0.00)	1 (11.11)	0 (0.00)	1 (3.6)
Total female siblings	11	3	4	18
All high level	2 (18.18)[2]	0 (0.00)	0 (0.00)	2 (11.1)[3]
All low level	6 (54.55)[2]	1 (33.33)	3 (75.00)	10 (55.6)[3]
Older > younger	1 (9.09)	1 (33.33)	0 (0.00)	2 (11.1)
Younger > older	1 (9.09)	0 (0.00)	1 (25.00)	2 (11.1)
Other	1 (9.09)	1 (33.33)	0 (0.00)	2 (11.1)

[1]Low HBV DNA level, < 1×10^5 cps/mL; high HBV DNA level, ≥ 1×10^5 cps/mL. [2]OR (95%CI) = 21 (1.50-293.25), $P = 0.024$; [3]OR (95%CI) = 29.96 (2.54-353.17), $P = 0.007$; logistic regression. HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen.

self-limited infection at age 20. A similar situation can be found for chronic HBV infection in that such patients usually enter the immune clearance phase by age 30. We suspect that generational differences might be associated with differences in maternal immunity at the time of an offspring's birth[36]. Further study will be needed.

Better nutrition is another potential reason for reduced HBV replication in younger generations, and long-term follow-up studies revealed that hepatic steatosis is a good prognostic indicator for chronic HBsAg carriers[28,29]. Hepatic steatosis correlated with a lower risk of HCC, lower mortality rate, and higher chance of spontaneous HBsAg clearance. A recent PNPLA3 polymorphism study on non-alcoholic fatty liver disease found that those SNP genotypes favoring hepatic steatosis development were associated with lower HBV DNA level[37].

During the time frame of our study, we did not have data on the nutritional habits of individuals, but for most participants we obtained body height data, which may reflect long-term nutritional status during the major growth period of humans[24,38]. In our cohort, the mean body height remained < 159 cm for individuals born before 1945. From about 1955 to 1965, however, mean body height increase rapidly to > 164 cm (Figure 2). These findings indicate a significant change in socioeconomic status of the Taiwanese population after the Second World War. Hence, increased food consumption and decreased physical activity may have contributed to the observed increase in the prevalence of hepatic steatosis[39]. Therefore, lifestyle and nutritional habits are factors that may have contributed to our observed shortened HBV replication phase in the younger generation.

We conclude that the generation of the family member, index HBsAg, and maternal HBsAg are important factors for predicting HBV persistence in HCC families. Sex and generation are factors associated with HBV replication. Perinatal infection substantially influences the duration of HBV replication in male offspring.

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COMMENTS

Background

Hepatitis B virus (HBV) replication is critical for disease progression. Multiple inconsistent genetic factors have been identified to be involved in the disease progression. Therefore, the non-genetic factors concerning persistent HBV replication should be clarified.

Research frontiers

Among 729 relatives enrolled, parent generation, index generation, maternal hepatitis B surface antigen (HBsAg), and index cases HBsAg status were factors associated with persistent HBV infection. Factors associated with HBV viral load were evaluated among 303 HBsAg-positive relatives. Generation and sex were independent factors associated with HBV viral load. The intra-familial HBV viral load was evaluated in families clustered with HBsAg-positive siblings. An intra-family trend of similar HBV viral load was found for 27 of 46 (58.7%) families. Male offspring of HBsAg-positive mothers and older siblings were associated with high viral load.

Innovations and breakthroughs

Based on the finding that older generation and older siblings have higher viral load, the authors suspect that maternal age at birth and nutritional status might be related to generational differences on viral load. HBsAg-positive mothers usually associated with high viral load on male offspring, but not on female offspring.

Applications

Sex, generation, maternal age at birth and maternal HBsAg status are factors that should be taken into consideration when genetic factors associated with HBV-related outcome are evaluated.

Peer-review

The manuscript from Hsieh *et al* reported the sex and generation associated with HBV load in hepatocellular carcinoma family. And perinatal infection is a major effect factor for male offspring's HBV replication. The entire sets of data

are nicely presented, and highly supportive of the conclusion.

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ORIGINAL ARTICLE

Observational Study

Impact of humic acids on the colonic microbiome in healthy volunteers

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Author contributions: Swidsinski A, Krüger M and Schrödl W designed the study; Dörffel Y, Neuhaus J conducted the study; Loening-Baucke V and Göktas Ö critically revised and wrote the manuscript; Reißhauer A performed FISH, and Gille C analyzed the data staistically.

Institutional review board statement: The investigations were approved by the ethical review board of the Charite Hospital and Institut für Bakteriologie und Mykologie An den Tierkliniken Leipzig.

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Abstract

AIM

To test the effects of humic acids on innate microbial communities of the colon.

METHODS

We followed the effects of oral supplementation with humic acids (Activomin®) on concentrations and composition of colonic microbiome in 14 healthy volunteers for 45 d. 3 × 800 mg Activomin® were taken orally for 10 d followed by 3 × 400 mg for 35 d. Colonic microbiota were investigated using multicolor fluorescence *in situ* hybridization (FISH) of Carnoy fixated and paraffin embedded stool cylinders. Two stool samples were collected a week prior to therapy and one stool sample on days 10, 31 and 45. Forty-

one FISH probes representing different bacterial groups were used.

RESULTS

The sum concentration of colonic microbiota increased from 20% at day 10 to 30% by day 31 and remained stable until day 45 (32%) of humic acid supplementation ($P < 0.001$). The increase in the concentrations in each person was due to growth of preexisting groups. The individual microbial profile of the patients remained unchanged. Similarly, the bacterial diversity remained stable. Concentrations of 24 of the 35 substantial groups increased from 20% to 96%. Two bacterial groups detected with Bac303 (*Bacteroides*) and Myc657 (mycolic acid-containing *Actinomycetes*) FISH probes decreased ($P > 0.05$). The others remained unaffected. Bacterial groups with initially marginal concentrations ($< 0.1 \times 10^9$/mL) demonstrated no response to humic acids. The concentrations of pioneer groups of *Bifidobacteriaceae*, *Enterobacteriaceae* and *Clostridium difficile* increased but the observed differences were statistically not significant.

CONCLUSION

Humic acids have a profound effect on healthy colonic microbiome and may be potentially interesting substances for the development of drugs that control the innate colonic microbiome.

Key words: Fluorescence *in situ* hybridization; Colonic microbiota; Colonic bioreactor; Humic acids; Healthy volunteers; Oral supplementation

Core tip: Modern patients are increasingly interested in natural medicinal products, which are often not scientifically evaluated. Humins arise from organic microbial degradation and are an important mediator of microbial interactions in nature. Although used for medical indications since ancient times, no data exist on the impact of humins on the human microbiome. Our investigations in healthy volunteers show that orally applied humic acids increase the sum concentrations of preexisting colonic microbiota from 20% to 30% without changes in the bacterial diversity of the individual microbiome and may be a serious amendment/alternative to fecal transplantation or probiotics.

Swidsinski A, Dörffel Y, Loening-Baucke V, Gille C, Reißhauer A, Göktas Ö, Krüger M, Neuhaus J, Schrödl W. Impact of humic acids on the colonic microbiome in healthy volunteers. *World J Gastroenterol* 2017; 23(5): 885-890 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i5/885.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i5.885

INTRODUCTION

All great ancient cultures were based on agriculture for which soil quality and prevention of its exhaustion were absolutely critical. Humus as an organic fecundity substrate of the earth excited thinkers from the ancient times and stimulated both solid research and charlatanry. First descriptions of medical applications can be found in Sanskrit and also ancient writings of Rome and China. Despite nearly mystic reverence and enormous interest, it is not before the early 1800s that chemical characterization and description of humic acids took place.

Humic substances are complex organic substances of soil, which are formed in the process of humification. Humification involves natural chemical and microbial activity that transforms the dead remains of living things into humic substances. It is the second greatest organic process on earth after photosynthesis and is responsible for fossil coal, oil deposits and others. Microorganisms utilize and break down organic substances and lead to accumulation of recalcitrant molecules. When microorganisms die, they are themselves broken down and added to the recalcitrant humic mass. The concurrent chemical-physical polymerization modifies humic substances in an unpredictable matter. In all, the genesis of humic substances can take hundreds or even thousands of years and leads to high variety, unique composition and extreme difficulties in characterization of these[1,2].

The growing interest of the modern society for environmental and biological welfare refreshed the attractiveness for implementation of humins. Gastroenterologists are often confronted with a wish of patients to be treated with "natural" products and asked for opinion on humic acids. The study of the scientific literature reveals a large number of medical trials with dietary supplements of humic acids conducted all over the world. The reported effects include different, partially incoherent properties such as anti-inflammatory and immune-stimulatory as well as analgesic, antimicrobial, antiviral/anti-HIV activity, antioxidant and even stroke protective effects[1-3]. The striking eclecticism of the findings and the lack of systematic studies make it difficult to build an unbiased opinion. Furthermore humic substances are distributed under a wide variety of trade names and descriptions in an unregulated market.

The colon is a central bio-fermenting organ degrading digestive leftovers. Since microbial activity is central in genesis and processing of humic acids, the innate human microbial communities should be the main object on which the effects of humic acids will be apparent. Astonishingly, we found no data to this topic in the literature. In order to close this gap, we investigated the impact of orally applied Activomin® (Pharmawerk Weinboehla, Weinboehla, Germany) on concentrations

and diversity of the human colonic microbiome. Activomin® is the only registered and standardized humic acids preparation in Germany.

MATERIALS AND METHODS

Patients, subjects and samples

Fourteen healthy volunteers from the Laboratories of Centre for Infectious Diseases, Faculty of Veterinary Medicine, University of Leipzig and Laboratory for Polymicrobial Infections and Biofilms, Charité Universitäts Medizin Berlin (24-64 years of age, mean 39 years, 5 males and 9 females) have taken 3 × 2 capsules (3 × 800 mg) Activomin® orally for 10 d followed by 3 × 1 capsule (3 × 400 mg) for 35 d. Two stool samples were collected a week prior to therapy and one stool sample on days 10, 31 and 45.

The study was approved by the ethics commission of University of Leipzig. The collection of fecal samples for fluorescence *in situ* hybridization (FISH) diagnosis of dysbiosis was approved by the ethics commission of the Charité University Hospital.

FISH

Colonic microbiota were investigated using FISH analysis of Carnoy fixated and paraffin embedded stool cylinders[4]. Multicolor FISH simultaneously using 3 differently stained FISH probes (C3 - orange, FITC-dobe - green, C5 - dark red) and counterstained with DAPI for DNA structures was performed on 4 μm longitudinally cut sections of punched-out stool cylinders. Sections were placed on SuperFrost plus slides.

A Nikon e600 fluorescence microscope was used. The images were photo-documented with a Nikon DXM 1200F color camera and software (Nikon, Tokyo, Japan).

Bacteria were quantified using group specific C3 probes. The FITC marked universal probe was used in each hybridization to evaluate the number of all bacteria, C5 marked probes with a different to C3 probes specificity were used to exclude unspecific binding. Only signals that hybridized with a specific FISH probe and the universal FISH probe, but did not hybridize with specific FISH probes from unrelated bacterial groups, were evaluated.

Bacterial concentrations of homogeneous populations were enumerated visually in one of the 10 × 10 fields of the ocular raster corresponding to 10 μm × 10 μm of the section surface at magnification of 1000. This number was assigned to concentration of 1 × 10^9 bacteria/mL, which was most equivalent to the calculation formula, which we had used previously[4].

In case of uneven distribution of bacteria over the microscopic field, the positive signals were enumerated in ten fields of the ocular raster along the gradient of distribution and divided by ten.

Investigated bacterial groups/FISH probes

Forty-one bacterial FISH probes were applied, Table 1. The exact specification of the FISH probes and hybridization conditions are available in public resources[5]. The names of the FISH probes are listed according to abbreviations of the probeBase online resource (http://www.microbial-ecology.net/probebase/credits.asp). The Fprau probe is described in 6[6].

The FISH probes were arranged in Table 1 to four functional groups described previously: essential bacteria, individual pioneer bacteria, individual substantial and individual marginal or accidental bacteria[7].

Bacteria detected with EREC (mainly *Roseburia*), Bac303 (*Bacteroides*), Fprau (*Faecalibacterium prausnitzii*) probes are always present in healthy human subjects and together contribute about half of the colonic microbiome. They are obviously essential for colonic bio-fermentation.

All other bacterial groups are individual, present only in some of the subjects in substantial concentrations (mean ⩾ 0.1 × 10^9/mL) or marginal concentrations (mean < 0.1 × 10^9/mL).

Four FISH probes including Bif153 (*Bifidobacteriaceae*), Cdif198 (*Clostridium difficile*), Ebac1790 (*Enterobacteriaceae*) and Clit135 (*Clostridium lituseburense*) represent individual bacterial groups with pioneer function, which are found prevalent in newborns, after antibiotic treatment and convalescence patients, but are seldom found in low concentration in healthy persons.

Statistical analysis

Differences between groups were evaluated using the twosided *t*-Student *U* test. Data are presented as mean ± SD, P <0.05 was considered statistically significant.

RESULTS

All participants completed the stool collection, even the one man, who developed loose stools and bloating. No other side effects were reported.

Humic acids induced changes of the microbiome

Table 1 summarizes changes in the mean concentrations of single bacterial groups prior to and during supplementation with Activomin®. Bacteria in the Table 1 are arranged to sets of essential, individual pioneer, individual substantial and individual marginal bio-fermenting groups.

The mean microbial concentration after 45 d of supplementation of humic acids increased 14% in the essential groups (P < 0.01), 28% (NS) in the individual pioneer groups and 41% (P < 0.002) in the individual substantial groups. The accidental bacterial groups with initially marginal concentrations demonstrated no response to humic acids.

Table 1 Mean microbial concentrations (± SD) as detected with applied fluorescence *in situ* hybridization probes (10^9 bacteria/mL)

	Day 0	Day 10	Day 31	Day 45	Change in % from day 0 to day 45	*P* value
Mean sum concentrations of all detected bacteria	85.4 ± 25.6	107.4 ± 15.6	123.7 ± 34.1	126.1 ± 50.1	↑ 32%	< 0.001
Essential all (*n* = 3)	36.2 ± 14.7	44.0 ± 5.1	42.7 ± 7.7	42.8 ± 9.0	↑ 14%	< 0.01
Erec (*Eubacterium rectale, Clostridium coccoides* group)	11.7 ± 6.9	17.1 ± 2.5	19 ± 4	17.7 ± 4.8	↑ 30%	< 0.001
Bac303 (*Bacteroides*)	12.9 ± 5.3	12.2 ± 5.7	9.5 ± 4.4	9.9 ± 5.0	↓ 30%	ns
Fprau (*Faecalibacterium prausnitzii*)	11.6 ± 5.9	14.7 ± 3.8	14 ± 3.6	14.7 ± 6.9	↑ 21%	ns
Individual pioneer						
All (*n* = 4)	7.8 ± 5.0	11.5 ± 9.3	9.9 ± 6.8	10.9 ± 8.5	↑ 28%	ns
Ebac1790 *Enterobacteriaceae*	0.25 ± 0.8	0.6 ± 2.1	1.2 ± 2.3	1.1 ± 2.0	↑ 72%	ns
Cdif198 *Clostridium difficile*	0.04 ± 0.09	0.01 ± 0.03	0.3 ± 0.93	0.10 ± 0.03	↑ 96%	ns
Bif153 Genus *Bifidobacterium*	7.1 ± 5.5	9.1 ± 8.6	7.7 ± 5.3	9.7 ± 7.2	↑ 27%	ns
Clit135 *Clostridium lituseburense* group including *C. difficile*	0.5 ± 0.86	0.7 ± 1.05	0.4 ± 1.08	0.4 ± 0.8	↔	ns
Individual substantial mean > 0.1 × 10^9/mL						
All (*n* = 28)	41.7 ± 17.3	51.4 ± 14.0	70.4 ± 28.8	71.6 ± 36.8	↑ 41%	< 0.002
ACI623 *Acidaminococcaceae* sp. (not the Selenomonas species)	1.4 ± 1.9	0.7 ± 0.8	1.6 ± 2.2	1.2 ± 1.6	↔	ns
AKK406 *Akkermansia*	2.3 ± 3.7	2.8 ± 4.6	1.9 ± 2.9	2.6 ± 4.1	↔	ns
Ato291 *Atopobium cluster*	3.8 ± 2.9	4.9 ± 3.5	6.1 ± 4.5	6.4 ± 3.6	↑ 41%	0.01
Bbif186 *B. bifidum*	0.3 ± 0.9	0.3 ± 0.6	0.2 ± 0.5	0.3 ± 0.5	↔	ns
Blon1004 *B. longum*	0.7 ± 1.2	0.9 ± 1.5	1.0 ± 1.5	0.6 ± 0.9	↔	ns
Bputre698 *Bacteroides* putredinis	0.8 ± 1.6	0.8 ± 1.3	1.8 ± 2.1	1.6 ± 1.8	↑ 50%	ns
Burkho *Burkholderia* spp.	0.7 ± 0.7	1.4 ± 1.0	1.3 ± 1.4	1.3 ± 1.2	↑ 46%	0.01
Ceut705 *C. eutactus, Coprococcus* sp.	3.0 ± 4.4	4.5 ± 5.1	5.6 ± 6.7	4.1 ± 5.2	↑ 32%	ns
Chis150 *Clostridium histolyticum*	0.6 ± 1.2	1.4 ± 3.6	2.5 ± 4.5	1.5 ± 2.1	↑ 60%	ns
Cor653 *Coriobacterium* group	0.5 ± 0.8	0.8 ± 1.0	1.2 ± 2.2	1.2 ± 1.5	↑ 42%	ns
Cvir1414 *Clostridium* viride group	1.9 ± 2.1	3.4 ± 2.2	4.2 ± 2.5	4.0 ± 2.1	↑ 53%	< 0.001
Ecyl387 *Eubacterium cylindroides*	0.7 ± 0.5	0.7 ± 0.4	1.4 ± 1.1	1.2 ± 0.7	↑ 42%	0.01
Ehal1469 *Eubacterium hallii*	0.6 ± 0.9	0.6 ± 1.1	0.7 ± 0.8	0.7 ± 0.8	↔	ns
Eram997 *Eubacterium ramulus*	0.3 ± 1.3	0.03 ± 0.04	0.7 ± 1.4	1.0 ± 1.5	↑ 70%	ns
Lab158 *Lactobacillus* sp., *Enterococcus* sp.	0.1 ± 0.2	0.8 ± 1.1	1.7 ± 3.0	0.5 ± 0.9	↑ 80%	0.02
Muc1437 *Akkermansia muciniphila*	2.8 ± 3.9	1.8 ± 3.2	6.5 ± 7.4	7.8 ± 8.9	↑ 64%	0.015
Myc657 *Mycobacterium* subdivision (mycolic acid-containing *Actinomycetes*)	3.1 ± 1.5	2.5 ± 1.3	1.6 ± 1.1	1.9 ± 1.9	↓ 39%	ns
Phasco741 *Phascolarctobacterium faecium*	0.6 ± 0.9	0.9 ± 0.8	0.8 ± 0.8	1.1 ± 1.1	↑ 45%	ns
Pnig657 *Prevotella nigrescens*	2.2 ± 3.7	0.7 ± 1.3	2.6 ± 3.1	1.7 ± 2.3	↔	ns
ProCo1264 *Ruminococcus productus*	0.7 ± 2.0	1.5 ± 2.6	1.4 ± 2.3	1.9 ± 3.7	↑ 63%	ns
Rfla729 *Ruminococcus albus*	2.2 ± 3.2	5.5 ± 5.0	4.7 ± 5.0	3.9 ± 4.6	↑ 44%	0.02
SFB1 *Segmented filame(14%ntous bacteria*	2.3 ± 3.3	1.6 ± 2.7	2.3 ± 1.6	2.9 ± 1.9	↔	ns
SNA *Sphaerotilus natans*	4.3 ± 3.7	6.1 ± 5.4	6.8 ± 5.9	5.9 ± 5.8	↑ 27%	ns
Strc493 most *Streptococcus* spp.	1.3 ± 3.3	0.5 ± 1.1	1.9 ± 3.9	3.7 ± 4.9	↑ 65%	ns
SUBU1237 *Burkholderia* spp., *Sutterella* spp.	1.7 ± 2.6	3.4 ± 2.6	5.6 ± 4.4	5.6 ± 4.2	↑ 69%	0.001
Urobe63a *Ruminococcus obeum*-like	1.6 ± 2.3	2.2 ± 2.3	2.5 ± 2.9	3.2 ± 2.4	↑ 50%	0.05
Veil223 *Veilonella*	0.1 ± 0.3	0.1 ± 0.4	0.6 ± 1.4	0.9 ± 2.1	↑ 88%	ns
Ver620 *Verrucomicrobium*	1.7 ± 3.9	0.5 ± 1.6	1.1 ± 3.2	1.9 ± 5.3	↔	ns
Individual marginal or accidental mean 150 (*n* = 6)						
Cperf191 *Clostridium perfringens*	0.001	0	0	0.001	↔	ns
Efaec *Enterococcus faecalis*	0.01 ± 0.02	0.01 ± 0.03	0.01 ± 0.02	0.01 ± 0.03	↔	ns
MIB724 mouse intestinal bacteria	0.01 ± 0.06	0.001 ± 0.002	0.01 ± 0.03	0.07 ± 0.1	↔	ns
Pce *Burkholderia* spp.	0.09 ± 0.30	0.03 ± 0.10	0.3 ± 0.7	0.07 ± 0.20	↔	ns
Rbro730 *Clostridium sporosphaeroides, Ruminococcus bromii, Clostridium leptum*	0.04 ± 0.20	0.08 ± 0.30	0.8 ± 3.0	0.1 ± 0.5	↑	ns
Urobe63b *Ruminococcus obeum*-like	0.01 ± 0.04	0.0001	0.001	0.6 ± 1.1	↔	ns

The response to humic acids of single bacterial groups was principally the same as in all functional sets of substantial bacteria. The concentrations of most bacterial groups within essential (2 of 3) pioneer (3 of 4) and individual substantial groups (19 of 28) increased in rates of 20% to 60%. In most cases, the increase was observed already at day 10 and continued to day 45. In groups with comparatively low initial mean concentrations (Ebac1790, Cdif198, Chis150, Eram997, Lab158, Veil223) an increase could be higher than 70% and up to 96%, but the contribution of these groups to the overall bacterial numbers was relatively low. Only the concentrations of bacteria detected with Bac303 (*Bacteroides*) and Myc657 (mycolic acid-containing *Actinomycetes)* FISH probes decreased under humic acids supplementation, but was statistically not significant, because of the high variance and low number of probands.

The increase in concentrations of microbiota was caused by preexisting groups, and not due to emerging new microorganisms. The individual microbial profile remained constant. In none of the test persons did

the ratio of positive/negative individual groups change more than 5%.

Humic acid supplementation did not affect microbial diversity. Mean percent of substantial individual bacterial groups positive for bacteria for each person was nearly the same over time with 72%; 74%; 76%; 72% at the control days accordingly.

The patterns in distribution of single bacterial groups over the stool cylinder differed depending on the species but remained the same in the mucus close transient zone and in the center of the fecal cylinder regardless of humic acids supplementation.

DISCUSSION

The dietary supplementation of humic acids for medical purposes and for promotion of health is deeply rooted in cultural traditions. The humus and its components are regarded as something purely biological, nature promoting and positive. However, the mechanisms how humic acids may work are purely understood. The sheer indefinite number of chemically active functional groups within the extreme complex chemical structure of humic substances makes biochemical investigations elaborate, costly and difficult to reproduce[1]. Even apparent effects of humic acids on the quality of soil and its microbiome remain vague and general as to ancient times and are up to now not disclosed in specific verifiable details[8].

Our data first demonstrate that the humic acids are indeed global fertilizers of microbial growth as proposed by traditional view and lead to an increase of more than 30% in the mean concentrations of the colonic microbiome ($P < 0.001$). The promotion of microbial growth involved 24 of 35 investigated substantial bacterial groups. The only investigated microbial groups that were negatively affected by humic acids were *Bacteroides* (Bac303) and *Mycobacterium* subdivision mycolic acid-containing *Actinomycetes* (Myc657). All other investigated groups were either increased or not affected.

In newborns, during stress, convalescence or disease, pioneer bacteria increase exponentially up to ranges otherwise typical for essential bacterial groups[7]. We did not observe such a reaction in our study. The most profound increase in concentrations to 41% ($P < 0.002$) was that of the individual substantial bacteria. The increase in concentrations of the pioneer groups was lower (28%) and statistically not significant, indicating that host stress and convalescence of the colonic microbiome are not present. Lack of functional stress is also supported by the fact that the individual microbial profiles in all subjects remained stable over the observation period and that the patterns in distribution of bacteria over the fecal cylinder did not change under humic acids application.

The comparatively low increase (14%, $P = 0.02$) of the essential bacterial groups observed in our study was due to suppression of *Bacteroides*, and probably further resulted from the fact, that essential bacterial groups are already normally maximal promoted by the host and their growth cannot be endlessly boosted.

Aside of the numeric impact on the microbiome, we do not know which clinical effects humic acids promoted, since all test persons were healthy and all, except one, tolerated Activomin® without negative or apparent positive health effects.

However, reduced diversity and concentrations of colonic microbiota were demonstrated in IBD[9], IBS and non-gastroenterological diseases such as obesity, diabetes, rheumatism and multiple sclerosis[10-13]. These changes in the microbiome are claimed responsible for pathogenies of multiple other diseases. To repair the disordered microbiome, fecal transplantation and probiotics have been recommended and clinically tested. However, such transfections are difficult to control and do not guarantee that the transferred microorganisms prevail, settle and proliferate in the colon[14].

Humic acids exert profound effects on the colonic microbiota and may be an interesting group of substances for the development of specific drugs, which deliberately influence colonic fermentation in an inflamed colon, obesity, rheumatic and neurologic disorders.

COMMENTS

Background

Patients demonstrate increasing interest in medical treatments that are not part of mainstream medicine. Critical argumentation is important, but difficult to do when not evaluated with scientific methods. Humins are a product of microbial metabolism and an important mediator of microbial interactions and activity. As natural fertilizers humins are used for medical indications since ancient times. It is believed that the human microbiome is the main target of humic activity. However no data exist on the impact of humins on the human microbiome.

Research frontiers

An evergrowing number of studies demonstrate the involvement of the colonic microbiome in obesity, digestive, endocrine, inflammatory and auto-immune and neurologic disorders. Different approaches are proposed to consolidate and improve the colonic microbiome. The research hotspot is to move beyond description and to introduce substances and therapies with proven controlled graduate effects on the microbiome.

Innovations and breakthroughs

The presented results show, that orally applied humic acids have a profound effect on the healthy colonic microbiome. Although the effects on single microbial groups were multidirectional, the sum concentrations of all colonic microbiota increased 20% to 30%. The increase occurred in the preexisting microbial groups without changes in the bacterial diversity of the microbiome.

Applications

The main message of our study is, that humic acids may be an interesting substrate for the development of defined drugs, which deliberately control colonic fermentation in conditions where it is suppressed (post-antibiotics, convalescence) or altered (metabolic disorders, inflammation, obesity *etc.*), and are a serious amendment/alternative to fecal transplantation or probiotics.

Terminology

FISH - fluorescence *in situ* hybridization Cy3, FITC, Cy5, DAPI - different fluorescent dyes corresponding to orange, green, dark red and blue colours. Fluorescence *in situ* hybridization (FISH) combines the specific identification of microorganisms and the morphological aspect and is as a consequence especially helpful for these purposes. Each single bacterium possesses 10^3-10^5 ribosomes of which each ribosome owns the same copy of ribosomal RNA. Some of the regions of the rRNA are strain-specific, others are universal for species, families or even kingdoms. Oligonucleotides synthesized complimentary to rRNA sequences and labelled with fluorescent dye are called FISH probes. When added to samples containing bacteria, FISH probes hybridize with the rRNA of the bacterial ribosomes. No additional enhancement of fluorescence is necessary and bacteria can be visualized directly due to the large number of ribosomes in each bacterium.

Peer-review

Although the scientific literature reveals a large number of medical trials with dietary supplements of humic acids conducted all over the world. None of the previous studies investigated effects of humic acids on the colonic microbiome.

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ORIGINAL ARTICLE

Observational Study

Early thrombomodulin-α administration outcome for acute disseminated intravascular coagulopathy in gastrointestinal surgery

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Author contributions: Konishi H, Okamoto K, Ichikawa D, Fujiwara H and Otsuji E designed the research; Konishi H, Shoda K, Arita T, Kosuga T, Morimura R, Komatsu S, Murayama Y, Shiozaki A, Kuriu Y, Ikoma H, and Nakanishi M performed the research and treated the patients; Konishi H and Okamoto K analyzed the data; Konishi H, Okamoto K and Otsuji E wrote the manuscript.

Institutional review board statement: We did not seek individual ethical approval by the Facility of Science Committee at Kyoto Prefectural University of Medicine because this study was a retrospective observational study without interpositions and with the medical practice necessary for therapeutic purposes.

Informed consent statement: All study participants provided informed written consent prior to their treatments and study enrollment.

Conflict-of-interest statement: All authors declare no conflict of interest related to this study or its publication.

Data sharing statement: The technical appendix, statistical code and dataset are available from the corresponding author at h-koni7@koto.kpu-m.ac.jp.

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Abstract

AIM

To investigate the efficacy of thrombomodulin (TM)-α for treatment of disseminated intravascular coagulopathy (DIC) in the field of gastrointestinal surgery.

METHODS

Thirty-six peri-operative DIC patients in the field of gastrointestinal surgery who were treated with TM-α were retrospectively investigated. The relationships between patient demographics and the efficacy of TM-α were examined. Analysis of survival at 28 d was also performed on some parameters by means of the Kaplan-Meier method. Relationships between the ini-

tiation of TM-α and patient demographics were also evaluated.

RESULTS

Abscess formation or bacteremia was the most frequent cause of DIC (33%), followed by digestive tract perforation (31%). Twenty-six patients developed DIC after surgery, frequently within 1 wk (81%). TM-α was most often administered within 1 d of the DIC diagnosis (72%) and was continued for more than 3 d (64%). Although bleeding tendency was observed in 7 patients (19%), a hemostatic procedure was not needed. DIC scores, systemic inflammatory response syndrome (SIRS) scores, quick-sequential organ failure assessment (qSOFA) scores, platelet counts, and prothrombin time ratios significantly improved after 1 wk ($P < 0.05$, for all). The overall survival rate at 28 d was 71%. The duration of TM-α administration ($\geqslant 4$, $\leqslant 6$) and improvements in DIC-associated scores (DIC, SIRS and qSOFA) at 1 wk were significantly better prognostic factors for 28-d survival ($P < 0.05$, for all). TM-α was administered significantly earlier to patients with severe clinical symptoms, such as high qSOFA scores, sepsis, shock or high lactate values ($P < 0.05$, for all).

CONCLUSION

Early administration of TM-α and improvements in each parameter were essential for treatment of DIC. The diagnosis of patients with mild symptoms requires further study.

Key words: Quick-sequential organ failure assessment; Thrombomodulin-α; Gastrointestinal surgery; Systemic inflammatory response syndrome; Acute disseminated intravascular coagulopathy

Core tip: The present study investigated the efficacy of thrombomodulin (TM)-α for treatment of disseminated intravascular coagulopathy (DIC) in the field of gastrointestinal surgery. DIC frequently developed within 1 wk of surgery. TM-α was frequently administered within 1 d of the DIC diagnosis and was continued for more than 3 d. The duration of TM-α administration and improvements in DIC-associated parameters at 1 wk were better prognostic factors for 28-d survival. TM-α was administered significantly earlier to patients with severe clinical symptoms. The early administration of TM-α and improvements in DIC parameters were essential for the treatment of DIC.

Konishi H, Okamoto K, Shoda K, Arita T, Kosuga T, Morimura R, Komatsu S, Murayama Y, Shiozaki A, Kuriu Y, Ikoma H, Nakanishi M, Ichikawa D, Fujiwara H, Otsuji E. Early thrombomodulin-α administration outcome for acute disseminated intravascular coagulopathy in gastrointestinal surgery. *World J Gastroenterol* 2017; 23(5): 891-898 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i5/891.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i5.891

INTRODUCTION

Disseminated intravascular coagulopathy (DIC) is characterized by the systemic activation of blood coagulation and continuous generation of intravascular fibrin, which contribute to multiple organ dysfunction syndrome or other life-threating conditions[1,2]. In the field of gastrointestinal surgery, DIC has been reported as a consequence of emergency surgery, severe complications or some types of malignancies[3-5], and as a frequent complicating factor for conditions leading to sepsis or a shock status. The acute DIC scoring system put forth by the Japanese Association for Acute Medicine (JAAM) is in widespread use for diagnosis of acute DIC[6,7]. In order to effectively treat DIC, the underlying causes need to be improved and appropriate drugs need to be administered intensively in the earliest stage possible. However, survival rates are never high, as has been previously reported[1,2].

In DIC, the activation of coagulation and inhibition of fibrinolysis lead to a hypercoagulable state and the deposition of fibrin in micro-vessels. Thrombomodulin-α (TM-α) is a recombinant human soluble thrombomodulin, which is a thrombin receptor on endothelial cell surfaces[8-10]. Thrombin binds to TM-α, and the thrombin-TM-α complex inactivates intravascular coagulation by activating the protein C pathway. The subsequent formation of thrombin and triggering of inflammatory reactions are regulated by TM-α, after which hypercoagulable DIC states become improved. Therefore, TM-α was approved as a curative medicine for the treatment of DIC in 2008, and its effects on DIC have been investigated in multicenter randomized clinical trials in Japan[8,11]. Resolution rates for DIC and bleeding symptoms were found to be significantly better for patients treated with TM-α than those treated with heparin[12,13].

Previous studies have determined the efficacy of TM-α treatments for DIC associated with gastroenterological surgery[4,5]; however, the data are still insufficient to establish the optimal therapeutic strategies for hematological malignancies or infections such as sepsis. The optimal initiation time or duration of the administration of TM-α and the predictive factors for therapeutic efficacy remain unclear for actual clinical practice. In the present study, the treatment of DIC by TM-α in the field of gastrointestinal surgery was retrospectively summarized, and outcomes were investigated.

MATERIALS AND METHODS

Patients and treatment courses

Thirty-six patients were retrospectively investigated. Each had been diagnosed with DIC in the peri-operative

Table 1 Baseline demographics

Subjects	*n* = 36
Sex, F/M	12/24
Age, median (range)	71 (48-86)
Underlying disease	
Perforation	
Gastric	1
Small intestine	2
Colo/rectal	8
Abscess/bacteremia	12
Ileus	3
Pancreatitis	2
Pneumonia	5
Drug-induced	3
Peri-operative, no/yes	10/26
Cancer-associated, no/yes	23/13
Post-operative day, ≤ 7/> 7	21/5
Combination treatment for DIC	
Unfractionated heparins	4
Anti-thrombin concentrates	28
γ-globulin agents	29
Vasopressors	26
Protease inhibitors	4
Sivelestat sodium hydrates	4
Steroid preparations	7
Dialysis	5
Blood transfusion	4

DIC: Disseminated intravascular coagulopathy.

Table 2 Patient characteristics

Subjects		*n* = 36
DIC scores	4	15
Before the treatment	5	9
(JAAM criteria)	6	8
	7	1
	8	3
SIRS scores	0/1	5
Before the treatment	2	9
	3	15
	4	7
qSOFA scores	0	10
Before the treatment	1	4
	2	17
	3	5
Duration of DIC	-2/-1	4
Before the administration of TM-α (d)	0	16
	1	10
	2	1
	3	4
	≥ 4	1
Duration of administration	1	5
	2	5
	3	3
	4	3
	5	9
	6	4
	≥ 7	7

DIC: Disseminated intravascular coagulopathy; JAAM: Japanese Association for Acute Medicine; qSOFA: Quick-sequential organ failure assessment; SIRS: Systemic inflammatory response syndrome. TM-α: Thrombomodulin-α.

period and treated with TM-α between January 2012 and December 2015 at the Division of Digestive Surgery in Kyoto Prefectural University of Medicine (Japan). The JAAM DIC scoring system was applied as the diagnostic criteria for DIC (DIC score ≥ 4)[6,7]. The baseline demographics and characteristics of patients are summarized in Tables 1 and 2. Some types of digestive cancers were the cause of DIC in 13 patients (cancer-associated).

TM-α (Recomodulin® injection; Asahi Kasei Pharma Corporation, Tokyo, Japan) was administered intravenously at a dose of 380 U/kg per day and continued as necessary[8-10]. This dose was decreased to 130 U/kg in a patient with severe renal failure, according to attending physicians' decision and the manufacturer's instructions. Particularly, the patients who needed dialysis or were considered to have increased creatinine and decreased eGFR were given a reduced dose of TM-α. The duration of the administration of TM-α, and its combined usage with other treatment drugs, were also decided by the attending physicians. Clinicopathological and laboratory data obtained at 1 wk and 2 wk after the initiation of TM-α administration were investigated, and the mortality rate at 28 d was determined.

This study was conducted in accordance with the principles of the Declaration of Helsinki, and written informed consent for the treatment and data collection was obtained from all patients. We did not seek individual ethical approval by the Facility of Science Committee at Kyoto Prefectural University of Medicine because this was a retrospective observational study without interpositions and with the medical practice necessary for therapeutic purposes.

Diagnostic criteria for DIC

In the present study, the following criteria were employed to diagnose DIC and compare treatment efficacies. Systemic inflammatory response syndrome (SIRS) scores were evaluated according to a previous study[14] and a SIRS score of ≥ 3 was converted to 1 point for the JAAM DIC score[7]. Quick sequential organ failure assessment (qSOFA) scores were determined by more than 1 point of altered mentation, systolic blood pressure of ≤ 100 mmHg, and respiratory rate of ≥ 22/min[15]. With respect to updates to the definitions for sepsis and septic shock criteria, sepsis was determined by more than 1 point of the qSOFA score in the present study[15], while traditional sepsis was defined by the existence of infection and SIRS. Shock was defined by a serum lactate level of > 2 mmol/L and a requirement for vasopressors to maintain mean arterial pressure despite adequate fluid resuscitation[15].

Statistical analysis

Statistical analyses were performed using the JMP 12 software program. The Wilcoxon signed-rank test was used to analyze the relationships between various biochemical measurements. A survival curve for overall survival was derived using the Kaplan-Meier method

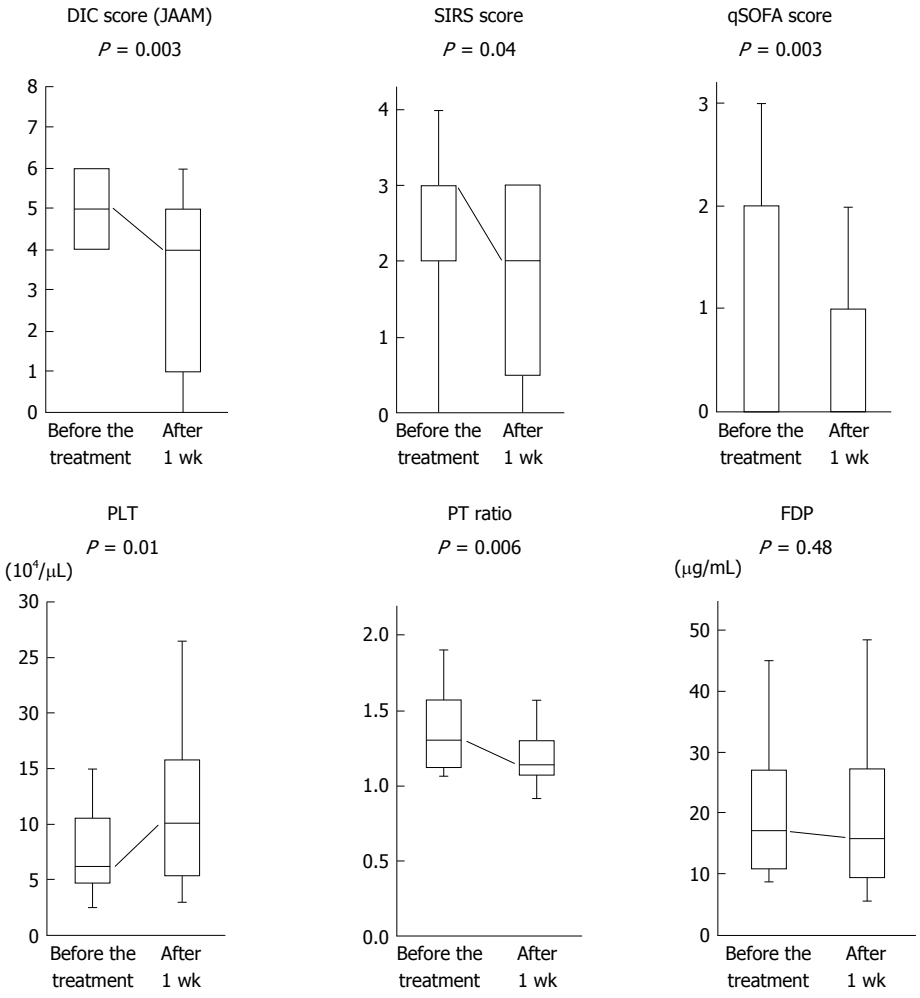

Figure 1 Alterations in disseminated intravascular coagulopathy-associated parameters between before and after 1 wk of treatment with thrombomodulin-α. Disseminated intravascular coagulopathy (DIC) scores [Japanese Association for Acute Medicine (JAAM)], systemic inflammatory response syndrome (SIRS) scores, quick-sequential organ failure assessment (qSOFA) scores, platelet (PLT) counts, prothrombin time (PT) ratios, and fibrin degradation products (FDP) values were compared for before and after 1 wk of thrombomodulin-α (TM-α) treatment. All parameters, except for FDP values, were significantly improved by TM-α administration after 1 wk. The Mann-Whitney *U*-test was used for the analysis.

and compared by the stratified log-rank test. A *P* value less than 0.05 was considered significant.

RESULTS

Baseline demographics and characteristics of patients

Clinical data of the 36 patients in this study are summarized in Tables 1 and 2. DIC was caused by a wide variety of diseases, with abscess formation or bacteremia after surgery being the most frequent cause (12/36, 33%), followed by perforation of the digestive tract (11/36, 31%). Twenty-six patients (72%) developed DIC after surgery, frequently within 1 wk of surgery (21/26, 81%). TM-α was frequently used in conjunction with other drugs and treatments, such as combined administration with anti-thrombin concentrates, γ-globulin agents, and vasopressors. Unfractionated heparins were administered to 4 patients (11%) as an alternative to TM-α.

A number of patients were diagnosed as having DIC with JAAM score of 4 or 5 (24/36, 67%). At the time of the DIC diagnosis, 5 (14%) and 14 (39%) patients did not fulfill the criteria of SIRS (≥ 2) and qSOFA (≥ 2), respectively. For most patients, TM-α was administered within 1 d of the DIC diagnosis (26/36, 72%) and was continued for more than 3 d (23/36, 64%). However, 5 patients (14%) were administered TM-α for only 1 d; the reasons for the discontinuation of its administration are listed in Table 3. Although bleeding tendency was observed in 7 patients (19%), severe bleeding was not observed and a hemostatic procedure was not required.

Effects of TM-α administration on DIC parameters

Figure 1 shows alterations in each DIC-associated parameter between before and after 1 wk of the treatment in patients administered TM-α for more than 1 d. DIC scores (*P* = 0.003), SIRS scores (*P* = 0.04), qSOFA scores (*P* = 0.003), platelet counts (*P* = 0.01) and prothrombin time ratios (*P* = 0.006) were significantly improved after 1 wk of the treatment.

Table 3 Reasons for discontinuation of thrombomodulin-α

Duration (d)	Total number	Cases	Reasons
1	5	2	Dialysis
		3	Bleeding tendency
2	5	3	Death
		2	Bleeding tendency
3	3	1	Resolved
		2	Bleeding tendency
4	3	3	Resolved

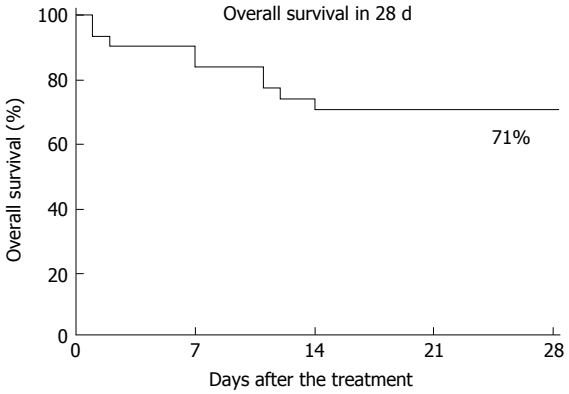

Figure 2 Survival analysis of the disseminated intravascular coagulopathy patients treated with thrombomodulin-α. Overall survival at 28 d was examined using the Kaplan-Meier method (n = 31) in order to evaluate the efficacy of thrombomodulin-α (TM-α) administration. The patients used in this analysis were treated with TM-α more than 1 d, and the overall survival rate was 71%.

C-reactive protein and creatinine values were also improved (data not shown).

Survival after TM-α administration

The overall survival at 28 d for all patients administered TM-α for more than 1 d is shown in Figure 2, and the overall survival rate was 71%. A survival analysis on some parameters is shown in Table 4. The duration of administration (≥ 4, ≤ 6; P = 0.03) and improvements in DIC scores (P = 0.01), SIRS scores (P = 0.09) and qSOFA scores (P = 0.001) at 1 wk were significant prognostic factors for 28-d survival.

Relationships between the initiation of treatment and patient demographics

In the survival analysis, patients administered TM-α within 1 d of the DIC diagnosis had slightly better prognoses than those administered it after 2 d (74% *vs* 50%; Table 4). TM-α was administered significantly earlier for patients with severe clinical symptoms at the time of DIC diagnosis, such as high qSOFA scores (P = 0.001), sepsis (P = 0.001), shock (P = 0.02) or high lactate values (P = 0.02) (Table 5).

DISCUSSION

DIC is not prevalent in the field of gastrointestinal surgery, but it is life-threating once it develops[4]. Early intensive care, including the administration of anti-thrombin concentrates and γ-globulin agents, has been shown to effectively improve the prognosis of patients with DIC[13,16]. The early administration of TM-α has also been reported to improve severe DIC and prognoses in the field of gastrointestinal surgery[3-6].

In the present study, peri-operative DIC patients in the field of gastrointestinal surgery who were treated with TM-α were retrospectively investigated. Some DIC-associated parameters, such as DIC (JAAM), SIRS and qSOFA scores, were significantly improved at 1 wk after the initiation of the TM-α treatment, and these improvements correlated with the better 28-d survival rate. On the other hand, in spite of the diagnosis of DIC, the administration of TM-α was significantly delayed in patients with mild symptoms, such as low SIRS or qSOFA scores, and the absence of sepsis or shock.

In order to achieve an early and accurate diagnosis of DIC, not only the counts of each parameter but also the changes in platelet counts are important. In the present study, the DIC scores of 4 patients were decided by changes in platelet counts measured in a 24-h period (data not shown). DIC scores were found to increase due to decrease in platelet counts of > 30% or 50% in a 24-h period, and further reductions of platelet counts were observed on the next day. Therefore, the rate of decreases in platelet counts is also important for reaching an early decision on the DIC score.

In the survival analysis, the overall survival rate at 28 d (71%) was similar to previous findings[4]. On the other hand, the survival rate of patients administered TM-α at 2 d after the DIC diagnosis was slightly worse (74% *vs* 50%). Early treatments, including TM-α, are generally considered to be advantageous for improving the prognosis of DIC patients[5,6,10]. In the present study, the administration of TM-α was significantly delayed (by more than 1 d after the DIC diagnosis) in patients who were less symptomatic (*i.e.*, not meeting the criteria of qSOFA, sepsis or shock and having low lactate values). Therefore, early and accurate diagnosis of DIC and initiation of treatments will be needed for all patients with mild symptoms who are suspected of having DIC.

The definitions of sepsis and shock were recently revised[15]. Previously, traditional sepsis had been defined as the presence of infection and SIRS, but it is now defined by an increase in the SOFA score (≥ 2). Moreover, shock is defined by a requirement for vasopressors and enhanced serum lactate levels (> 2 mmol/L). In the present study, we used qSOFA scores exclusively because we were unable to confirm all data to provide an accurate SOFA score.

The indication of TM-α administration is decided by the JAAM DIC score (≥ 4) only, and these DIC patients frequently present with accompanying severe complications such as shock or sepsis[2,13-16]. It remains controversial whether these severe conditions can

Table 4 Survival analysis at 28 d after thrombomodulin-α administration

Factor		*n* = 31	28-d survival rate	*P* value
Sex	Male	22	73%	0.83
	Female	9	67%	
Age	≤ 70	14	64%	0.54
	> 70	17	76%	
Duration of administration	≥ 4, ≤ 6	16	88%	0.03
	≤ 3, ≥ 7	15	53%	
Initiation of administration after DIC (d)	≤ 1	27	74%	0.43
	≥ 2	4	50%	
DIC scores before the treatment	≤ 5	21	67%	0.52
	≥ 6	10	80%	
Improvement in DIC scores at 1 wk	≤ 3	14	93%	0.01
	≥ 4	17	53%	
SIRS scores before the treatment	≤ 2	12	58%	0.2
	≥ 3	19	79%	
Improvement in SIRS scores at 1 wk	≤ 2	21	86%	0.09
	≥ 3	7	57%	
qSOFA scores before the treatment	≤ 1	11	73%	0.8
	≥ 2	20	70%	
Improvement in qSOFA scores at 1 wk	≤ 1	22	91%	0.001
	≥ 2	6	33%	
Sepsis	Present	20	70%	0.8
	Absent	11	73%	
Shock	Present	16	81%	0.24
	Absent	15	60%	
Lactate values before the treatment	≥ 2	8	75%	0.69
	< 2	18	83%	

DIC: Disseminated intravascular coagulopathy; qSOFA: Quick-sequential organ failure assessment; SIRS: Systemic inflammatory response syndrome.

Table 5 Relationships between treatment initiation and patient demographics

Factor		Treatment initiation after DIC		*P* value
		≤ 0 d	≥ 1 d	
Duration of administration	≥ 4, ≤ 6	9	7	0.94
	≤ 3, ≥ 7	11	9	
DIC scores before the treatment	≤ 5	12	12	0.34
	≥ 6	8	4	
SIRS scores before the treatment	≤ 2	6	8	0.22
	≥ 3	14	8	
qSOFA scores before the treatment	≤ 1	3	11	0.001
	≥ 2	17	5	
Sepsis	Present	17	5	0.001
	Absent	3	11	
Shock	Present	13	4	0.02
	Absent	7	12	
Lactate values before the treatment	≤ 3	6	10	0.02
	> 3	11	3	

DIC: Disseminated intravascular coagulopathy; qSOFA: Quick-sequential organ failure assessment; SIRS: Systemic inflammatory response syndrome.

influence the therapeutic effects of DIC. In the present study, the parameters showing severe conditions were also investigated, but were found to not significantly affect the efficacy of TM-α or prognosis of patients.

The present study had some limitations. The number of patients examined was small because DIC is not prevalent in the field of gastrointestinal surgery. Furthermore, DIC scores were retrospectively evaluated by only the JAAM acute DIC scoring system, and we did not confirm that the attending physicians gave accurate DIC scores at diagnosis. In the future, the comparisons with other criteria, such as the International Society for Thrombosis and Haemostasis DIC score, will be needed. In previous studies, DIC patients treated with TM-α for approximately 6 d have been commonly evaluated[4,10], while patients in the present study were treated for shorter or longer durations. Some of our patients who were administered TM-α for a shorter duration showed amelioration of the DIC, whereas many patients with a shorter or longer duration of administration had worse prognoses. A 6-d administration is needed if patient conditions permit it, and the advantages and disadvantages of the early discontinuation of administration due to improvements

in DIC require further investigations. Another limitation is that all patients in this study were treated with TM-α and other drugs, and comparisons of the efficacy of the treatments, prognosis of patients or development of side effects between TM-α and the other drugs were not performed.

In conclusion, although the number of patients examined in the present study was small, we herein demonstrated that the early diagnosis of DIC and initiation of the TM-α administration are effective for achieving improvements in DIC in the field of gastrointestinal surgery. The diagnosis of patients with mild symptoms requires further study.

COMMENTS

Background

Disseminated intravascular coagulopathy (DIC) has been reported in the field of gastrointestinal surgery as a consequence of emergency surgery, severe complications or some types of malignancies, and has been shown to frequently complicate conditions leading to sepsis or a shock status. In order to effectively treat DIC, the underlying causes need to be resolved, or at least improved, and appropriate drugs need to be administered intensively at earlier stage. However, survival rates are never high, as has been reported consistently.

Research frontiers

Thrombomodulin-α (TM-α) was approved as a curative medicine for the treatment of DIC, and previous studies have reported its efficacy for DIC associated with gastrointestinal surgery. However, the data are still insufficient to establish optimal therapeutic strategies. The research hotspot is the introduction of an optimized treatment with TM-α and identification and clinical application of predictive factors to improve therapeutic efficacy of DIC in the field of gastrointestinal surgery.

Innovations and breakthroughs

DIC is not prevalent in the field of gastrointestinal surgery, but is life-threating once it develops. In the present study, some DIC-associated parameters, such as DIC (Japanese Association for Acute Medicine), systemic inflammatory response syndrome (SIRS) and quick-sequential organ failure assessment (qSOFA) scores, were significantly improved at 1 wk after the initiation of TM-α treatment, and these improvements correlated with better 28-d survival. Early diagnosis of DIC and initiation of the TM-α administration are effective for achieving improvements in DIC in the field of gastrointestinal surgery. On the other hand, in spite of DIC diagnosis, administration of TM-α was significantly delayed in patients with mild symptoms, such as low SIRS or qSOFA scores, and the absence of sepsis or shock.

Applications

The data in this study suggested that early diagnosis of DIC and initiation of the TM-α administration are clinically effective for DIC treatment in the field of gastrointestinal surgery. Furthermore, this study also provided readers with important information regarding the delay of TM-α administration for less symptomatic DIC patients.

Terminology

DIC leads to a hypercoagulable state and the deposition of fibrin in microvessels. TM-α is a recombinant human soluble thrombomodulin, which is a thrombin receptor on endothelial cell surfaces. Thrombin binds to TM-α, and the thrombin-TM-α complex inactivates intravascular coagulation by activating the protein C pathway. Therefore, the further formation of thrombin and triggering of inflammatory reactions are regulated by TM-α, and hypercoagulable DIC states are improved.

Peer-review

This manuscript reports on the early diagnosis and earlier initiation of recombinant thrombomodulin for DIC patients in the field of digestive surgery. Identification of an optimized treatment with TM-α and clinical application of predictive factors will be very useful for DIC treatment, improving the therapeutic efficacy.

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ORIGINAL ARTICLE

Prospective Study

Frailty is independently associated with increased hospitalisation days in patients on the liver transplant waitlist

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Abstract

AIM

To investigate the impact of physical frailty on risk of hospitalisation in cirrhotic patients on the liver transplant waitlist.

METHODS

Cirrhotics listed for liver transplantation at a single centre underwent frailty assessments using the Fried Frailty Index, consisting of grip strength, gait speed, exhaustion, weight loss, and physical activity. Clinical and biochemical data including MELD score as collected at the time of assessment. The primary outcome was number of hospitalised days per year; secondary outcomes included incidence of infection. Univariable and multivariable analysis was performed using negative binomial regression to associate baseline parameters including frailty with clinical outcomes and estimated incidence rate ratios (IRR).

RESULTS

Of 587 cirrhotics, 64% were male, median age (interquartile range) was 60 (53-64) years and MELD score was 15 (12-18). Median Fried Frailty Index was 2 (1-3); 31.6% were classified as frail (fried frailty $\geqslant$ 3). During 12 mo of follow-up, 43% required at least 1

hospitalisation; 38% of which involved major infection. 107/184 (58%) frail and 142/399 (36%) non-frail patients were hospitalised at least once ($P < 0.001$). In univariable analysis, Fried Frailty Index was associated with total hospitalisation days per year (IRR = 1.51, 95%CI: 1.28-1.77; $P \leqslant 0.001$), which remained significant on multivariable analysis after adjustment for MELD, albumin, and gender (IRR for frailty of 1.21, 95%CI: 1.02-1.44; $P = 0.03$). Incidence of infection was not influenced by frailty.

CONCLUSION

In cirrhotics on the liver transplant waitlist, physical frailty is a significant predictor of hospitalisation and total hospitalised days per year, independent of liver disease severity.

Key words: Hospitalisation; Infection; Cirrhosis; Frailty; Transplantation

Core tip: This study demonstrates a significant independent link between bedside measures of physical frailty and risk for hospitalisation in cirrhotic patients on the liver transplant waitlist. This adds to previous data showing a link between frailty and mortality in cirrhosis, and therefore allows us to better select at-risk cirrhotic patients who are most in need of more intense chronic disease management programs.

Sinclair M, Poltavskiy E, Dodge JL, Lai JC. Frailty is independently associated with increased hospitalisation days in patients on the liver transplant waitlist. *World J Gastroenterol* 2017; 23(5): 899-905 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i5/899.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i5.899

INTRODUCTION

The most commonly used tool to prioritise patients for liver transplantation is the MELD or MELD-sodium score[1], which fails to capture the decline in systemic health suffered by many liver transplant candidates. This is particularly relevant as liver transplant recipients are ageing and accumulating comorbidities[1]. Muscle wasting and weakness are incredibly common, observed in up to 70% of waitlisted individuals[2]. Both quantitative measures of muscle mass and functional measures of muscle strength have been associated with waitlist mortality, infection and post-transplant complications[3-5].

Frailty is a multi-system disorder that is classically associated with ageing, disability and comorbidity, and is known to increase the risk for falls, hospitalisation and mortality[6]. Quantification of frailty is most commonly performed using the Fried Frailty Index. Initially designed for use in geriatric populations, the Fried Frailty Index encompasses handgrip strength, exhaustion, gait speed, unintentional weight loss and physical activity[7]. Some data suggest that functional measures of muscle strength may better predict outcomes in cirrhotics than CT-based measures of sarcopenia[8]. Furthermore, frailty measures can be performed at the bedside, without the need for ionising radiation, which makes them preferable for repeated measures to assess changes over time. This is important as progression of frailty is itself associated with poorer outcome[9].

Physical frailty as measured the by Fried Frailty Index has previously been identified as a risk factor for mortality in cirrhosis[3,4], yet there are little data investigating the impact of frailty on hospitalisation in cirrhosis. An independent link between low muscle mass and infection risk has been identified pre-liver transplantation[5,10], as well as increased hospitalisation days[11], and thus similar findings may be expected for frailty.

This study aims to evaluate the impact of frailty on total number of hospitalisation days. The ultimate goal is to identify an at-risk subset of the cirrhotic population to assist in the development of preventative strategies to improve outcomes in this vulnerable population.

MATERIALS AND METHODS

We report a single-centre prospective observational cohort study of 587 pre-transplant cirrhotics, performed at the University of California, San Francisco, between July 2012 and December 2014.

Subjects

All adult ($\geqslant$ 18 years) cirrhotic subjects actively listed for liver transplantation for cirrhosis are invited to enrol in the ongoing prospective Functional Assessment in Liver Transplantation (FrAILT) study. Ninety-seven percent of invited participants enrol in this study. Enrolment occurs in the outpatient setting as described previously[3]. All patients provided written informed consent. Major exclusion criteria include inability to consent due to severe encephalopathy (numbers count > 120 s), prior transplantation due to the impact of immunosuppressants on muscle function, as well as transplant listing for reasons other than cirrhosis. Patients with incomplete frailty testing measures at baseline or lost-to-follow-up at 12 mo were also excluded.

Baseline variables

At study entry, patient demographics including age, sex, disease aetiology, and medical comorbidities (including hepatocellular carcinoma, diabetes, coronary artery disease and HIV infection) were recorded. Standard baseline biochemical parameters were retrieved from electronic medical records including liver function

tests to calculate MELD score, coagulation profile, full blood count and electrolyte profile. Clinical information regarding the presence of ascites, ascertained by the patient's primary hepatologists, was recorded. Hepatic encephalopathy was assessed using the numbers connection test, with a score > 35 s indicating the presence of encephalopathy[12].

Frailty assessments

Assessments of physical frailty were performed in the outpatient setting using the Fried Frailty Index, consisting of grip strength, gait speed, exhaustion, weight loss, and physical activity. Frail was defined as Fried Frailty Index ⩾ 3 points out of a maximum of 5. These assessments have been validated in geriatric and cirrhotic populations[3,7,9,13].

Short physical performance battery (SPPB) assessment was also undertaken as a second measure to validate findings using the Fried Frailty Index. The SPPB comprises gait speed, standing balance (ability to perform a tandem stand) and chair stands (time taken to complete 5 chair stands. Frail is defined as a SPPB score ⩽ 9. This score has also been associated with poor outcome in cirrhosis[3].

Outcomes

The primary outcome was number of hospitalised days per year during the 12 mo follow-up period immediately following the frailty assessment. This was determined from medical records at the home institution and review of external medical records in the case of hospital admissions elsewhere. Patients who died or were transplanted within 12 mo were censored at this time (n = 82).

Secondary outcomes included number of hospitalisations over 12 mo, length of stay per hospitalisation, and hospitalisation for major infection. Infection was defined according to NACSELD (North American Consortium for Studies of End-Stage Liver Disease) criteria[14], to avoid inadvertent inclusions of subjects receiving empirical antibiotic therapy for liver decompensation.

Alternate causes of hospitalisation were listed as hepatic encephalopathy, acute kidney injury, ascites, gastrointestinal bleeding or other, according to hospital discharge records.

Statistical analysis

The statistical review of the study was performed by a biomedical statistician. Descriptive statistics are displayed as the median [interquartile range (IQR)] unless stated otherwise. Wilcoxon rank-sum and χ^2 tests compared frail *vs* non-frail and hospitalised *vs* non-hospitalised patients,

Univariable negative binomial regression evaluated the association of frailty with hospitalisation days per year and estimated incidence rate ratios (IRR) and 95%CI. Variables significant at the 0.2 level and below were included in the multivariate model. Backward elimination (P > 0.05 for removal) was used to select the final multivariable model.

Logistic regression was used to evaluate the relationship between hospitalisation for infection and frailty. Bivariable regression models estimated OR and 95%CIs for each factor while accounting for observation time. Characteristics with a bivariable P value below 0.2 were assessed in the multivariable model to allow for consideration of all possible contributing factors. Backward elimination (P > 0.05 for removal) identified the subset of variables associated with hospitalisation for infection while adjusting for observation time. Frailty (Fried Frailty Index ⩾ 3) was included in the final model as the predictor of interest.

A cut-off P value less than 0.05 was used to determine statistical significance. Analyses were performed in SAS 9.4 (SAS Institute, Cary NC).

RESULTS

616 consecutive cirrhotic patients were enrolled into the FrAILT study between July 2012 and February 2015. 587 (95%) of these patients had complete data for analysis in this study. The median (IQR) age was 60 (53-64) years, BMI 28.2 (24.8-33.1) cm/m^2, and median MELD score was 15 (12-18) and 64.2% were male. Fifty-seven percent were Caucasian, 26% Hispanic, 7% Asian, 4% African American, and 6% were of other ethnicity. Four patients had missing Fried Frailty Index. Thirty-one point six percent of patients were classified as frail, as defined by a Fried Frailty Index of 3 or above. Frail patients had more severe liver failure than non-frail patients as measured by MELD score and features of decompensation, and lower rates of hepatocellular carcinoma. Frail patients were slightly but significantly older than non-frail patients. Baseline demographics by frailty group are described in Table 1.

Outcomes

During the 12 mo study period, 43% of subjects required at least 1 hospitalisation. The primary reason for hospitalisation was infection in 39%, hepatic encephalopathy in 19%, acute kidney injury or ascites in 16%, GI bleeding in 8% or other miscellaneous cause in 19%. In those patients requiring hospitalisation (n = 243, for eight hospitalised patients the number of hospitalisations is unknown), 54% had a single hospitalisation, 33% had 2 or 3 hospitalisations, and 13% had 4 or more hospitalisations. The median (IQR) length of stay per hospitalisation was 4.5 (3.0-7.5) d.

Risk factors for hospitalisation

Using a Fried Frailty Index of ⩾ 3, frail patients were significantly more likely to be hospitalised, with 58% of frail and 36% of non-frail patients hospitalised at least once in the subsequent 12-mo period (P <

Table 1 Baseline demographics of waitlisted cohort enrolled into the FrAILT study stratified by frail (Fried Frailty Index ≥ 3) and non-Frail (Fried Frailty Index < 3)

Characteristic	Overall (*n* = 587)	Frail (*n* = 184)	Non-frail (*n* = 399)	*P* value
Age (yr)	60 (53-64)	60 (54-64)	59 (52-63)	0.03
Male	64%	60%	66%	0.12
BMI (kg/m^2)	28 (25-33)	28 (25-33)	28 (25-33)	0.74
MELD (points)	15 (12-18)	16 (14-20)	14 (12-17)	< 0.001
Albumin (g/dL)	3.0 (2.6-3.5)	2.9 (2.5-3.2)	3.2 (2.7-3.6)	< 0.001
Sodium (nmol/L)	137 (134-139)	135 (132-138)	137 (135-139)	< 0.001
HCC	33%	25%	36%	0.004
Numbers connection test (s)	40 (30-54)	46 (34-61)	38 (29-51)	< 0.001
Ascites	28%	40%	22%	< 0.001
HIV	2.9%	2.7%	3.0%	0.85
Diabetes	31%	32%	30%	0.46

BMI: Body mass index; MELD: Model for end stage liver disease; HCC: Hepatocellular carcinoma; HIV: Human immunodeficiency virus.

Table 2 Significant differences between patients hospitalised within 12 mo and non-hospitalised individuals

Characteristic	Hospitalised, *n* = 251	Non-hospitalised, *n* = 336	*P* value
Age (yr)	59 (53; 63)	60 (55; 64)	0.02
Male gender	57%	69%	0.003
MELD score	16 (13; 20)	14 (11; 17)	< 0.001
Albumin (g/dL)	2.8 (2.5; 3.2)	3.2 (2.8; 3.7)	< 0.001
Sodium (mmol/L)	136 (133; 139)	137 (135; 139)	0.002
Fried Frailty Index	2 (1; 3)	2 (1; 2)	< 0.001
SPPB score	11 (9; 12)	11 (10; 12)	< 0.001
Chair stands per second	0.4 (0.3-0.5)	0.5 (0.4-0.6)	< 0.001
Walk speed (m/s)	1.2 (0.9-1.4)	1.3 (1.1-1.6)	< 0.001
Handgrip strength (kg)	27.7 (21.7; 35.3)	32.7 (25.0; 40.9)	< 0.001
Encephalopathy (numbers connection score)	41.6 (31.7; 57.3)	38.3 (29.4; 52.1)	0.008

MELD: Model for end stage liver disease; SPPB: Short physical performance battery.

Table 3 Significant predictors of hospitalisation days per 12 mo on multivariable analysis

Characteristic	IRR (95%CI), *n* = 583	*P* value
Frail by Fried Frailty Index	1.21 (1.02, 1.44)	0.03
MELD score	1.10 (1.06, 1.15)	< 0.001
Albumin (g/L)	0.43 (0.31, 0.61)	< 0.001
Female sex	1.85 (1.22, 2.81)	0.004

Frail-Fried Frailty Index ≥ 3. IRR: Incidence rate ratios; MELD: Model for end-stage liver disease.

0.001). Frail patients had more hospitalisation [median 1 hospitalisation (0-2) *vs* 0 hospitalisations (0-1), *P* < 0.001] and spent more days in hospital than non-frail patients [median 3 (0-8.2) d *vs* 0 (0-4) d, *P* < 0.001].

The significant differences between patients who were hospitalised compared with not hospitalised within 12 mo are displayed in Table 2. Walk speed was slower (median 1.2 *vs* 1.3 metres/second), handgrip strength was lower (median 27.7 kg *vs* 32.7 kg) and chair stands completed per second was also lower in hospitalised patients (median 0.4 stands/s *vs* 0.5 stands/s), indicating greater frailty. Females were more likely to be hospitalised than men (51% *vs* 38%, *P* = 0.003). Subjects who were hospitalised were marginally younger than those who were non-hospitalised (median age 59 *vs* 60 years, *P* = 0.02).

Among patients with at least one hospitalisation, the median (IQR) length of stay per hospital admission was similar by frailty status [5 (3.0-8.0) d for frail *vs* 4 (3.0-6.3) d for non-frail, *P* = 0.24]. For patients hospitalised for infection, the median (IQR) length of stay was longer, at 6 (3.5-8.3) d, as compared to 3.5 (2.2-6.0) d for those hospitalised for other causes, *P* < 0.001.

On univariable analysis, the Fried Frailty Index, as a continuous variable, was associated with total hospitalisation days per year (IRR = 1.51, 95%CI: 1.28-1.77; *P* < 0.001). This remained significant on multivariable analysis after adjustment for MELD, albumin, and female sex (IRR = 1.21, 95%CI: 1.02-1.44; *P* = 0.03). Table 3 displays the significant results of the negative binomial regression model.

Risk factors for infection

Twenty percent of frail patients compared to 15% of non-frail patients experienced infection (*P* = 0.09). On bivariable analysis (incorporating time to outcome), the point estimates were suggestive of an increased odds of infection for frail *vs* non-frail patients although statistical significance was not achieved by SPPB or Fried Frailty Index (Table 4). There was however a significantly reduced odds of infection in patients who could complete 5 chair stands within 10 s (*P* = 0.046). In addition, the MELD score was significantly associated with the incidence of infection, and serum albumin and the presence of hepatocellular carcinoma were inversely associated with infection. There was no significant relationship for other factors, including the presence of ascites (OR = 1.15, *P* = 0.570), HIV infection (OR = 1.53, *P* = 0.464) or diabetes (OR = 0.98, *P* = 0.939).

On multivariable analysis, only serum albumin (OR = 0.39, 95%CI: 0.26-0.58, *P* < 0.001) and the

Table 4 Predictors of infection on bivariable analysis (accounting for observation time)

Characteristic	OR (95%CI)	*P* value
Albumin (g/dL)	0.40 (0.27-0.60)	< 0.001
MELD score	1.07 (1.02-1.11)	0.005
HCC (yes *vs* no)	0.60 (0.36-0.99)	0.046
Chair stands (completion of 5 chair stands within 10 s)	0.32 (0.11-0.98)	0.046
Age (yr)	0.98 (0.96-1.0)	0.05
Frail (SPPB score ≤ 9)	1.54 (0.94-2.50)	0.08
Frail (Fried frailty Index ≥ 3)	1.49 (0.94-2.36)	0.09
Sodium	0.96 (0.90-1.01)	0.10
Hepatic encephalopathy (numbers connection > 45 s)	0.72 (0.46-1.14)	0.16

MELD: Model for end stage liver disease; HCC: Hepatocellular carcinoma; SPPB: Short physical performance battery.

presence of hepatic encephalopathy (OR = 0.59, 95%CI: 0.36-0.95, *P* = 0.03) remained significant predictors of infection. Frailty, as measured by the Fried Frailty Index, was not significant in this model (OR = 1.30 Fried Frailty Index ≥ 3 *vs* < 3, 95%CI: 0.80-2.12, *P* = 0.29).

DISCUSSION

This prospective study, including nearly 600 patients with cirrhosis awaiting liver transplantation, demonstrates that functional measures of frailty are associated with increased hospitalisation days independent of the MELD score. The increase in hospitalisation observed in this study was due to an increased incidence of hospitalisation, predominantly for complications of end-stage liver disease. These data from the ongoing FrAILT study add critical information to our prior report on the significant association between frailty and waitlist mortality[3] by providing an intermediate outcome of hospitalisation. The clinical implication of this finding is that functional frailty measures performed in the outpatient setting can identify patients who may benefit from tailored interventions to reduce the risk of subsequent hospitalisations and ultimately, death.

Our data have important implications for the management of patients with cirrhosis, who impose a high burden on the health care system with frequent exacerbations of their chronic disease. In particular, implementation of a chronic disease model of care for decompensated cirrhotics has strong potential to improve outcomes in this population[15]. Indeed, in one study in Italy, case management was shown to significantly reduce both the 30 d re-hospitalisation rate as well as overall mortality, while reducing cost[16]. Given the resource requirements of such a program it is not practical or feasible to suggest that every cirrhotic should be enrolled, therefore a simple frailty measure such as the Fried Frailty Index is useful in its ability to select patients who are most in need of such an intensive management strategy. Perhaps even more importantly, frailty represents a potentially modifiable outcome through focused pre-habilitation programs that specifically target the individual components that contribute to the frail phenotype[17]. Demonstrating that frailty is associated with increased hospitalisations provides strong justification to develop and implement such programs early in the disease progression to prevent hospitalisation and subsequent deterioration in this vulnerable population.

In our cohort, frailty was not significantly associated with infection on multivariable analysis, although there was a trend to increased infection. This differs from previous research demonstrating that sarcopenia, a major contributor to the frail phenotype, has previously been shown to increase risk of infection-related hospitalisations[10]. It may be that frailty represents an early phenotype, whereas sarcopenia represents more established systemic disturbance that may be required for infection risk. However in the absence of longitudinal data incorporating frailty measures and body composition measurements, this cannot be confirmed. It may also be that quantification of hepatic encephalopathy in this cohort by the numbers count test, a feature recognised to be closely related to frailty[18], allowed us to better ascertain the relative contributions of different factors to infection risk than previous studies. The lack of an independent association between frailty and infection was somewhat surprising, and further studies are required to clarify this issue.

The majority of hospital admissions in our cohort were indeed for liver-related decompensation including infection, but also acute kidney injury, hepatic encephalopathy and hepatorenal syndrome (81%), and thus could be expected to accelerate the progression of liver disease. This sheds important insight into the potential mechanisms of the impact of frailty on adverse health outcomes in cirrhotics: instead of increasing the likelihood of any single complication, frailty decreases a patient's reserve to withstand any of the typical complications of cirrhosis, increasing the severity of the complications and the likelihood of inpatient admission.

Limitations of this study include its observational nature, albeit prospective, therefore the impact of reversing frailty cannot be proven. However the consistent associations between frailty and poor outcome suggest that it is indeed itself a contributing factor, and the finding that deterioration in frailty further increases risk of mortality adds further weight to this argument[9]. Furthermore, data from a study of patients undergoing TIPS insertion suggest that reversing muscle wasting can indeed improve survival[19]. An additional limitation is the inability to accurately classify the cause for non-infectious hospital admissions in this study, to determine whether the increase in hospitalisation was due to a specific factor, such as hepatic encephalopathy. Most episodes of decompensation of liver disease are multifactorial in nature, the specific triggering factor may never be identified, and standardised diagnostic

criteria are lacking. Finally, this study was single-centre, and thus validation in other cohorts is required.

Strengths of this study include a large study population that is representative of transplant waitlist cohorts with a high study uptake of 97% of patients assessed for transplantation, with near-complete (95%) longitudinal data. We therefore believe that these results are representative of a tertiary centre transplant waitlist. Despite the study limitations, our identification of a relationship between frailty - as measured by a simple tool that can easily and rapidly be performed in the clinic setting - a significantly increased risk of hospitalisation has important implications for the management of patients with cirrhosis. The Fried Frailty Index can help clinicians identify those at greatest risk of hospitalisation and thus in greatest need of chronic liver disease management programs to provide additional support.

In conclusion, physical frailty in subjects on the liver transplant waitlist, as measured by the Fried Frailty Index, is a significant predictor of 12 mo hospitalisation days independent of liver disease severity. This findings adds to the previously established link between frailty and mortality. These data provide us with a strong rationale to develop pre-habilitation and chronic disease management programs for frail patients on the transplant waitlist to reduce hospitalisation, reduce mortality and reduce healthcare costs.

COMMENTS

Background

Frailty has previously been associated with mortality in patients on the liver transplant waitlist. Its impact on hospitalisation however is no well described.

Research frontiers

As the liver transplant population is ageing and accumulating comorbidities, exploring the impact of systemic health by using measures such as the Fried Frailty Score, the authors are starting to better understand the impact of non-liver disease severity on outcome in this population.

Innovations and breakthroughs

This manuscript for the first time evaluates the impact of the Fried Frailty Score on hospitalisation days over a 12 mo period in subjects on the liver transplant waitlist. It identifies physical frailty as an independent predictor of hospitalisation risk regardless of MELD score. This adds to previous work linking frailty to mortality in cirrhosis, and is similar to previous findings relating to sarcopenia in cirrhosis, as measured by computerised tomography. Sarcopenia however has been linked with an increase in infection risk in cirrhosis, whereas in our study frailty was not significantly associated with infection.

Applications

This study provides a strong rationale to consider physical frailty as a measure of disease severity in cirrhotics on the liver transplant waitlist, and to consider prehabilitation or chronic disease management programs to minimise risk for frail cirrhotics.

Terminology

The Fried Frailty Score consists of handgrip strength, gait speed, exhaustion, weight loss, and physical activity. The score ranges from 0 to 5, with 0 being normal. Frail is generally defined as Fried Frailty Index $\geqslant$ 3 points out of a maximum of 5. These assessments have been validated in geriatric and cirrhotic populations.

Peer-review

The study is interesting because authors found that frailty is an independent factor (independent from MELD most importantly) for hospitalisation. Future results of this prospective may show that frailty might be a contributing factor for listing of transplantation priority.

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ORIGINAL ARTICLE

Prospective Study

Percutaneous electrochemotherapy in the treatment of portal vein tumor thrombosis at hepatic hilum in patients with hepatocellular carcinoma in cirrhosis: A feasibility study

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Author contributions: Tarantino L, Busto G and Nasto A designed the study; Tarantino L, Busto G and Nasto A performed the operations; Tarantino L, Talamo M, Accardo C, and Bortone S performed and evaluated imaging data; Fristachi R and Cacace L performed and analyzed pathology data; Tarantino L, Di Minno MND, and Ambrosino P wrote the manuscript; Gallo P, Tarantino P and Nasto RA collected clinical records and were also involved in editing the manuscript.

Institutional review board statement: This study was approved by the Institutional Board of the Umberto I, A.Tortora Hospital, Nocera inf., Pagani.

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Abstract

AIM

To treated with electrochemotherapy (ECT) a prospective case series of patients with liver cirrhosis and Vp3-Vp4- portal vein tumor thrombus (PVTT) from hepatocellular carcinoma (HCC), in order to evaluate the feasibility, safety and efficacy of this new non thermal ablative technique in those patients.

METHODS

Six patients (5 males and 1 female), aged 61-85 years (mean age, 70 years), four in Child-Pugh A and two in Child-Pugh B class, entered our study series. All patients were studied with three-phase computed tomography (CT), contrast enhanced ultrasound (CEUS) and ultrasound-guided percutaneous biopsy of the thrombus before ECT. All patients underwent ECT treatment (Cliniporator Vitae®, IGEA SpA, Carpi, Modena, Italy) of Vp3-Vp4 PVTT in a single session. At the end of the procedure a post-treatment biopsy of the thrombus was performed. Scheduled follow-up in all patients entailed: CEUS within 24 h after treatment; triphasic contrast-enhanced CT and CEUS at 3 mo after treatment and every six months thereafter.

RESULTS

Post-treatment CEUS showed complete absence of enhancement of the treated thrombus in all cases. Post-treatment biopsy showed apoptosis and necrosis of tumor cells in all cases. The follow-up ranged from 9 to 20 mo (median, 14 mo). In 2 patients, the follow-up CT and CEUS demonstrated complete patency of the treated portal vein. Other 3 patients showed a persistent avascular non-tumoral shrinked thrombus at CEUS and CT during follow-up. No local recurrence was observed at follow-up CT and CEUS in 5/6 patients. One patient was lost to follow-up because of death from gastrointestinal hemorrage 5 wk after ECT.

CONCLUSION

In patients with cirrhosis, ECT seems effective and safe for curative treatment of Vp3-Vp4 PVTT from HCC.

Key words: Hepatocellular carcinoma; Portal vein tumor thrombosis; Electrochemotherapy

Core tip: Six patients with portal vein tumor thrombus (PVTT) at hepatic hilum underwent electrochemotherapy (ECT), a new non thermal ablative technique. The follow-up ranged from 9 to 20 mo (median, 14 mo). In 2 patient, the follow-up computed tomography (CT) demonstrated complete patency of the treated portal vein. Three patients showed a persistent avascular non-tumoral shrinked thrombus at CT during follow-up. No local recurrence was observed in 5/6 patients. One patient, was lost to follow-up because of death from gastrointestinal hemorrage 5 wk after treatment. ECT seems effective and safe for curative treatment of PVTT at hepatic hilum.

Tarantino L, Busto G, Nasto A, Fristachi R, Cacace L, Talamo M, Accardo C, Bortone S, Gallo P, Tarantino P, Nasto RA, Di Minno MND, Ambrosino P. Percutaneous electrochemotherapy in the treatment of portal vein tumor thrombosis at hepatic hilum in patients with hepatocellular carcinoma in cirrhosis: A feasibility study. *World J Gastroenterol* 2017; 23(5): 906-918 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i5/906.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i5.906

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third most frequent oncologic cause of death worldwide[1]. HCC is often diagnosed at intermediate or advanced stages, when portal vein (PV) and its branches have already been involved by the tumoral process[2,3]. It has been reported that approximately 10%-40% of HCC patients are diagnosed with portal vein tumor thrombus (PVTT)[4,5]. According to the Barcelona Clinic Liver Cancer classification system[6], HCC patients with PVTT are classified as advanced stage (or Stage C), with a rather poor prognosis and an expected median survival span of about 2.7-3.0 mo[3,6]. Current guidelines only recommend systemic chemotherapy with Sorafenib in such patients[6].

The Liver Cancer Study Group of Japan has recently proposed a macroscopic classification for PVTT categorized into five grades, defined as follows: Vp0, no PVTT; Vp1, PVTT distal to the 2nd order branches of the PV; Vp2, PVTT in the 2nd order branches of the PV; Vp3, PVTT in the 1st order branches of the PV; Vp4, PVTT in the main trunk of the PV or a PV branch contralateral to the mainly involved lobe (or both)[7].

In recently published papers[8-14], surgical hepatic resection (HR) and loco-regional therapies, such as trans-arterial chemoembolization (TACE), radiofrequency ablation (RFA), microwawe (MW) ablation, gamma-knife radiosurgery, 90Y-based transarterial radioembolization (TARE), proved to be feasible and moderately effective treatments to improve survival of HCC patients with peripheral PVTT (Vp1 and Vp2). However, all these therapies did not substantially modify prognosis in patients with PVTT in the main PV and/or its 1st order branches (Vp3 and Vp4). Some previous isolated reports advocated a possible role of percutaneous ethanol injection (PEI)[15], RFA[16,17] or interstitial laser therapy (ILT)[18] in the treatment of Vp3-Vp4 PVTT. However, the use of PEI remains unreliable in such clinical settings because of the easy

escaping of the ethanol in the blood stream and the high probability of producing neoplastic thrombi. On the other hand, thermal ablation with RF and ILT are harmful techniques when applied near the hepatic hilum because of the high risk of irreversible damage to main bile ducts, main hepatic artery, gallbladder, duodenum or gastric wall[19-23]. As a matter of fact, with the only exception of the above cited studies, no other group recommended the use of these techniques in the treatment of Vp3-Vp4 PVTT.

A potential ideal ablation technique for Vp3-Vp4 PVTT should be able to kill tumor cells in the portal vessels without heat generation and without affecting patency of main bile ducts, arterial vessels and even without any damage to PV walls. Electrochemotherapy (ECT) is a non-thermal local tumor ablation modality using electroporation[24]. This is a physical method that enhances cell membrane permeability, and enables non-permeant or poorly permeant chemotherapeutic agents to enter cells, greatly enhancing their efficacy[25-28]. Safety and efficacy of the use of ECT in proximity of vascular and ductile structures of liver and pancreas have been already demonstrated in several studies[29-33].

In order to evaluate the feasibility, efficacy and safety of ECT in the treatment of Vp3-Vp4 PVTT, we present a prospective case series of patients with liver cirrhosis and extensive Vp3-Vp4 PVTT from HCC treated with ECT.

MATERIALS AND METHODS

From December 2014 to December 2015, 42 patients (29 male, 13 female, age range 48-91 year) with Vp3-Vp4 PVTT were observed at our Unit of Hepatology and Interventional Ultrasound in a tertiary care Institution - A. Tortora Cancer Hospital. To be included in the present study, patients had to fulfil the following basal inclusion criteria: (1) Malignant PV thrombosis of the right and/or left portal branch and/or of the distal portion (last 2 cm) of the main PV; (2) synchronous naive HCC nodule or recurrence after previous treatments for HCC not exceeding 5 cm in size and not more than 3 nodules in number. The presence of nodules abutting the gastrointestinal tract or hepatic hilum, the diaphragm, gall-bladder, kidney and right or left branch of the PV were not considered exclusion criteria; (3) Karnofky performance status > 70; (4) severity of liver function impairment not exceeding Child-Pugh class B-8, platelet count > 50.000/mm^3, INR < 1.5, total bilirubin < 1.5 mg/dL; (5) absence of ascites at the time of treatment; (6) absence of gastric varices and/or esophageal varices not exceeding grade F2 at endoscopy; and (7) absence of indications to Sorafenib therapy (Child B/C class) or intolerance to previous Sorafenib therapy.

Causes for exclusion from the treatment were: (1) extrahepatic HCC metastases; (2) heart impairment, arrythmias or presence of a cardiac pace-maker; (3) severe lung or renal or hepatic insufficiency; (4) epilepsy; (5) allergy to bleomycin; and (6) presence of prosthesis.

Seven patients in Child-Pugh A class had already been treated with Sorafenib at other Institutions and had stopped the treatment because of severe side effects. These patients had been addressed to our Institution for evatuation of eligibility to ECT. Other 9 Child-A-class patients were advised for systemic therapy with Sorafenib at our Institution. Two out of them stopped Sorafenib because of side effects. Seven patients remained on Sorafenib therapy. Seven Child-Pugh-C-class patients were excluded from ECT treatment because of severe impairment of hepatic function. Twenty-two patients were excluded from ECT treatment because of the presence of one or more of the following conditions (Figure 1): multinodular HCC (14 patients), extensive hepatic infiltration from HCC (5 patients), large esophageal (F3) or gastric varices (3 patients). Therefore, 36 out of 42 patients (85.7%) were excluded from ECT, and six patients with liver cirrhosis and Vp3-Vp4 PVTT from HCC fulfilled the selection criteria and were included in our series. Baseline characteristics of the six patients are reported in Table 1.

This study was approved by the Institutional Board of our Institute and was conducted according to the declaration of Helsinki. All patients provided written, informed consent.

According to the EFSUMB (European Federation of Societies for Ultrasound in Medicine and Biology) guidelines[34,35], HCC was diagnosed on the basis of characteristic enhancement patterns (arterial enhancement and late wash-out) at three-phase contrast-enhanced computed tomography (CT) and contrast enhanced ultrasound (CEUS). In order to assess the nature of the thrombosis, pre-treatment CEUS and ultrasound (US) guided percutaneous biopsy of the thrombus were performed in all patients[36-38]. Intraoperative biopsy of the treated PVTT was also scheduled in all patients at the end of the treatment.

To be included in the study, patients had to fulfil the following inclusion criteria: (1) absence of indications to Sorafenib therapy (Child B/C class) or intolerance to previous Sorafenib therapy; (2) severity of liver function impairment not exceeding Child-Pugh class B-8; (3) platelet count > 50.000/mm^3, INR < 1.5, total bilirubin < 1.5 mg/dL; (4) absence of ascites at the time of treatment; (5) absence of gastric varices and/or esophageal varices grade > F2 at endoscopy; (6) absence of extrahepatic HCC metastases; and (7) synchronous first HCC nodule or recurrence after previous treatments for HCC not exceeding 5 cm in size and not more than 3 nodules in number. The presence of nodules abutting the gastrointestinal tract or hepatic hilum, the diaphragm, gall-bladder, kidney and right or left branch of the PV were not considered exclusion criteria.

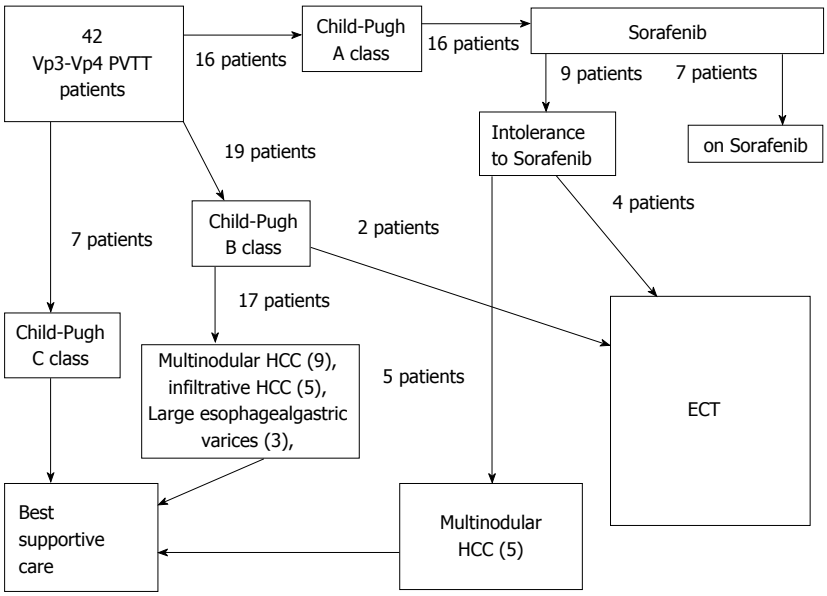

Figure 1 Selection of eligible patients to electrochemotherapy among a consecutive series of 42 patients with VP3-VP4 portal vein tumor thrombosis. ECT: Electrochemotherapy; PVTT: Portal vein tumor thrombosis; HCC: Hepatocellular carcinoma.

Table 1 Baseline characteristics of patients with portal vein tumor thrombosis in our series

Patient	Age/sex	PS:E/K	Cirrhosis etiology	Child-Pugh class	AFP (ng/mL)	Albumin (g/100 mL)	INR	Bilirubin (mg)	PLT ($n/\mu L$)	EGDS EV, GV, RS	Comorbidities
1	64 yr/M	2/70	HCV	A6	14	3.7	1.10	1.20%	75000	F1-EV, GV-, RS-	Diabetes
2	54 yr/M	1/80	HCV	B7	84	3.2	1.40	1.40%	62000	F2-EV, GV+, RS-	Diabetes, MI, COPD
3	85 yr/M	1/80	HCV	A5	32	4.2	1.14	1.20%	125000	F!-EV, GV-, RS-	None
4	75 yr/M	2/70	HCV	A6	65	3.6	1.32	1.00%	88000	F2-EV, GV-, RS-	Thyroid carcinoma, COPD
5	61 yr/F	0/100	HCV	A5	47	4.0	1.25	0.90%	74000	EV-, GV-, RS-	Diabetes, hypertension
6	77 yr/M	2/70	HCV	B7	16	3.5	1.55	1.30%	56000	F1-EV, GV-, RS-	Diabetes

PS E/K: Performance Status ECOG/Karnoffsky; AFP: Pre-treatment alphafetoprotein; INR: International normalized ratio; PLT: Platelets; EGDS: Esophagastroduodenoscopy; EV, GV, RS: Esophagealvarices, gastric varices, red spots.

Before ECT procedure, all patients underwent: (1) serum blood tests for liver function and haemocoagulation and serum alpha-fetoprotein dosage; (2) upper gastrointestinal tract endoscopy (esophagogastroduodenoscopy) for evaluation of esophageal varices; (3) electrocardiogram; (4) chest X-ray; (5) abdominal US with a commercially available US equipment (EPIQ 7, Philips SpA, Amsterdam, The Netherlands) and a 3.5 and 5.0 MHz convex electronic probe; and (6) triphasic contrast-enhanced CT [Iomeron® 400 (iomeprol), Bracco SpA, Milan, Italy].

ECT procedure

ECT is a combined use of chemotherapeutic drugs and electric pulses applied to the treated tumour nodule. Local application of electric pulses to the tumour increases drug delivery into cells, specifically at the site of electric pulse application (Figure 2). Drug uptake by delivery of electric pulses is increased for only those chemotherapeutic drugs whose transport through the plasma membrane is impeded. Among many drugs that have been tested so far, bleomycin and cisplatin found their way from preclinical testing to clinical use. We performed ECT under general anesthesia, with intubation. In order to avoid strong muscle contractions induced by electric pulses, the myorelaxant cisatracurium besylate (Nimbex®, Glaxo-SmithKline, Brentford, United Kingdoms) was used. All patients underwent US guided percutaneous biopsy of the PVTT with a 21 gauge Chiba needle (ecojekt, HS Hospital service, Rome, Italy) before the start of ECT procedure. Than, under US guidance, four to six electrode-needles were inserted percutaneously along the external margin of the thrombosed portal vessel (Figure 3). The electrodes were connected to independently controlled generator outputs of the Cliniporator Vitae® (IGEA SpA, Carpi, Modena, Italy). The Cliniporator Vitae® device is a pulse generator with

Table 2 Hepatocellular carcinoma patterns, treatments and results in 6 patients with portal vein tumor thrombosis treated with electrochemotherapy

Patient	Previous treatments	Site of PVTT	HCC nodules/size	Results of ECT	Follow-up
1	Surgery, RFA	Right PV, distal main PV	-	Complete necrosis of Vp3-Vp4 PVTT Complete recanalization	20 mo
2	None	Right PV, distal main PV	1 nodule/3.5 cm	Complete necrosis of Vp3-Vp4 PVTT Complete recanalization	Death for rupture of esophageal varices 14 mo after ECT
3	None	Right PV	1 nodule/3 cm	Complete necrosis of Vp3-Vp4 PVTT Persistent shrinked bland thrombosis	Death for Liver failure 14 mo after ECT
4	RFA	RightPV, left PV, distal main PV	2 nodules/1.5 and 2.5 cm	Complete necrosis of Vp3-Vp4 PVTT Persistent shrinked bland thrombosis	Death 5 wk after ECT for rupture of esophageal varices
5	None	Left PV	1 nodule/3.5 cm	Complete necrosis of Vp3-Vp4 PVTT Persistent shrinked bland thrombosis	12 mo
6	Surgery, RFA, TACE	Right PV	-	Complete necrosis of Vp3-Vp4 PVTT Persistent shrinked bland thrombosis	9 mo

ECT: Electrochemotherapy; PV: Portal vein; PVTT: Portal vein tumor thrombosis; HCC: Hepatocellular carcinoma; RFA: Radiofrequency ablation; TACE: Trans-arterial chemoembolization; MI: Myocardial infarction; COPD: Chronic obstructive pulmonary disease.

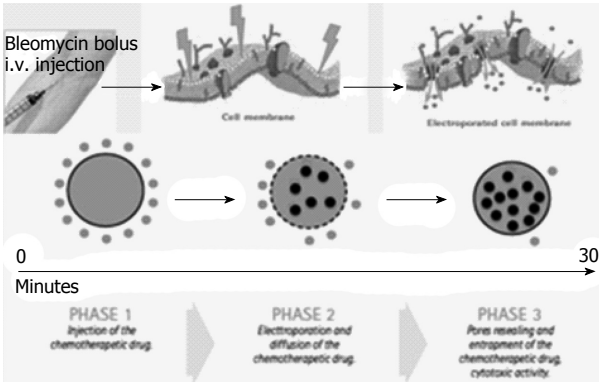

Figure 2 Illustrative sketch of the mechanism with which electrochemotherapy operates: After insertion of the electrodes in the tissue, a bleomycin bolus is administered intravenously. Eight minutes after injection, bleomycin diffuses in the tumor tissue. At this point the electric pulses are started in order to alter permeability of cells' membranes. The electroporation process greatly increases the bleomycin intracellular entrance. Therefore by self repair of the membranes, pores reseal and the bleomycin is entrapped in the cells.

6 independently controlled and electrically isolated outputs, each providing up to 3000 Volts (maximum current: 50 Amperes) and delivering 8 rectangular electrical pulses (rise time: 1 μs) of 100 μs duration at a pulse repetition frequency of 4 Hz. Eight minutes after intravenous bolus injection (15000 IU/m^2) of Bleomycine sulfate (Bleoprim®, Sanofi Aventis, Paris, France), electric pulses were delivered. After electric pulses delivery, the electrodes were partially or completely withdrawn and repositioned around the HCC nodule associated with the PVTT in order to treat the tumor with ECT. Hemostasis of liver capsule, peritoneum, abdominal wall and skin was performed with a thermal track ablation by connecting every single electrode to an activated electric scalpel during the complete withdrawal of the electrodes.

US guided percutaneous cutting needle biopsy of the treated PVTT with an 18 gauge modified Menghini's needle (Biomol, HS Hospital service, Rome, Italy) was performed in all patients after ECT. CEUS control of PVTT was performed within 24 h after the end of ECT procedure.

After procedure, all patients started Enoxaparine (4000 IU, daily). The scheduled follow-up in all patients entailed: monthly color-Doppler US (CDUS) in the first 3 mo after treatment; triphasic contrast-enhanced CT and CEUS 3 mo after treatment; afterwards, CT and CEUS controls every six months.

RESULTS

Overall patients

Six patients (5 males and 1 female), aged 54-85 years (mean age, 69 years) entered our study series. Baseline characteristics of the patients are reported in Table 1.

Four patients were in Child-Pugh A class and two in Child-Pugh B class. Four patients showed 1 (3 cases) or 2 (1 case) HCC nodules synchronous with Vp3-PVTT (all patients) and Vp4-PVTT (one case). The size of HCC nodules ranged from 2.5 to 4.5 cm (mean = 3.4 cm). Two patients, previously treated with surgery and thermal ablation, showed an isolated right branch Vp3-PVTT, without any residual or recurrent HCC nodule in the liver. Treatments' results in the single patient are reported in Table 2.

Intraoperative pre-treatment biopsy of PVTT was adequate and showed viable HCC in 5/6 cases (Figure 4A). In one patient the fine-needle biopsy only showed blood material. However, pre-treatment CEUS demonstrated hypervascular PVTT with characteristic enhancement patterns (arterial enhancement and late wash-out) (Figure 5B) in all cases (6/6), thus confirming the malignant nature of thrombosis in all patients[36,39].

Two out of six patients underwent ECT of the isolated Vp3-PVTT. Other three patients underwent ECT of PVTT and of the associated HCC nodule in the same session. In these patients, after ECT of PVTT, the electrodes were partially or completely withdrawn and

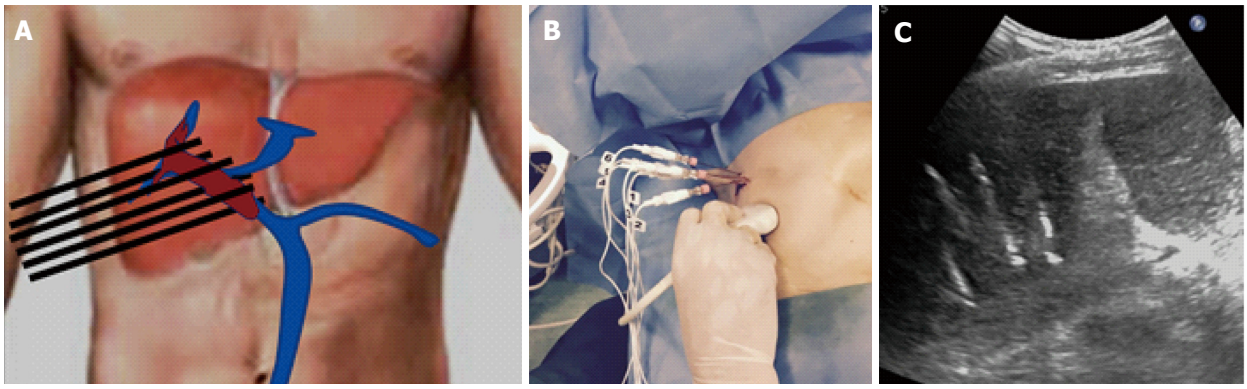

Figure 3 Insertion of six electrodes for electrochemotherapy treatment of right portal vein. A: Illustrative sketch showing schematically the position of electrodes around the external margin of the Tumor thrombosis; B: Up to six electrode-needles are inserted percutaneously; C: The position of electrodes can be monitored with US during the procedure.

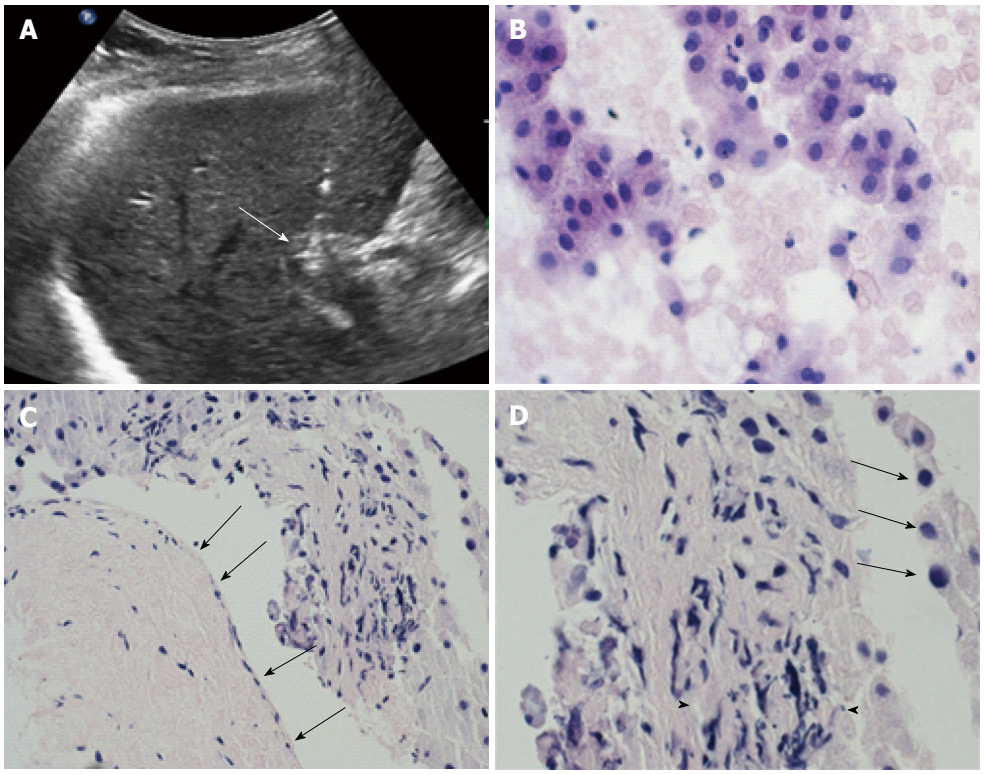

Figure 4 All patients underwent pre- and post-treatment biopsy of the portal vein tumor thrombus. A: ultrasound scan demonstrate the correct positioning of the needle tip (arrow) in the thrombus; B: Intraoperative pre-treatment biopsy of the thrombus was adequate and showed viable cells from hepatocellular carcinoma in 5/6 cases; C: High magnification of biopsy specimen showed severe involutive changes of tumor cells with cellular apoptosis (arrows) and areas of necrosis (arrowheads) in all six cases. Low magnification in the same specimen, beside the altered tumor thrombus, showed absence of damage from the procedure to portal vein wall (arrows); D: Portal endothelium shows normal appearance with regular wall layers.

were reinserted around the tumor. In one patient with 2 HCC nodules, we only treated the PVTT and planned a subsequent session of thermal ablation to treat the 2 HCC nodules (Figure 1).

Post-treatment biopsy of PVTT showed severe involutive changes of tumor cells with cellular apoptosis and areas of necrosis in all cases. In 3 cases, PV wall and periportal tissues were present in the specimen; in these portions of the specimens, PV wall showed normal findings with normal endothelium; no distortion or necrosis was detectable in the periportal structures (Figure 4B and C).

Post treatment intraoperative CEUS demonstrated complete absence of enhancement of the thrombosis (Figuer 5C) and of the treated HCC nodule in all cases.

The procedure was well tolerated in all cases. No intraoperative or post-operative major complication was reported. All patients were discharged from the hospital the day after the treatment.

The follow-up ranged from 9 to 20 mo (median, 14 mo). In two patients, CDUS showed complete re-canalization of the treated PVTT at 2 and 3-mo CDUS

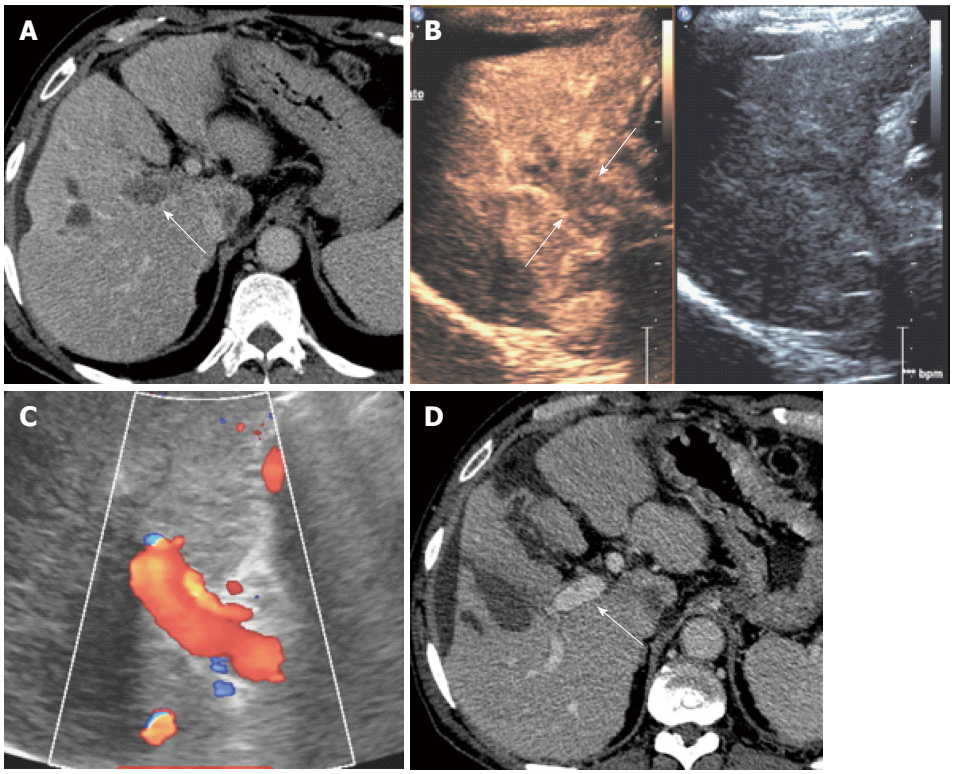

Figure 5 M.D.S. 64 years hepatitis C virus related cirrhosis. A: On september 2014 computed tomography (CT) showed complete malignant thrombosis of right portal vein (arrows); B: Pretreatment contrast enhanced ultrasound showed diffuse enhancement of the thrombus (arrows) consistent with malignant thrombosis; The patient underwent electrochemotherapy with insertion of 6 electrode-needles and *i.v.* Bleomicin bolus; C: Three months monthly color-Doppler ultrasound control showed complete patency of the right portal vein; D: Persistent patency of right portal vein (arrows) and absence of local recurrence was confirmed at 3, 9, 15 mo follow-up CT control.

follow-up, respectively. In these two patients, CEUS and CT confirmed complete patency of the vessel without any intravascular or perivascular recurrence during follow-up. In three patients (14, 12, and 9 mo follow-up, respectively), CT and CEUS showed permanent complete thrombosis with a persistent, shrinked, avascular thrombus into the treated vessels. In all three cases, no intravascular or perivascular enhancement consistent with residual tumor or local recurrence was detected at CT and CEUS during follow-up. In the remaining patient, 24 h post-treatment CEUS showed absence of enhancement of the treated thrombus. However, the patient was lost to follow-up because of death from gastrointestinal hemorrage five weeks after ECT treatment.

During follow-up, no local recurrences, at the site of the treated PVTT, occurred. However, 3 distant recurrences in other segments were detected in 2 patients. All 3 new lesions were treated by RFA in 2 cases and ECT in 1 case.

Patients' histories

Patient 1: Male, 64-year-old with HCV-related Child-Pugh A6 cirrhosis. Previous RFA of a HCC nodule (diameter = 3 cm) in the Ⅳ segment in december 2012. In November 2013, laparotomy and intraoperative MW ablation for 2 HCC recurrences in Ⅰ and Ⅴ-Ⅷ segments and PVTT of the segmental portal branch (Vp2) for segment Ⅴ. Post treatment CT, on January 2014, demonstrated complete necrosis of the HCC nodules and of the Vp2-PVTT. In september 2014, CT showed and extensive and complete right Vp3-PVTT without any intraparenchymal recurrence of HCC. The patient started Sorafenib (Nexavar®, Bayer, Leverkusen, Germany) 400 mg daily but was forced to stop therapy because of severe side effects. In December 2014, the patient accepted ECT, as alternative therapy. Pretreatment biopsy proved PV infiltration from "moderately differentiated HCC" (Grade 2 Edmondson). The patient underwent ECT with insertion of 6 electrode-needles. The 2-mo CDUS showed complete patency of the right PV (Figure 4C). The result was confirmed at 3-mo CEUS and CT (Figure 4D). CT control at 9, 15 and 18 mo still showed a completely patent PV without any intravascular recurrence. During follow-up, US and CT controls showed recurrence of HCC in the Ⅷ segment and in the Ⅵ segment at 6 and 12 mo respectively. Recurrences were treated by ECT and RFA respectively.

Patient 2: Male 54-year-old with HCV-related Child-Pugh B7 cirrhosis, obesity, chronic obstructive pulmonary disease (COPD), and previous myocardial infarction. The patient had been treated ten years before with coronary stents, and was on secondary

ticlopidine prophylaxis. In February 2015, abdominal US, CEUS and CT showed HCC in the V segment and extensive malignant portal thrombosis of the right PV. The patient underwent ECT by percutaneous insertion of 4 electrodes. The intraoperative post treatment CEUS showed a completely avascular PV thrombus. The 3-mo CEUS and CT showed a subtotal recanalization. The 6-mo and 12-mo CT confirmed absence of local recurrence and complete recanalization of the PV. Unfortunately, the patients were lost to follow-up at 14 mo because of death after severe hemorrhage from gastroesophageal varices.

Patient 3: Male, 85-year-old with HCV-related Child-Pugh A5 cirrhosis. In december 2014, diagnosis of large infiltrating HCC (size: 4, 5 cm) in the VII segment. The patient started systemic therapy with Sorafenib (400 mg/daily) at another Institution. In April 2015, he underwent CT and MRI at our institution that detected a large infiltrating HCC in the Ⅶ segment and extensive and complete thrombosis of the right PV from its origin on the main trunk up to its bifurcation. CEUS and MRI were consistent with malignant thrombosis. The evident progression of the disease forced to stop Sorafenib therapy and the patients was advised for ECT treatment as alternative. Pretreatment biopsy proved PV infiltration from "moderately differentiated HCC" (Grade 2 Edmondson). The patient underwent ECT of both HCC nodule and Vp3-PVTT by insertion of six electrodes. Six months and 12-mo CT showed persistence of avascular, shrinked right PV thrombosis. During follow-up, a new HCC nodule was detected in the Ⅳ segment at 6 mo follow-up. The new lesion was treated by RFA. Post treatment CT demonstrated complete necrosis of the recurrence.

Patient 4: Male, 75-year-old, with HCV-related Child-Pugh A6 cirrhosis and COPD. The patient had been previously treated with radioiodine for thyroid papillary carcinoma that was in complete remission at the time of admission. Pre-treatment EGDS showed grade F2 esophageal varices and absence of gastric varices. CT in May 2015 showed 2 HCC nodules in the VIII and VII segment and Vp3-Vp4 PVTT, involving both right and left PV and the distal portion of main PV. The patient started Sorafenib (Nexavar®, Bayer, Leverkusen, Germany) 400 mg daily. Three weeks later the patient was forced to stop therapy because of severe side effects. The patient was offered a two-step treatment schedule, entailing ECT of Vp3-Vp4 PVTT as first step and RFA of HCC nodules as second step. In this patient we inserted simultaneously six electrodes in two sets of three electrodes each, in order to cover the right and main PV with three electrodes and the left PV with the other three. Post treatment CEUS showed complete absence of enhancement of thrombosis in the right, left and main PV. One month CEUS confirmed absence of enhancement in the treated Vp3-Vp4 PVTT, and also showed bland complete thrombosis of main portal trunk and upper mesenteric vein. Then, Enoxaparine dosage was increased to 8000 UI daily. The patient underwent weekly controls with CDUS that showed unchanged findings in portal vessels overtime. Unfortunately, five weeks after treatment the patient died because of a severe hemorrage from rupture of esophageal varices.

Patient 5: Female, 61-year-old, with HCV-related Child-Pugh A5 cirrhosis. In September 2011 the patient underwent liver resection for a single HCC nodule in the Ⅲ segment. In may 2015, CT and CEUS showed HCC nodule in the Ⅳ segment and complete left Vp3-PVTT. The patient started systemic therapy with Sorafenib (400 mg/daily); the treatment was stopped after 3 wk because of severe side effects (weakness, diarrhea). Therefore he accepted ECT treatment. Six electrodes were inserted along the wall of the PVTT. Post-treatment intraoperative CEUS showed a large avascular area (8 cm × 7 cm) including the treated vessel and the perivascular liver parenchyma. Three months CEUS and CT follow-up showed avascular, shrinked thrombosis of the left PV. Six-months CT and nine-months CEUS confirmed the persistence of avascular thrombosis and absence of any recurrence.

Patient 6: Male, 77-years-old, with HCV-related Child-Pugh B7 cirrhosis and recurrent HCC. The patient had undergone multiple locoregional procedures (liver resection, TACE, RFA) in September 2005, June 2006, June 2008, January 2009, and July 2013 to treat multiple HCC recurrences. In August 2015, CT showed complete malignant thrombosis of the right PV. Six electrodes were inserted along the wall of the Vp3-PVTT. Post treatment CEUS, 3-mo and 9-mo CT and CEUS showed persistent, complete, shrinked avascular thrombus.

DISCUSSION

The present case series is the first, to our best knowledge, evaluating safety and efficacy of ECT for the treatment of extensive PVTT at hepatic hilum (Vp3-Vp4) in patients with HCC. We found that this mini-invasive interventional technique is feasible, safe and effective and it could represent a suitable option for the management of PVTT in this clinical setting.

With an annual incidence of more than 620000 new cases, HCC is the third most frequent cause of cancer death worldwide[1]. Its incidence is progresively increasing[2] and, despite surveillance programs, HCC is often diagnosed at an intermediate or advanced stage[3]. It has been reported that up to 44% of HCC patients are complicated with PVTT at the time of death[40]. PVTT severely affects prognosis of cirrhotic patients with HCC. The median survival time of patients with unresectable HCC without PVTT ranges from 10 to 24 mo while it

is significantly reduced in cases with associated PVTT (2-4 mo). PVTT is related with poor prognosis probably because of the intensified risk of tumor spread, increased portal pressure inducing variceal bleeding and reduced portal flow and subsequent jaundice, ascites, hepatic encephalopathy and hepatic failure[4].

For HCC patients with PVTT, current guidelines recommend systemic therapy with Sorafenib, an oral multiple tyrosine-kinases inhibitor that suppresses angiogenesis and tumor cell proliferation[6]. In clinical studies[41,42]. Sorafenib proved to improve of several months survival of patients with advanced HCC. Nevertheless, subgroup analyses only showed a marginal survival benefit for Sorafenib as compared with placebo in patients with PVTT[42,43]. All currently accepted HCC treatment guidelines consider PVTT a contraindication for transplantation, HR and TACE[44,45]. The hilar position of PVTT (Vp3-Vp4), in contrast with peripheral PVTT (Vp1-Vp2), further worsen the prognosis, also because represents a controindication to loco-regional therapies. When feasible, surgical resection of the tumor and associated PVTT might be the best possible treatment, but only a small percentage of patients can benefit of this approach[9]. Mild-moderate improvement in survival of patients with Vp1-Vp2 PVTT have been reported with TACE[10,11] and TARE[12]. However, both TACE and TARE demonstrated only minimal or absent advantages in patients with Vp3-Vp4 PVTT[11,12]. Ablation techniques such as PEI and RFA are usually not indicated in the management of intermediate and advanced HCC[44,45]. In 1990, Livraghi *et al*[15] reported complete ablation and absence of recurrence at 4-12 mo follow-up in 4 patients with segmental (V1p-V2p PVTT) treated with PEI. Several papers reported synchronous ablation of segmental PVTT and associated HCC nodule by RFA, MW ablation or ILT[14-18]. However, most authors excluded patients with involvement of main branches or main portal trunk (Vp3-Vp4 PVTT) because ablation of nodules next to hepatic hilum is considered unsafe. The high risk of damage to main bile ducts (stricture, rupture, obstruction) and to the hepatic artery (bleeding, pseudoaneurysm, arterio-portal fistula) after ablation of tumors in the center of the liver has been well described and demonstrated both *in vivo* experimental studies and in patients series[19,23]. Several measures have been suggested to avoid complications from damage to hepatic hilum structures during RFA and MW ablation[46], however none of these approaches have been extensively applicated in large series. In 2009 and in 2014 Giorgio *et al*[16,17] published their long-term results of RFA in 35 HCC patients with Vp3 and/or Vp4 PVTT. They reported absence of major complications, complete recanalization of main portal trunk in 26/35 (74%) patients and cumulative survival rates at 1, 3, and 5 years of 63%, 30% and 20%, respectively. Lu *et al*[18] demonstrated similar results in 108 patients treated with ILT for Vp3-Vp4 PVTT, reporting a 3-year survival rate of 22.4%. Both authors did not describe, in their procedures, any safety measure in order to avoid damage to hepatic hilum structures. In our knowledge, no other Author followed this high risk strategy.

A potential ideal ablation technique for Vp3-Vp4 PVTT should be able to kill tumor cells in the portal vessels without heat generation and without affecting patency of main bile ducts, arterial vessels and even without any damage to PV wall. The electroporation is a process in which electric impulses can cause structural changes in biological membranes[24,47,48]. Depending on pulse amplitude, duration, and the number of pulses, two possible results can be achieved. At subcritical electric fields, electroporation leads to transient pore formation with increase of membranes permeability to macromolecules that hardly could penetrate the cells in absence of electroporation. The average pore size is stationary and very small and, subsequently, a complete membranes' recovery occur (reversible electroporation)[47]. At supercritical field strengths, the pore radius increases reaching a critical pore size. Therefore, the membrane disgregates without any possibility of recovery [irreversible electroporation (IRE)][49]. In the last 20 years, two electroporation-based therapeutic techniques have been introduced: IRE and ECT. IRE uses high intensity electric pulses to obtain death of all cells in the electric field through irreversible permeabilization of cell membranes[49-52]. ECT is a local tumor ablation modality that, through reversible electroporation, enhances cell membrane permeability, and enables non-permeant or poorly permeant chemotherapeutic agents to enter cells, greatly enhancing their efficacy in killing tumor cells[24,31].

ECT and IRE can be used to treat tumours surrounded by vital structures such as larger blood vessels, nerves, and viscera without subsequent damage to these structures[24,47,48]. The safety and efficacy of their use around vascular, hollow viscera and ductile structures in liver and pancreas have been already demonstrated in many published papers[29-33]. However, few papers have evaluated the feasibility and effectiveness of percutaneous ECT on deep tumours[31-33] and to our knowledge we are the first to evaluate the safety and effectiveness of percutaneous ECT in the treatment of PVTT from HCC.

The analysis of pathologic findings in specimens obtained with biopsies of PVTT performed before and after ECT procedures is particularly interesting. Pretreatment biopsies of thrombosis gave adequate material for definitive diagnosis of PVTT from HCC in 5/6 patients. Post-treatment biopsies of the treated thrombus were performed in all patients. Necrosis and apoptotic aspects of tumor cells were detected in post-treatment biopsies in all specimens. In some patients the post-treatment biopsy material was particularly adequate to describe what actually happens when tumor tissue and normal cells are exposed to ECT. Infact,

three out of six post-treatment biopsy specimens included PV wall and periportal tissues around the tumor thrombus. In these cases, the damage of tumor cells (necrosis and apoptosis) was clearly showed, while no involutive aspect was detected in perivascular tissue and even in the portal endothelium. This selective effectiveness of ECT technique to damage only tumor cells is based on the intrisic mechanism of the chemotherapeutic agent used in the procedure. In particular, bleomicyn cytotoxicity is specific for cell cycle G2 phase[53]. As a result, only rapidly growing tissues, with cells in mitosis, are the target of the drug. However, when bleomycin is administered in a peripheral vein, only a low antitumoral efficacy is expected because of a poor diffusion through cells membranes and, therefore, only slight availability of the drug in the site of action (intracellular DNA)[53]. The electroporation process, obtained by the electric field through electrodes insertion in the target tissue, allows the bleomicyn to concentrate in the cells and operate the cell cycle block with subsequent cell death[24]. ECT ablation is not a physical process but a biochemical therapy, physically induced and amplified, highly selective for tumor cells[25]. ECT does not destroy nor modify the stromal architecture of the target tissue and damages normal cells only partially or not at all[25]. As a result, ECT treatment can be safely indicated in tumor next to, or even inside, the hepatic hilum, a condition that represent a contraindication to all the other locoregional therapies. In our opinion, Vp3-Vp4 PVTT represents the ideal condition to evaluate the ECT safety and efficacy. On this assumption, we started this prospective study on a series of consecutive cirrhotic patients affected from HCC and Vp3-Vp4 PVTT.

Our results showed that ECT is highly effective and safe for Vp3-Vp4 PVTT. In five patients, we observed the complete necrosis of the PVTT. In cases with an associated HCC nodule we also achieved the complete necrosis of the tumor. In two patients we observed at 2-3 mo follow-up the complete recanalization of the treated portal vessel. In no case, during a mediane follow-up of 12 mo, we observed a local recurrence. ECT of PVTT can be performed safely. No intraoperative and early postoperative complication occurred. A late complication occurred in one patient who underwent ECT for a complete tumor thrombosis of terminal part of main portal trunk and both right and left PV. Postoperative CEUS showed complete necrosis of all portions of PVTT. He left the hospital the day after treatment. In two weeks, he developed moderate ascites and Color Doppler US showed bland complete thrombosis of the whole axis of main trunk of PV. To treat the progression of bland thrombosis, dosage of LMW heparin was increased (from 4.000 UI to 8000 UI daily). At weekly Color Doppler US examination, the thrombus seemed to remain stable. However, five weeks after ECT treatment, the patient died for a severe hemorrage from rupture of esophageal varices. Another patient died for rupture of esophageal varices at 14 mo follow-up. In this patient, all imaging studies performed during follow-up excluded local o distant recurrence of HCC.

Four patients are alive and no local recurrence of the treated PVTT or the treated HCC nodule have been detected at 9-20 mo follow-up. In two patients distant recurrence occurred and were treated with percutaneous ablation in all cases.

This study has some limitations, represented by the small number of treated patients and the short-term follow-up. However, the preliminary results of our case series might represent the proof of concept for a future prospective study on consecutive patients and a long-term follow-up. Actually, our study is a prospective series, still going on. In this initial experience, we selected advanced HCC patients not eligible to systemic therapy with Sorafenib. The efficacy and safety profile of ECT procedure could encourage a combination treatment of ECT plus Sorafenib even in naive patients, in which systemic therapy is indicated. The main concern with the results in this study is the occurrence of a bland thrombosis of main portal trunk and fatal late hemorrhage from esophageal varices. In our short series (6 patients) a single fatal complication account for 16%-17% of cases. This rate, if confirmed on larger series, would be inacceptable, and patients with gastric varices or with esophageal varices even < F2 staging, should be excluded from treatment, or alternatively, advised of the high risk of the occurrence of this complication.

In conclusion, results of our prospective series suggest that ECT is a feasible effective and safe therapy for Vp3-Vp4 PVTT from HCC in cirrhotic patients not eligible to other therapeutic approaches. A high risk of hemorrhage from gastroesophageal varices after ECT treatment of main, right or left PV must be considered in the pre-treatment evaluation of the patients.

COMMENTS

Background

In patients with hepatocellular carcinoma (HCC) on liver cirrhosis and portal vein tumor thrombus (PVTT) at hepatic hilum, Current guidelines only recommend systemic chemotherapy with Sorafenib. Thermal ablation techniques are harmful when applied near the hepatic hilum because of the high risk of irreversible damage to main bile ducts and other structures. A potential ideal ablation technique in these cases should be able to kill tumor cells in the portal vessels without heat generation and without affecting patency of main bile ducts, arterial and venous vessels.

Research frontiers

Electrochemotherapy (ECT) is a non-thermal local tumor ablation that enhances cell membrane permeability, and enables non-permeant or poorly permeant chemotherapeutic agents to enter cells, greatly enhancing their efficacy. Safety and efficacy of the use of ECT in proximity of vascular and ductile structures of liver and pancreas have been already demonstrated in several studies.

Innovations and breakthroughs

ECT can be used to treat tumours surrounded by vital structures such as

larger blood vessels, nerves, and viscera without subsequent damage to these structures. The safety and efficacy of their use around vascular, hollow viscera and ductile structures in liver and pancreas have been already demonstrated in many published papers. However, few papers have evaluated the feasibility and effectiveness of percutaneous ECT on deep tumours and to our knowledge we are the first to evaluate the safety and effectiveness of percutaneous ECT in the treatment of PVTT from HCC.

Applications

If this experience will be confirmed, many tumors at hepatic hilum, not susceptible of surgical resection or systemic therapy, could be effectively treated in the future, even percutaneously.

Peer-review

HCC patients with PVTT usually have poor prognosis for absence of effective satisfying treatment measures. This article entitled by Tarantino *et al* showed that "In patients with cirrhosis, ECT seems effective and safe for curative treatment of Vp3-Vp4 PVTT from HCC". This paper provides new method of the PVTT treatment in patients with advanced HCC.

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CASE REPORT

Pancreaticoduodenectomy: Secondary stenting of the celiac trunk after inefficient median arcuate ligament release and reoperation as an alternative to simultaneous hepatic artery reconstruction

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Abstract

In patients undergoing pancreaticoduodenectomy (PD), unrecognized hemodynamically significant celiac axis (CA) stenosis impairs hepatic arterial flow by suppressing the collateral pathways supplying arterial flow from the superior mesenteric artery and leads to serious hepatobiliary complications due to liver and biliary ischemia, with a high rate of mortality. CA stenosis is usually due to an extrinsic compression by a previously asymptomatic median arcuate ligament (MAL). MAL is diagnosed by computerized tomography in about 10% of the candidates for PD, but only half are found to be hemodynamically significant during the gastroduodenal artery clamping test with Doppler assessment, which is mandatory before any resection. MAL release is usually efficient to restore an adequate liver blood inflow and prevent ischemic complications. In cases of failure in MAL release, postponed PD with secondary stenting of the CA and reoperation for PD should be considered as an alternative to immediate hepatic artery reconstruction, which involves the risk of postoperative thrombosis of the arterial reconstruction. We recently used this two-stage strategy in a patient undergoing surgery for pancreatic adenocarcinoma.

Key words: Pancreaticoduodenectomy; Celiac axis stenosis; Median arcuate ligament

Core tip: In patients undergoing pancreaticoduodenectomy (PD), hemodynamically significant celiac axis (CA) stenosis has the potential to cause vascular insufficiency leading to serious hepatobiliary complications with a high rate of mortality. CA stenosis is usually due to an extrinsic compression by a previously asymptomatic median arcuate ligament (MAL). MAL release is usually efficient to restore an adequate liver blood inflow and

prevent ischemic complications. In cases of failure in MAL release, postponed PD with secondary stenting of the CA and reoperation for PD should be considered as an alternative to immediate hepatic artery reconstruction, which involves the risk of postoperative thrombosis.

Guilbaud T, Ewald J, Turrini O, Delpero JR. Pancreaticoduodenectomy: Secondary stenting of the celiac trunk after inefficient median arcuate ligament release and reoperation as an alternative to simultaneous hepatic artery reconstruction. *World J Gastroenterol* 2017; 23(5): 919-925 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i5/919.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i5.919

INTRODUCTION

Pancreaticoduodenectomy (PD) involves a division of the gastroduodenal artery (GDA) and resection of the pancreaticoduodenal arcades, which depend on both the GDA and the superior mesenteric artery (SMA). In the case of hemodynamically significant celiac axis (CA) stenosis, PD suppresses the collateral pathways supplying arterial flow from the SMA into the branches of the CA and impairs hepatic arterial flow. Thus, in patients undergoing PD, CA stenosis has the potential to cause vascular insufficiency leading to serious hepatobiliary complications with a high rate of mortality[1-9]. With more than 95% accuracy, multidetector computed tomography (CT) with routine arterial reconstruction and sagittal views is currently the standard examination to allow for preoperative detection of CA stenosis[1,9-12]. Most cases of CA stenosis are related to an extrinsic compression by the median arcuate ligament (MAL). MAL is diagnosed by CT in about 10% of the candidates for PD, but only half are found to be hemodynamically significant during the GDA clamping test with Doppler assessment, which is mandatory before any resection[1,12-15]. MAL release is usually successful in restoring liver blood flow and preventing ischemic complications[1,2,9]. In cases of failure in MAL release, postponed PD with secondary stenting of the CA and reoperation for PD should be considered as an alternative to immediate hepatic artery reconstruction, which involves the risk of postoperative thrombosis[1,16-21]. We recently used this strategy in a patient undergoing surgery for pancreatic adenocarcinoma.

CASE REPORT

A 68-year-old male patient, presenting with insulin-requiring diabetes which evolved over 30 years, was referred with a pancreatic cephalic ductal adenocarcinoma after prosthetic drainage for jaundice and biopsy by echoendoscopy. Thoracic and abdominal CT showed no metastases. Liver magnetic resonance imaging was normal. According to the guidelines of the National Comprehensive Cancer Network, the tumor was considered resectable[22]. Abdominal CT without contrast showed multiple calcifications in the aorta and visceral arteries, as well as calcifications in the pancreaticoduodenal arcade (Figure 1). In addition to the calcifications, the arterial phase of the CT showed: (1) a focal narrowing in the proximal celiac trunk with a "hooked" appearance characteristic of a MAL; and (2) arterial supply from the SMA to the common hepatic artery *via* the GDA, as well as a dorsal pancreatic artery (Figure 2A and B).

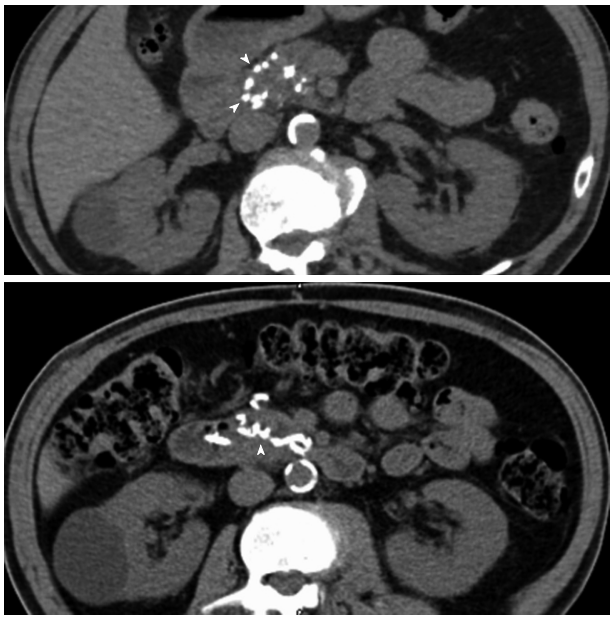

Figure 1 Computerized tomography scan without contrast showing calcifications in the pancreaticoduodenal arcade.

Exploratory laparotomy showed no contraindication to resection. Para-aortic lymph node biopsy showed no metastasis. Peroperative ultrasound showed a large pancreaticoduodenal arcade and a large dorsal pancreatic artery. However, the preoperative CT scan had underestimated the local extension because evidence of tumor abutment on the mesenteric vein existed. We performed a MAL division using a lateral approach allowing for a progressive division of the right diaphragmatic crus on the right side of the abdominal aorta, and the right side and the upper edge of the CA was progressively freed of all dense fibrous tissue. An additional GDA clamping test with Doppler ultrasound monitoring showed unsatisfactory restoration of the liver blood flow through the CA. Thus, considering the tumor as "borderline" resectable, revascularization of the hepatic artery and PD were both postponed. The postoperative course was uneventful. The in-hospital stay was 7 d.

The patient received 4 cycles of neoadjuvant FOLFIRINOX before imaging reassessment and endovascular management. Endovascular revascularization

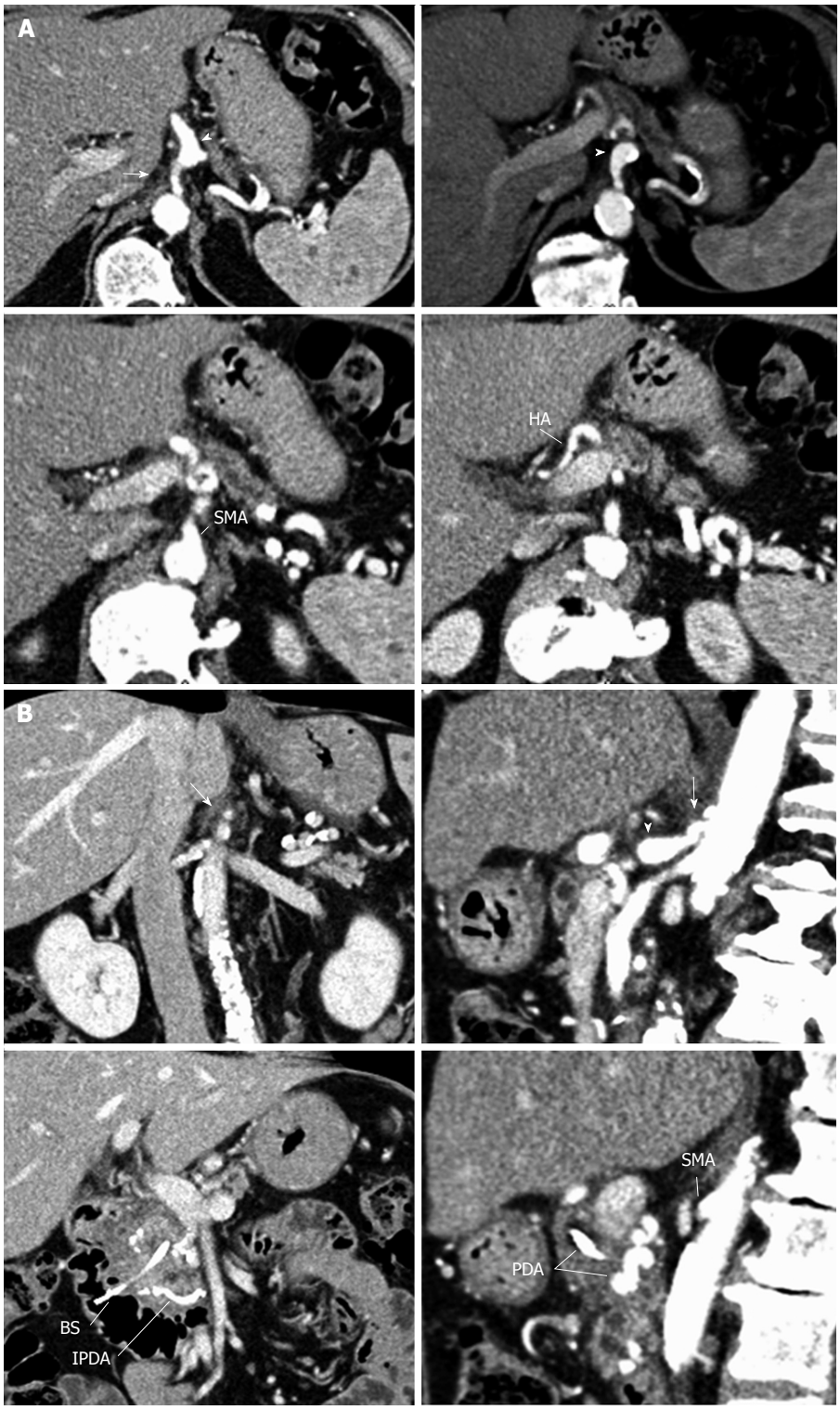

Figure 2 Features of median arcuate ligament. Arterial phase of the axial computerized tomography (CT) scan (A), along with coronal and sagittal CT-scan reconstructions (B) showing severe stenosis of the celiac trunk from extrinsic compression by dense fibrous tissue (arrow) and poststenotic dilation of the proximal celiac trunk (arrowhead). SMA: Superior mesenteric artery; HA: Hepatic artery; PDA: Pancreaticoduodenal arcade; IPDA: Inferior pancreaticoduodenal artery; BS: Biliary stent.

was performed 45 d after the first surgical step, during the interval between 2 cycles of chemotherapy. A CT scan showed modification of the CA/aorta "*angle*" after MAL release, which allowed for the possibility of a much easier stenting. Selective arteriography of the CA showed a short and significant remaining proximal stenosis of the CA. A careful crossing of the stenosis allowed angioplasty followed by stenting (Figure 3A and B). Subsequently, the CA blood flow was restored and the duodenopancreatic arterial supply disappeared.

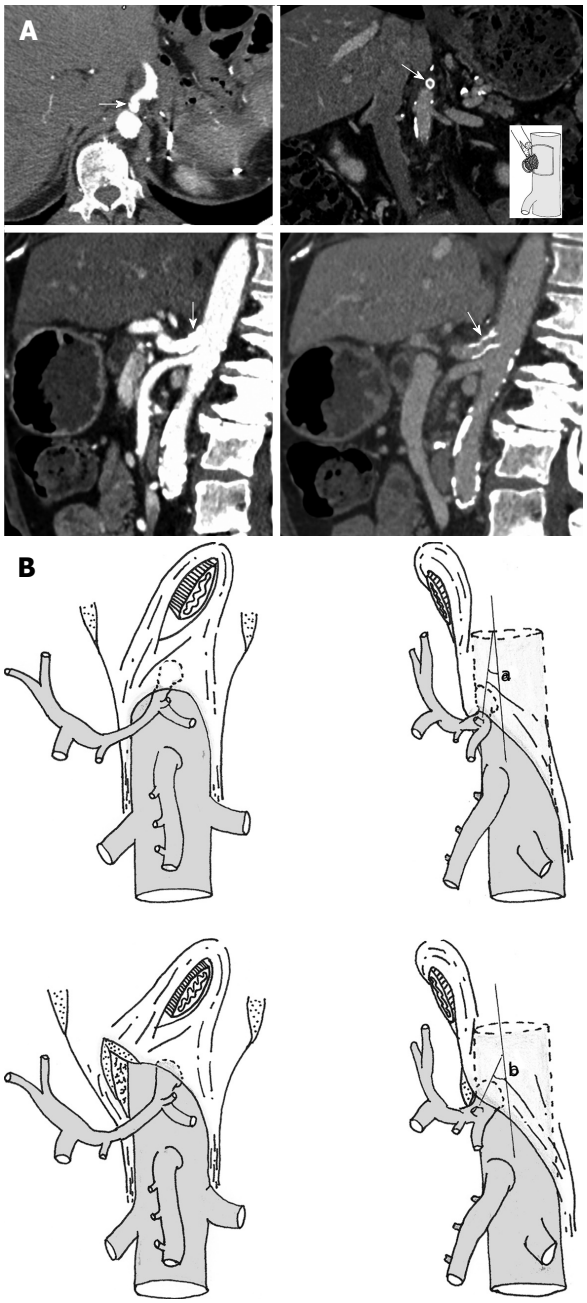

Figure 3 Computerized tomography scan. Computerized tomography scan after stenting of the celiac trunk (A), drawings showing the angle between the aorta and celiac trunk before and after median arcuate ligament release (B).

After 6 cycles of chemotherapy with a normalization of CA19-9 and an objective response on CT, a PD was performed without vein resection (Figure 4). The hepatic arterial inflow was preserved after GDA and dorsal pancreatic artery clamping and division. The divided common bile duct was well vascularized.

The standardized pathological examination of the specimen showed a 20 mm yp T3N1 poorly differentiated pancreatic adenocarcinoma with perineural involvement (6/10 positive nodes; lymph node ratio: 0.6). The resection was R0 as the inked margins were all negative; SMA, venous and posterior inked margins were free of tumor with a more than 1 mm clearance.

The postoperative course was uneventful. The patient was discharged on day 15 after equilibration of the diabetes. Adjuvant chemotherapy was performed for 6 mo. After 18 mo of follow-up, the patient was well and recurrence-free.

DISCUSSION

In recent years, mortality after PD has continuously decreased and today is less than 3% in high-volume centers[3,23]. However, ischemic complications are underestimated[1], and preoperative unrecognized visceral artery stenosis (*e.g.*, CA, SMA) may severely alter the early postoperative outcomes of PD with a high mortality rate[1-9]. In cases with CA stenosis, PD results in an interruption of the alternate pathway for blood supply to the liver. After biliary anastomosis, hepaticojejunostomy leakage, liver abscesses with sepsis, liver insufficiency and subsequent multiple organ failure represent a major cause of death[3-5,24,25].

CA stenosis is detected during the preoperative staging of pancreatic cancer in 4%-11% of patients scheduled for PD[1,2,9,10,18]. The majority of these detected stenoses are asymptomatic before the diagnosis of the tumor. Despite an increased number of aged patients undergoing PD in recent years, atherosclerotic intrinsic stenosis is rare; Gaujoux *et al*[1]. reported a rate of 0.04% in a large series of 545 patients undergoing PD [2/57 (3.5%) visceral artery stenosis]. Instead, MAL is the major cause of CA stenosis. The liver blood supply is provided mostly by the pancreaticoduodenal arcades (95%) and the dorsal pancreatic artery (77%)[10-12,26]. Most cases are asymptomatic (10, 16, 19 and 20) and hemodynamically significant stenosis during the GDA clamping test has been reported to be present in about 40% of the cases, which represents 5% of the patients submitted to PD[1,13].

Atherosclerotic stenosis should be treated preoperatively by endovascular stenting. In contrast, for cases with MAL, a ligament release is the first mandatory procedure and a lateral approach is less risky than a medial approach, which may result in an arterial injury: MAL-induced CA stenosis is reported to be successfully treated by ligament release in nearly 90% of cases[1,2,9,20]. In the recent series reported by Gaujoux *et al*[1], 20 out of 23 MAL releases were successful with no postoperative mortality[1]. If there is not restitution of flow in the hepatic artery after MAL release, then a vascular reconstruction prior to division of the GDA should be considered before proceeding with the resection. Indeed, preoperative MAL stenting is technically challenging and proves mostly ineffective by the default of expanding and restenosis. In the current report, stenting was easier after MAL release and this strategy should be considered as an alternative to vascular bypass, avoiding the risk of postoperative thrombosis[13,16-21]. Secondary stenting should proceed with cautious regard for the potential risk of injury

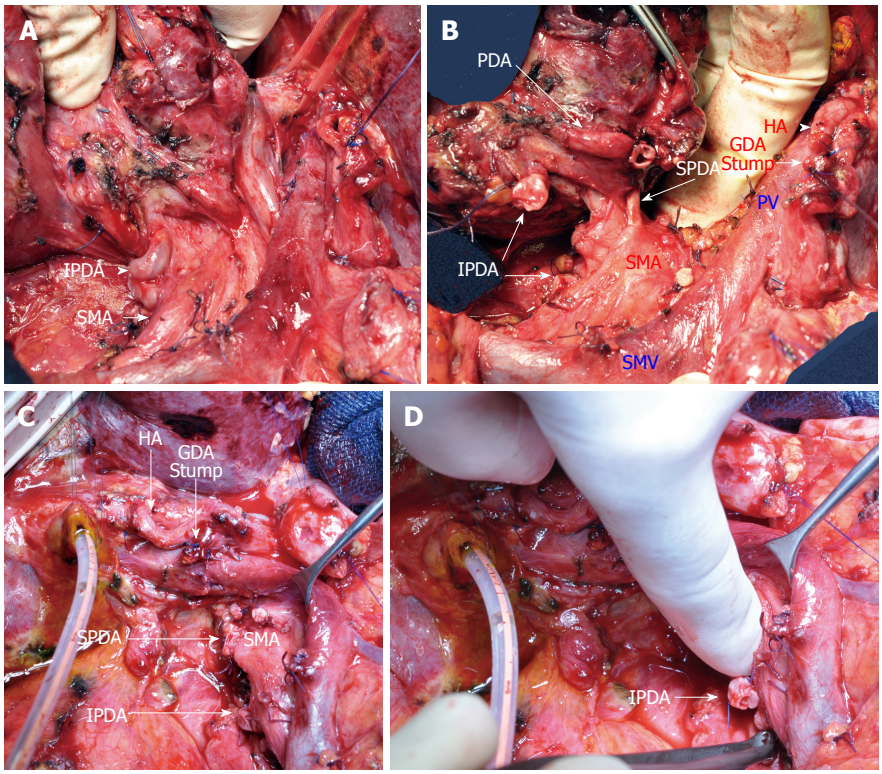

Figure 4 Pancreaticoduodenectomy. Operative views (A, B) after mobilization of the specimen showing a very large inferior pancreaticoduodenal artery (IPDA) and the pancreaticoduodenal arcade. After resection (C, D), the stumps of the pancreaticoduodenal arteries (superior pancreaticoduodenal artery and IPDA) are shown. SMA: Superior mesenteric artery; HA: Hepatic artery; GDA: Gastroduodenal artery; SMV: Superior mesenteric vein; PV: Portal vein.

during the procedure due to the fragility of the artery after ligament release. A successful laparoscopic approach for a MAL division has been reported in symptomatic patients with MAL syndrome but without pancreatic tumor. In cases of CA stenosis detected by a multidetector CT scan before PD of a clearly resectable pancreatic adenocarcinoma, the following strategy should be considered as an option: (1) in cases with a hemodynamically significant stenosis, as assessed by GDA clamping and Doppler ultrasound, perform a first-step, including MAL release, with abdominal exploration and a para-aortic lymph node biopsy (currently recommended in order to avoid futile resection)[27,28]; (2) in cases involving a failure of MAL release to restore the liver blood inflow, perform secondary cautious endovascular stenting of the CA in order to avoid the risk of postpancreatectomy arterial reconstruction thrombosis and postpone PD. A laparoscopic or robotic approach should be considered for the first step[29].

In conclusion, CA stenosis is usually due to a previously asymptomatic MAL which induces a major risk of post-PD complications due to liver and biliary ischemia if unrecognized before the resection. The GDA clamping test is mandatory in order to detect hemodynamically significant stenosis. MAL release is usually efficient to restore an adequate liver blood inflow. Postponed PD after inefficient MAL release, followed by secondary stenting, should be considered as a two-stage option that avoids hepatic artery bypass and the postoperative risk of thrombosis of the arterial reconstruction.

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COMMENTS

Case characteristics

Failure in median arcuate ligament (MAL) release during pancreaticoduodenectomy (PD) for pancreatic adenocarcinoma

Clinical diagnosis

Celiac axis (CA) stenosis due to MAL in a patient with pancreatic adenocarcinoma.

Imaging diagnosis

Postoperative computed tomography scan showed modification of the CA/aorta "angle" after MAL release, which allowed for the possibility of a much easier stenting.

Treatment

Postponed PD and secondary stenting of the CA as an alternative to hepatic artery reconstruction

Related reports

MAL release is usually efficient (90%) to restore an adequate liver blood inflow and prevent ischemic complications; in case of inefficient release hepatic artery reconstruction is usually indicated.

Experiences and lessons

Preoperative MAL stenting is technically challenging and proves mostly ineffective by the default of expanding and restenosis; in the current report, stenting was easier after MAL release and this strategy should be considered as an alternative to vascular bypass, avoiding the risk of postoperative thrombosis; secondary stenting should proceed with cautious regarding the potential risk of injury during the procedure due to the fragility of the artery after ligament release.

Peer-review

Great work on a theme really important in our diary practice.

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CASE REPORT

Peroral endoscopic myotomy for treatment of Guillain-Barre syndrome-associated achalasia: A rare case

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Author contributions: Shin SK wrote the paper; Kim KO designed the report and reviewed the paper; Kwon KA and Park DK supervised the initial manuscript; Kim SY, Kim JH, Kim YJ and Chung JW contributed cases and edited the manuscript; Kim EJ participated to the medical care of the patient.

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Informed consent statement: The patient involved in this study gave his written informed consent authorizing use and disclosure of his protected health information.

Conflict-of-interest statement: Authors have no conflict of interest to disclose.

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Abstract

Guillain-Barre syndrome (GBS)-associated achalasia is a very rare disease of uncertain cause. We report the case of a patient diagnosed with GBS-associated type Ⅰ achalasia who was successfully treated with peroral endoscopic myotomy (POEM). A 30-year-old man who was diagnosed with GBS 3 mo before was referred to our department with dysphagia and meal-related regurgitation. The results of esophagography, endoscopy, and high-resolution manometry (HRM) revealed type Ⅰ achalasia. POEM that utilized a submucosal tunneling technique was performed to treat the GBS-associated type Ⅰ achalasia. After POEM, smooth passage of a contrast agent into the stomach was shown in follow-up esophagography, and follow-up HRM revealed a decrease in the mean integrated relaxation pressure 22.9 mmHg to 9.6 mmHg. The patient remained without dysphagia for 7 mo, even though the patient's neurological problems were not fully resolved. POEM may be a safe and effective treatment for GBS-associated type Ⅰ achalasia.

Key words: Peroral endoscopic myotomy; Achalasia; Guillain-Barre syndrome

Core tip: Guillain-Barre' syndrome (GBS)-associated achalasia is a rare disease of uncertain cause. Previously, a case of GBS-associated achalasia that was treated with pneumatic dilation was reported. However, pneumatic

dilatation has about a 25%-50% chance that the patient will require another procedure within five years. Here, we present a case of a patient diagnosed with GBS-associated type Ⅰ achalasia who was successfully treated with peroral endoscopic myotomy.

Shin SK, Kim KO, Kim EJ, Kim SY, Kim JH, Kim YJ, Chung JW, Kwon KA, Park DK. Peroral endoscopic myotomy for treatment of Guillain-Barre syndrome-associated achalasia: A rare case. *World J Gastroenterol* 2017; 23(5): 926-930 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i5/926.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i5.926

INTRODUCTION

Guillain-Barré syndrome (GBS) is an immune-mediated polyradiculoneuropathy characterized by rapidly progressive, symmetrical, ascending weakness and sensory loss. It is usually triggered by an infection, such as upper respiratory tract infection or gastroenteritis[1]. GBS-associated achalasia is a very rare condition of uncertain cause. Previously, a case of GBS-associated achalasia that was treated with pneumatic dilation was reported[2]. However, pneumatic dilatation has about a 25%-50% chance that the patient will require another procedure within five years[3,4]. Recently, peroral endoscopic myotomy (POEM) has emerged as a safe and effective technique that can adequately treat achalasia. Here, we present a case in which a patient diagnosed with GBS-associated type Ⅰ achalasia was successfully treated with POEM.

CASE REPORT

A 30-year-old man was referred to our department with dysphagia and meal-related regurgitation, with a baseline Eckardt symptom score of 11. Three months before, he had suffered from progressive quadriparesis, dysarthria, and facial palsy following gastrointestinal illness that he had about 10 d prior. With the characteristic protein-cell count differentiation of albuminocytologic dissociation detected *via* cerebrospinal fluid analysis (protein 73.4 mg/dL, WBC 8 /mm^3) and high titer of the serum IgM antibody to GD1b, in addition to nerve conduction studies, a diagnosis of GBS was made. Despite receiving five days of intravenous immunoglobulin therapy immediately after the diagnosis of GBS, the patient's neurological problems had not recovered, and swallowing difficulty was worsening. His family history was unremarkable. Abdominal examinations showed normal bowel sounds, no palpable masses, and no organomegaly. Laboratory tests revealed normocytic normochromic anemia, with a hemoglobin level of 11.1 g/dL. The results of liver and renal function tests were within normal limits. A videofluoroscopic swallowing study (VFSS) on day 30 showed dysfunctional oropharyngeal transfer of the barium paste, subglottic aspiration of the liquid barium, and decreased esophageal peristaltic movement. Oromotor facilitation and electrical stimulation therapy to the pharyngeal muscles were performed for the swallowing difficulty. A follow-up VFSS on day 71 showed slight improvement of the oropharyngeal transfer of barium paste. However, decreased esophageal peristaltic movement was still present, and the dysphagia was aggravated. On day 93, subtle dilated distal esophageal lumen with acute tapering at the lower esophageal sphincter (LES) and narrowing at the esophagogastric junction was shown through esophagography (Figure 1), and dilation of the esophageal lumen and retention of food remnants in the esophagus were identified during endoscopy (Figure 2). A mean integrated relaxation pressure (IRP) of 22.9 mmHg over test swallows with absent peristalsis was shown in high-resolution manometry (HRM) (Figure 3). POEM was performed in the operating room under general anesthesia to treat GBS-associated type Ⅰ achalasia (Figure 4). To summarize, it consisted of four steps: longitudinal mucosal incision, creation of a submucosal tunnel extending towards the gastroesophageal junction, selective myotomy of the circular muscle fibers, and mucosal closure by clip. After the treatment, smooth passage of a contrast agent into the stomach was shown in follow-up esophagography (Figure 5), and follow-up HRM showed that the mean IRP decreased to 9.6 mmHg (Figure 6). The endoscope could be passed without resistance at 2 mo after the procedure. The patient's Eckardt symptom score decreased to 2. Although the patient's neurological problems were not fully recovered at 10 mo after diagnosis of GBS, he could eat a soft diet without problems for 7 mo after POEM.

DISCUSSION

GBS is an acute, immune-mediated polyneuropathy that often leads to severe weakness[5]. Required criteria for diagnosis include progressive weakness of more than two limbs, areflexia, and progression for no more than four weeks. Other causes of acute neuropathy, such as lead poisoning, vasculitis, botulism, and porphyria, require exclusion. Supportive criteria include relatively mild sensory signs, raised protein levels in the cerebrospinal fluid while maintaining a relatively normal cell count, and neurophysiological evidence of conduction block. Weakness is frequently proximal and distal, unlike dying-back axonopathies, and respiratory involvement occurs in about a quarter of cases[6]. Cranial nerve involvement can be observed in 45%-75% of patients with GBS[7]. In cases of cranial nerve involvement, facial and oropharyngeal weakness can appear, and dysphagia can develop[8].

A definite mechanism of GBS has not been established, but it has been found that the antibodies generated by the activation of immune responses after infections makes a cross-bridge *via* molecular mimicry

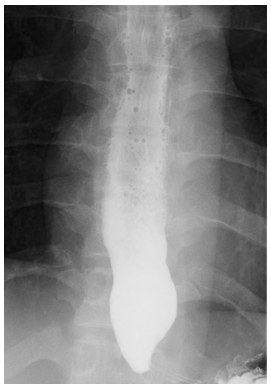

Figure 1 Subtle dilated distal esophageal lumen with acute tapering at the lower esophageal sphincter and narrowing at the esophagogastric junction was shown in esophagography.

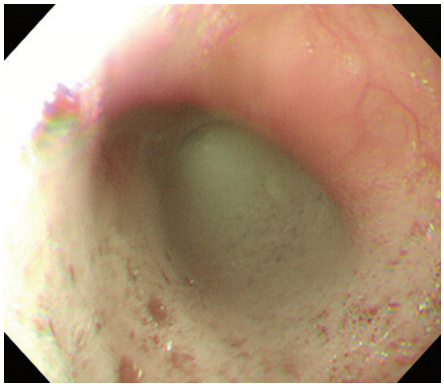

Figure 2 Dilation of the esophageal lumen and retention of food remnants in the esophagus was identified during endoscopy.

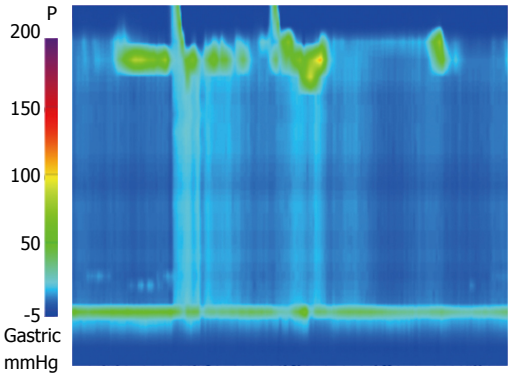

Figure 3 Mean integrated relaxation pressure 22.9 mmHg over test swallows with absent peristalsis was shown in high-resolution manometry.

with antigens in the peripheral nerves, which destroys them[9,10]. This hypothesis is supported by the fact that anti-anglioside antibodies have been found in GBS patients' serum. In our case, high titer of the serum IgM antibody to GD1b was found.

On the other hand, GBS-associated achalasia is a very rare disease of uncertain cause. Achalasia is an esophageal motor disorder characterized by the absence of peristalsis and a defective relaxation of the LES which results in impaired bolus transport and

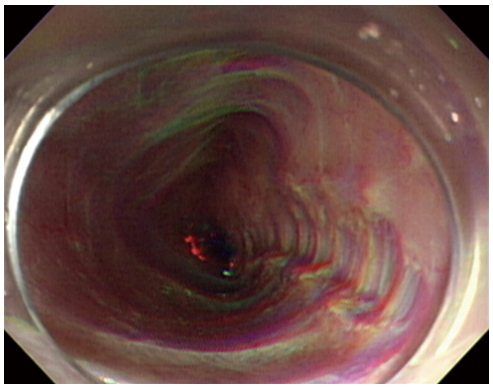

Figure 4 Peroral endoscopic myotomy was performed to treat Guillain-Barre syndrome-associated type I achalasia.

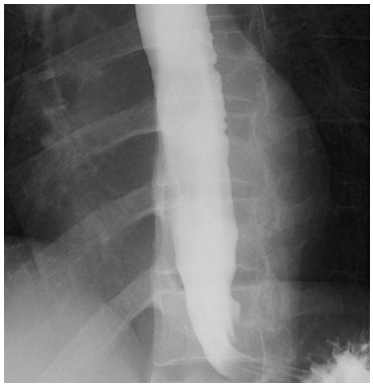

Figure 5 After peroral endoscopic myotomy, smooth passage of a contrast agent into the stomach was shown in follow-up esophagography.

food stasis in the esophagus[11,12]. In 1994, Firouzi *et al*[2] presented a case of GBS with achalasia. An autoimmune response triggered by an infection such as gastroenteritis or respiratory tract infection is discussed as one possible cause of the loss of inhibitory ganglion cells within the myenteric plexus in patients with GBS-associated achalasia[13]. Previously, it was reported that GD1b antibodies preferentially stained the large dorsal root ganglion neurones in animal models[14], and degenerative changes in the ganglion cells of the dorsal motor nucleus of the vagus have been reported in some patients with achalasia[15]. In our case, changes in the ganglion cells of the dorsal motor nucleus of the vagus by GD1b antibodies may have induced GBS-associated achalasia.

Although intravenous immunoglobulin and plasma exchange have proved effective, many patients with GBS still develop severe weakness and have a long disease course, often with incomplete recovery, pain, and fatigue[16]. In our case, despite receiving five days of intravenous immunoglobulin therapy immediately, the patient's neurological problems had not recovered and swallowing difficulty was worsening.

Treatment of achalasia is currently aimed at decreasing the resting pressure in the LES. In a previous report, GBS-associated achalasia was subsequently diagnosed by manometry and was successfully treated

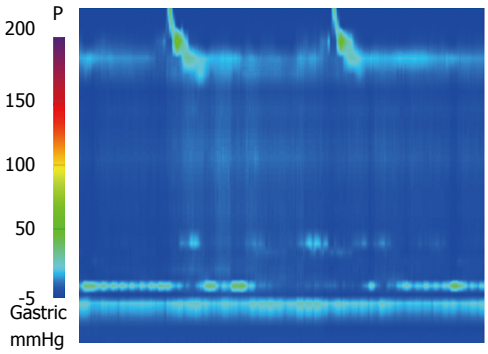

Figure 6 Mean integrated relaxation pressure decreased to 9.6 mmHg in follow-up high-resolution manometry.

with pneumatic dilation[2]. However, the pneumatic dilatation has about a 25%-50% chance that the patient will require another procedure within five years[4]. An alternative to dilation in patients with achalasia is endoscopic injection of the botulinum toxin. The symptomatic response to this treatment is often short lived, with a greater than 50% recurrence rate within 6 mo[17].

Recently, POEM has been developed as an alternative endoscopic treatment that is effective and less invasive[18]. A major benefit of POEM may be the ability to avoid the risk of unexpected and uncontrolled perforation that may occur during balloon dilation. The clinical effects of the POEM treatment were immediate, and the later HRM showed improvement in the LES pressure and pressurization disappeared[19].

In the present case, we performed POEM for treatment of GBS-associated type Ⅰ achalasia. After POEM without any complications, mean IRP decreased from 22.9 mmHg to 9.6 mmHg and Eckardt symptom score was reduced from 11 to 2 points. Our initial experience suggests that POEM is a safe and effective treatment for GBS-associated type Ⅰ achalasia.

COMMENTS

Case characteristics

A 30-year-old man was referred to our department with dysphagia and meal-related regurgitation, with a baseline Eckardt symptom score of 11.

Clinical diagnosis

After the diagnosis of Guillain-Barré syndrome (GBS), a mean integrated relaxation pressure of 22.9 mmHg over test swallows with absent peristalsis was shown in high-resolution manometry, which refers to GBS associated type Ⅰ achalasia.

Differential diagnosis

Cranial nerve involvement in GBS.

Laboratory diagnosis

Laboratory tests revealed normocytic normochromic anemia, with a hemoglobin level of 11.1 g/dL, and other laboratory findings were within normal limits.

Imaging diagnosis

Subtle dilated distal esophageal lumen with acute tapering at the lower esophageal sphincter and narrowing at the esophagogastric junction was shown through esophagography.

Pathological diagnosis

Pathologic confirmation was not performed in our case.

Treatment

Peroral endoscopic myotomy (POEM) was performed to treat GBS-associated type Ⅰ achalasia.

Related reports

Previously, a case of in GBS-associated achalasia that was treated with pneumatic dilation was reported.

Term explanation

GBS is an immune-mediated polyradiculoneuropathy characterized by rapidly progressive, symmetrical, ascending weakness and sensory loss.

Experiences and lessons

The authors' initial experience suggests that POEM is a safe and effective treatment for GBS -associated type Ⅰ achalasia.

Peer-review

It is an interesting and well-written case report.

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